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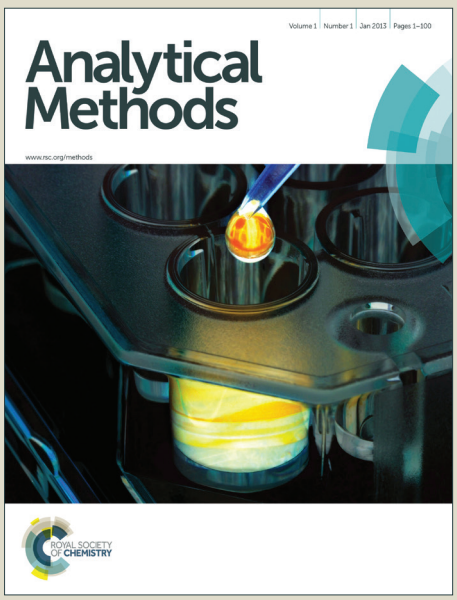

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Development of a green method for separation and identification of the degradation impurity of Isoniazid by SFC-MS/MS

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ABSTRACT

To produce an eco-friendly stability indicating method, the reaction solutions containing degradation products of isoniazid were used for separation and optimized by varying the supercritical fluid chromatography (SFC) conditions. Supercritical carbon dioxide (SC-CO2) (85%) and modifier dichloromethane: methanol: ethyl acetate: formic acid (70:30:0.5:0.1 v/v/v/v) (15%) is used as mobile phase and separation was achieved using a C-18 column. The degradation products separated at relative retention times (RR_T) of 1.58 and 1.82, respectively. The method was validated as per international standards in terms of selectivity, linearity, precision and recovery. The drug shows a linear response at concentrations between 1 and 100 μgml−1. The mean values (±%RSD) of slope, intercept and correlation coefficient were 179009 (±0.3%) 151382 (±0.6%), respectively. The mean %RSD values for intra and inter-day precision were 0.63 and 1.29, respectively. The recovery of the drug ranged between 98.82 and 100.68%, when it was spiked to a mixture of solutions in which sufficient degradation was observed. The study was extended to SFC-MS/MS and FT-IR which were carried out to identify the degradation products. The FT-IR spectra and *m*/*z* values of the peaks at RR_T 1.58 and RR_T 1.82 matched with isonicotinic acid and isonicotinamide, respectively.

Key Words: Supercritical Fluids, Degradation products, Isoniazid, Isonicotinic acid, Isonicotinamide, Chromatography.

1. INTRODUCTION

Isoniazid (INH, isonicotinyl hydrazine) (Figure 1) is most widely used drug for treatment of tuberculosis [1]. It is a first line agent in tuberculosis, which have to be taken for longer periods at a prescribed time interval and hence the quality of the product should be maintained for longer period. A safe, reliable and economical method has to be incorporated to find out the concentration of these drugs simultaneously as they are available in combined as well as single dosage.

Figure-1 Molecular structure of (a) Isoniazid and their degradant impurity (b) Isonicotinic acid and (c) Isonicotinamide

Extended review reveals that various analytical methods based on amperometric [2], voltametric [3, 4], spectrophotometric [5-11], spectrofluorometric [12], HPLC [13-21], HPTLC [22-24], RP-HPTLC [25] have been developed for determination of INH in pharmaceutical dosage forms and biological fluids. Several methods viz. HPLC and LC/MS are reported for the stability of the INH and acid degradation products [26-32]. Only single eco-friendly method available for determination of INH in fixed dose combination [33]. Besides this method, method based on capillary zone electrophoresis (CZE) was reported [34] for detection of INH and acid degradation products, which can be consider to be eco-friendly method. However, reported method has disadvantage as it has lots of parameter to optimize and detection time was more than 10 min. Although, there were lots of methods available, we have developed eco-friendly, simple, accurate and precise method using supercritical CO_2 and MS/MS and FT-IR study was done for identification of degradants of the INH.

2. EXPERIMENTAL

2.1 Instruments:

2.1.1 Supercritical Fluid Chromatograph

A JASCO-2000 series (Japan Spectroscopic Co. Ltd., Hachioji, Japan) of supercritical fluid chromatograph was used for the separations in this study. It was equipped with two pumps (PU-2080 and PU-2080 CO_2), which were capable to adjust the flow rate (0.001 to 10 ml/min.) for both Supercritical CO_2 and modifier. The system pressure was maintained electronically by back-pressure regulator (BP-2070), which allowed the flow rate and pressure to be controlled independently. An external loop with a capacity of 20 µL was equipped with rheodyne injector, capable to inject liquid sample accurately into the analytical column. The temperature of the column was thermostatically controlled in a column oven (Jasco-Co-2060), while inbuilt with a cooling circulator. Detection of analyte was done by using a UV detector (Jasco-UV-2070). The effluent coming from the SFC was injected in the MS/MS for detection of any impurity if present.

2.1.2 Mass spectrometer

An AB Sciex (Canada) QTRAP-4500 series mass spectrometer was used in the present investigation. It was outfitted with exclusive TurboV™ source contained TurboIonSpray probes which provide advanced linear ion trap technology for the highest degree of sensitivity. The TurboV ion chamber has embedded ceramic heater technology with improved gas dynamics. Data acquisition and integration were done by Windows-based analyst software.

2.1.3 SFC/MS/MS conditions

Analysis was performed on fused silica column (inertsil ODS-5 µm C18, 150mm×4.6mm) protected by precolumn filter cartridges. After optimization, mobile phase consisting of

dichloromethane: methanol: ethyl acetate: formic acid (70:30:0.5:0.1 v/v/v/v) (15%) and supercritical CO2 (85%) at back pressure of 10 MPa were used.

The optimized value for MS/MS analyses were as follows: ESI positive ion mode; capillary voltage, 3.5 kV; cone voltage, 40 V; Gas 1 (nebulizing gas) and Gas 2 (cone gas) were set to 50 units each and the source temperature was set at 550°. High-purity nitrogen gas was used as nebulizer and cone gas.

The injection volume and column temperature were set at 20 µL and 40 °C, respectively. Full-scan SFC–MS/MS spectra were obtained by scanning from m/z 50 to 500.

2.1.4 FT-IR Study

A JASCO FT-IR 4100 was used to record the spectra of separated impurities. Spectra were acquired using the diffuse reflectance cell supplied by the JASCO (model- DR 81) at auto scan rate. Data integration was performed using the spectra manager software.

2.1.5 NMR Study

400 MHz ^{1}H NMR spectra were recorded on Bruker's Advance III 400MHz FT NMR with solid multi nuclei probe. Chemical shifts were measured relative to TMS (tetramethyl silane) as an internal reference. All measurements were made at T= 298 K.

2.2 Materials

Isoniazid standard was received as gift specimen from Sunij Pharma Pvt. Ltd. (Vatva GIDC, Ahmedabad) and tablet containing isoniazid was procured from local market. HPLC grade dichloromethane, ethyl acetate and methanol were procured from Merck, Germany – and whatmann filter paper no. 42 (0.45 µm) (Sigma Aldrich) were used.

2.3 Method:

Selection of analytical wavelength

The spectra taken at λ_{max} 260nm of INH in proposed mobile phase was found to be linear and degraded products were well separated. Hence, 260nm was chosen as detection wavelength in SFC.

2.3.1 Preparation of modifier phase:

A blend of 30 ml methanol, 70 ml of dichloromethane, 0.5 ml ethyl acetate and 0.1 ml formic acid was filtered through 0.45 µm filter paper and the blend was sonicated for 10 min to degas the mixture and used as modifier phase.

2.3.2 Conduct of stress degradation studies

The stress degradation studies were carried out as reported [35, 36]. Acid and alkali degradation studies were done in 0.1N HCl and 0.1N NaOH respectively, at drug concentration of 1mg/ml. The solution was heated at 60 ◦C for 5 days. The peroxide decomposition behavior was also checked for drug concentration of 1mg/ml in 3% H_2O_2 solution. This solution was stored at room temperature for 24 hrs. The photodegradation was carried out by distributing drug on a flat glass surface and exposed it to the UV in photostability chamber. The thermal decomposition was executed by heating the drug powder in sealed glass vials at 60 ◦C for 5 days. All the samples were also stored and prepared under dark with same stress conditions. The samples were withdrawn at a time interval of 1, 3 and 5 days.

2.3.3 Preparation of INH Standard and Working Solutions:

The INH stock standard was prepared by dissolving 10 mg of INH in 10 ml modifier containing Dichloromethane: Methanol: Ethyl Acetate: Formic acid (70:30:0.5:0.1 v/v/v/v). This solution was kept in refrigerator at 2°C - 8°C. The working solutions were obtained by suitably diluting the INH stock solution.

2.3.4 Preparation of Calibration Standards and Quality Control (QC) Samples:

The appropriate volume of aliquots from standard INH was transferred to volumetric flasks of 10 ml capacity to prepare six calibration standards. The volume was adjusted to the mark with mobile phase give a solution in the range of 1-100 µg/ml for INH.

2.3.5 FT-IR Spectroscopy

After careful collection of the effluent, samples were allowed to dry at room temperature and then mixed with previously dried KBr in an agate mortar and pestle to make fine and uniform mixture of sample and KBr.

2.4 Method validation

The optimized method was validated by using ICH guidelines [37] for determining selectivity, limits of quantification (LOQ) and detection (LOD), linearity, precision and recovery. To assess the selectivity of the proposed method, spiked and non-spiked samples were injected into the SFC/MS system. The detection (LOD) and quantification limits (LOQ) were experimentally determined by injecting a number of non-spiked samples (n = 6) and measuring the magnitude of the background analytical response. The LOD and LOQ were estimated as three and ten times the signal-to-noise (S/N) ratio, respectively. The precision of the method was evaluated by injecting three different concentrations in triplicate, on the same day and on consecutive three days. The recovery was determined in 6 replicates at 3 concentrations (low, medium and high QC levels).

2.5 Characterization of degradation product(s)

FT-IR, MS-MS and NMR studies were carried out to identify the functional groups and m/z values of the major degradation products formed under various stress test conditions. The obtained values were compared with the known degradation products of isoniazid.

3. RESULTS AND DISCUSSION

The goal of this work was to provide an ecofriendly selective alternate method for identification and determination of the INH and related substance by SFC-MS/MS. It is reported that when a volatile acid (like, formic acid) added to the mobile phase will increase the positive ion of the analyte, which is most helpful in MS/MS [33, 35], therefore, added 0.1% v/v formic acid to the Dichloromethane: Methanol: Ethyl Acetate (70:30:0.5 v/v/v) as a modifier in the SFC/MS work.

3.1 Selection of mobile phase

For the selection of mobile phase, we have varied the concentration of modifier methanol and dichloromethane with the addition of Ethyl acetate and 0.1% of formic acid ranging from 10% to 30% and supercritical carbon dioxide (SC-CO_2) and chromatograms were recorded

Figure 2: Chromatogram of A) standard INH and B) related compound (I- Isonicotinic acid and II- Isonicotinamide) in proposed mobile phase

The results prove that the optimized system containing dichloromethane: methanol: ethyl acetate: formic acid (70:30:0.5:0.1 v/v/v/v) (15%) and supercritical CO_2, was found to be satisfactory and gave well separate peak for INH and degradant product (Figure 2).

3.2 Degradation studies

Under the different condition of stress degradation, two degradant were separated and identified. The retention time and relative retention time (RRT) of the drug and degraded products are shown in table 1.

3.2.1 Thermal condition

The drug exposed to 60 ◦C for 5 days didn't show significant degradation, which suggest the drug is stable to the stress heat condition.

3.2.2 Hydrolytic condition

The drug under the acidic condition (0.1N HCl and heated at 60 ◦C for 5 days) degraded with the time and gave two peaks at RRT 1.58 and 1.82 respectively (Figure 3). Degradation in acidic condition was greater as compared with the alkali degradation.

The alkali stress condition (0.1N NaOH and heated at 60 ◦C for 5 days) shows little fragmentation ~5% in five days. Degradation shows a main degradant peak at RRT 1.58 and a peak at RRT 1.82 (Figure 3).

3.2.3 Oxidation

The drug under the oxidative condition (3% H2O2 at room temperature) have no significant degradation with little peak at RRT 1.58 (Figure 3).

3.2.4 Photolytic condition

The results obtained from the samples prepared under dark and normally prepared samples were same, which suggest that there was no additional effect of light on samples (Figure 3).

Figure 3: Chromatogram of INH degradation in a) acidic b) basic c) oxidation and d) photolytic condition

Table 1 Retention time and relative retention times of various peaks

Peak	Retention time (RT)	Relative retention time (RRT)
Isoniazid (INH)	2.91min	1.00
I	4.58min	1.58
II	5.28min	1.82

3.3 Validation of the developed method

The information obtained from linearity studies are presented in Table 2. The response of the drug was linear in the QC sample range between 1 to 100 µgml−1. The mean (±%RSD) values of slope, intercept and correlation coefficient were 179009 (±0.4), 151382 (±0.6) and 0.9985 (±0.04), respectively. The mean quality coefficient of calibration curve (%QC_{mean}) was 3.46% which is less than 5%, indicate method was linear. The residual plot also shows a pretty random pattern (Figure 4). This random pattern indicates that a linear model provides a

decent fit to the data. The %RSD values for intra-day and inter-day precision studies (Table 3) were ranging from 0.32% to 0.94% and 0.46% to 1.98%, respectively, confirming that the method was sufficiently precise. Good separation was accomplished even when the process was duplicated by two different people, therefore confirming the reproducibility of the method. As shown Table 4, good recoveries were made at the three different levels, i.e. 50%, 100%, 150% with the mean recovery being 99.68%. Fig. 2 shows that the method was selective to the drug as well as the degradation products.

Figure 4: Residual plot showing the random pattern

Table 2 Linearity data obtained in three different days (n=6)

Regression parameters	Day 1	Day 2	Day 3	Mean±S.D. (%R.S.D.)
Slope	178623	178686	179719	179009±615 (0.34)
Intercept	151136	150652	152359	151382±880 (0.58)
R2	0.9982	0.9985	0.9989	0.9985±0.0003 (0.04)

Table 3: Precision data obtained during intra-day (n = 6) and inter-day (n = 3) studies

Actual concentration (µgml−1)	Intra-day measured concentration (µg/ml)±S.D.; %R.S.D.	Inter-day measured concentration (µg/ml)±S.D.; %R.S.D.
1	1.06±0.01; 0.94	1.01±0.02; 1.98
30	30.09±0.19; 0.63	29.92±0.17; 0.57
80	80.06± 0.26; 0.32	80.41± 0.37; 0.46

Table 4: Results of recovery (*n=6) study of INH

Amount of Drug	Amount of Drug Added	Total Amount Recovered		% Recovery	
INH (µg/mL)	INH (µg/mL)	INH Mean±S.D.* (µg/mL)	%RSD	INH Mean±S.D.* (µg/mL)	%RSD
3	2.4	5.42±0.05	0.92	100.01±0.36	0.36
3	3	6.03±0.07	1.16	100.20±1.24	1.24
3	3.6	6.58±0.06	0.91	98.82±0.81	0.82

3.4 Characterization of degradation products

To increase the quantity of degradant impurity, INH was exposed to the higher acid concentration (1N HCl for 5 days). And then the impurities with retention time at 4.58 and 5.28 were carefully collected from the SFC system.

3.4.1 MS/MS Study

Mass chromatograms in the positive electron spray ionization (ESI) mode for the drug and degradation products are shown in Fig. 5. Evidently, the m/z value of 138 for isoniazid corresponded to its molecular weight of 137, thus validating the output of the mass spectrometer. The m/z values obtained for the degradation products, resolving at RRT 1.58 and 1.82 in the same run were 124 and 123, respectively. It was revealed that the separated degradation impurities at m/z values of 124 and 123 were isonicotinic acid and isonicotinamide respectively, when compared to the mass spectrum of known degradation products of isoniazid.

Figure. 5. Mass profiles for a) isoniazid and degradation products appearing at b) RRT 1.58 (Isonicotinic acid) and c) RRT 1.82 (Isonicotinamide)

Figure 6. FT-IR spectra of degradation products of INH appearing at a) RRT 1.58 (Isonicotinic acid) and b) RRT 1.82 (Isonicotinamide)

Figure 7. NMR spectra degradation products appearing at a) RRT 1.58 (Isonicotinic acid) and b) RRT 1.82 (Isonicotinamide)

3.4.2 FT-IR Study

FT-IR spectra of the impurities collected from the SFC at RRT 1.58 and 1.82 were taken. The spectra were matched with standard spectra of isonicotinic acid and isonicotinamide which indicate the separated compound may be isonicotinic acid and isonicotinamide, which were further confirmed the data obtained from the MS/MS. FT-IR spectra were shown in figure 6.

3.4.3 NMR Study

The collected effluent from the SFC at RRT 1.58 and 1.82 were used to take the NMR spectra. NMR spectra matched with the known impurity suggests isonicotinic acid (a) and isonicotinamide (b), respectively (Figure 7).

4. CONCLUSION

To the best of our knowledge this is the first method that uses the eco-friendly approach with the support of tandem mass spectroscopy to determine Isoniazid and related compounds by stress degradation. The method has been successfully applied to stability samples of pharmaceutical dosage forms of different manufacturer. The reported method offers the vantage of the usage of ecofriendly method which distinguishes all the components within the 6 min without interference from the excipient.

5. ACKNOWLEDGEMENT

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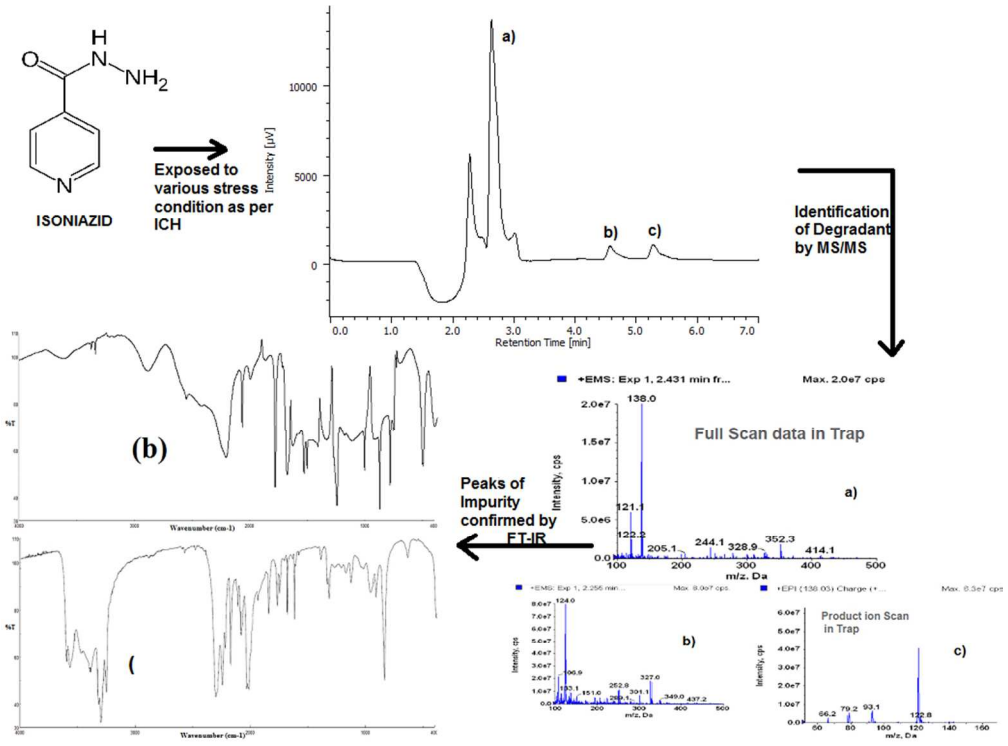

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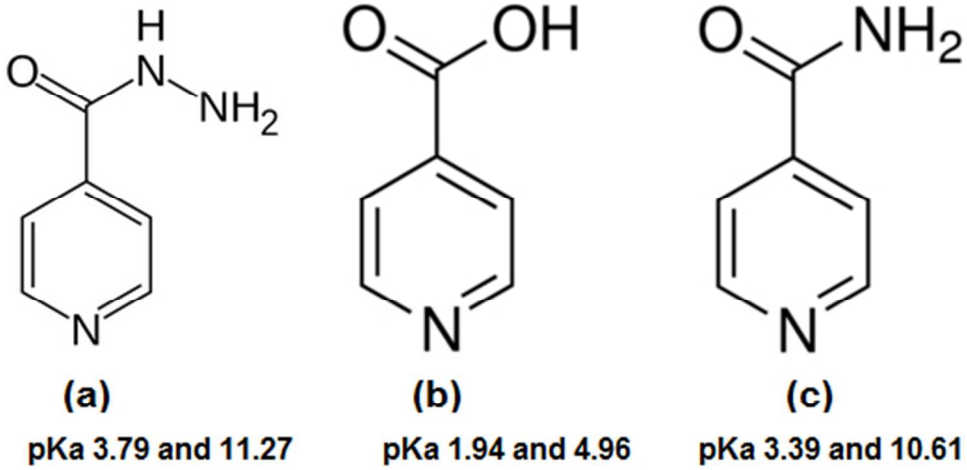

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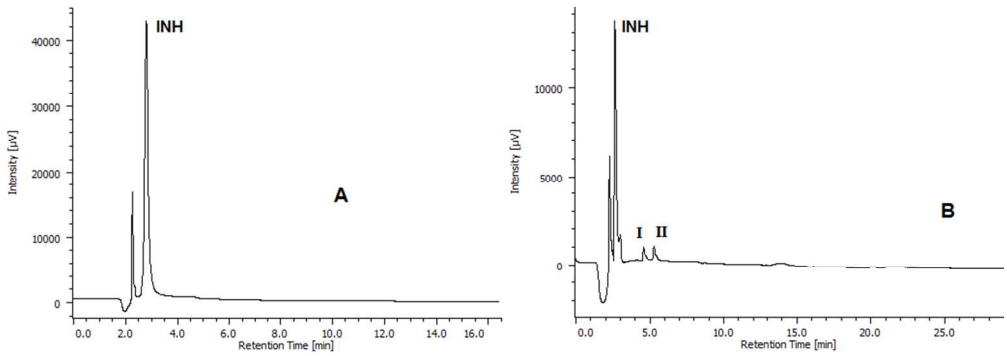
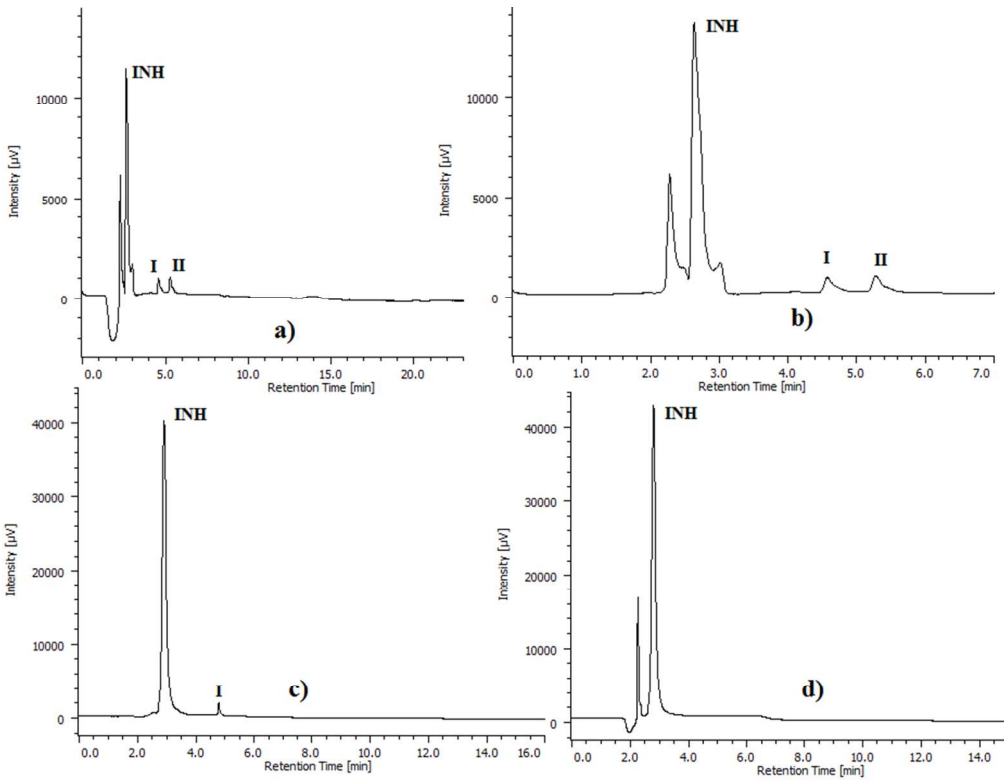
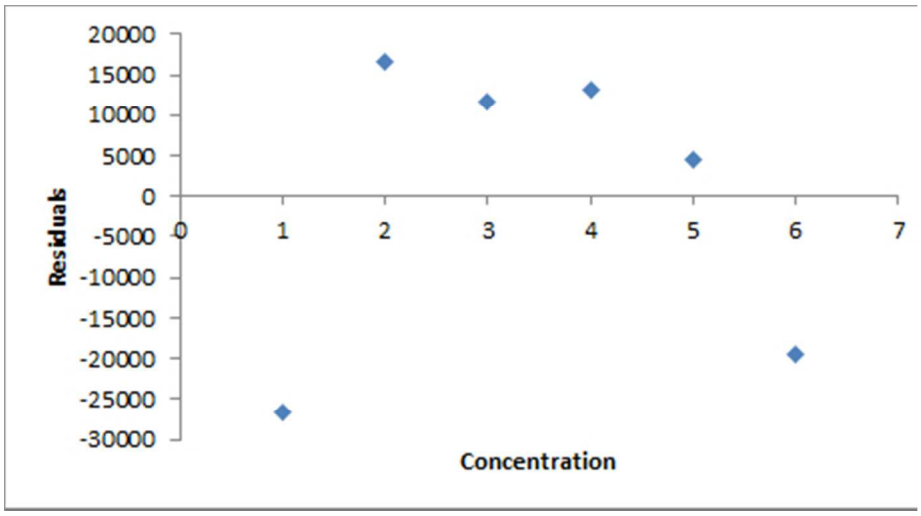


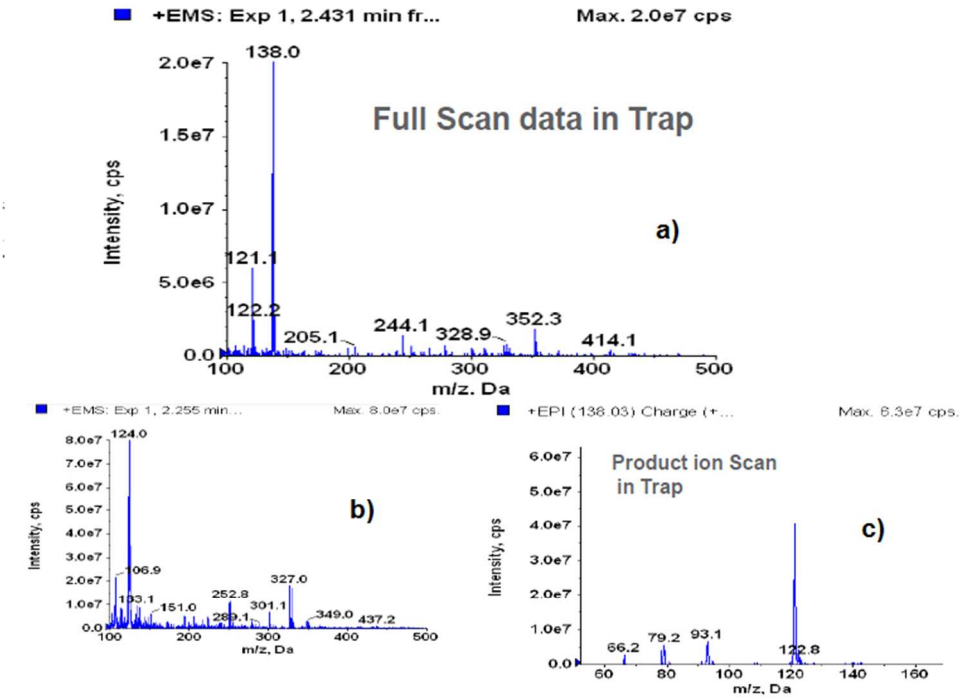
Chromatogram of A) standard INH and B) related compound (I- Isonicotinic acid and II- Isonicotinamide) in proposed mobile phase
274x94mm (96 x 96 DPI)



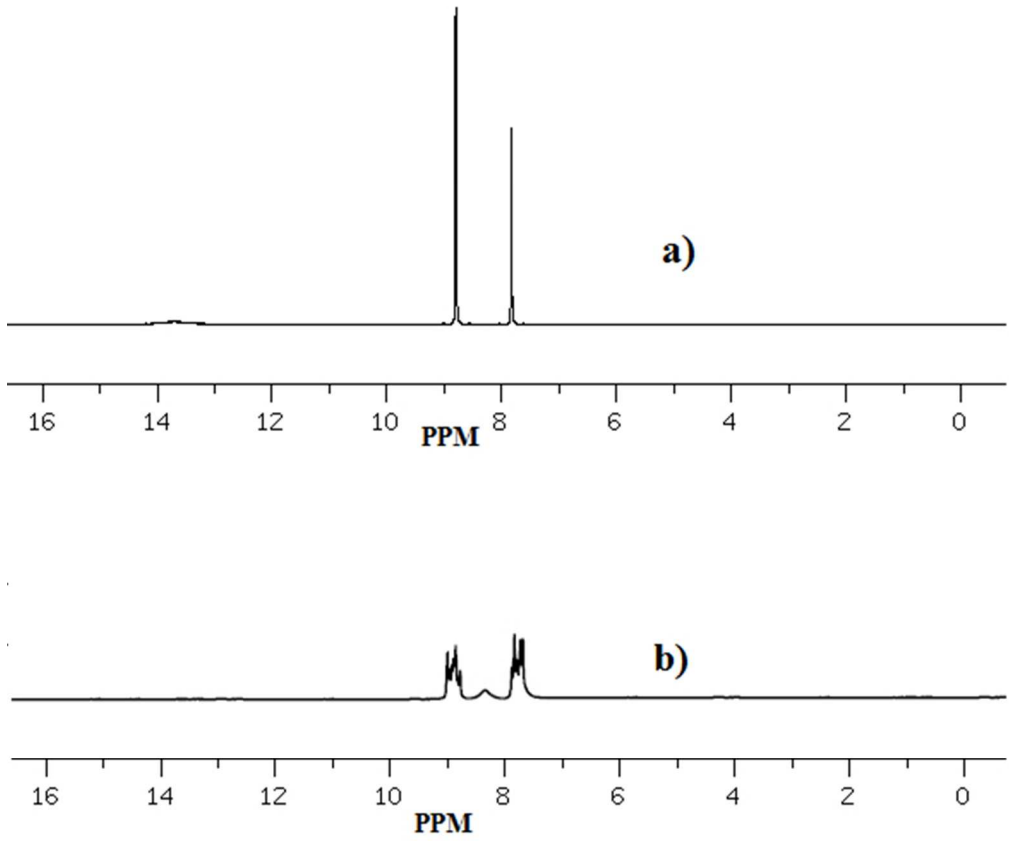
Chromatogram of INH degradation in a) acidic b) basic c) oxidation and d) photolytic condition
244x188mm (96 x 96 DPI)



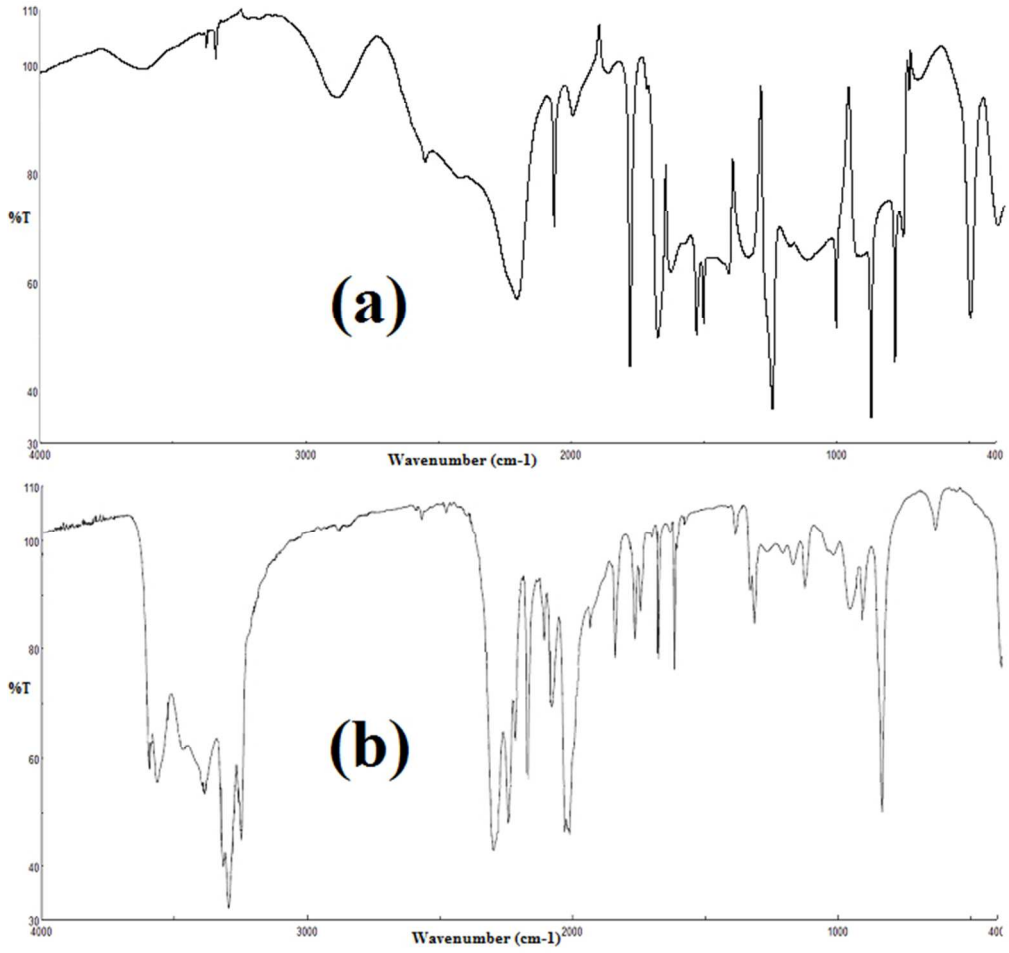
Residual plot showing the random pattern
120x66mm (96 x 96 DPI)



Mass profiles for a) isoniazid and degradation products appearing at b) RRT 1.58 (Isonicotinic acid) and c) RRT 1.82 (Isonicotinamide)
210x146mm (96 x 96 DPI)



NMR spectra degradation products appearing at a) RRT 1.58 (Isonicotinic acid) and b) RRT 1.82 (Isonicotinamide)
154x133mm (96 x 96 DPI)



FT-IR spectra of degradation products appearing at a) RRT 1.58 (Isonicotinic acid) and b) RRT 1.82 (Isonicotinamide)
187x180mm (96 x 96 DPI)