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Research Article

Comparative Study on Suitability of Columns for Efficient Recovery of Nitroimidazole Compounds in Chicken Meat Using UPLC-MS/MS Instrumentation

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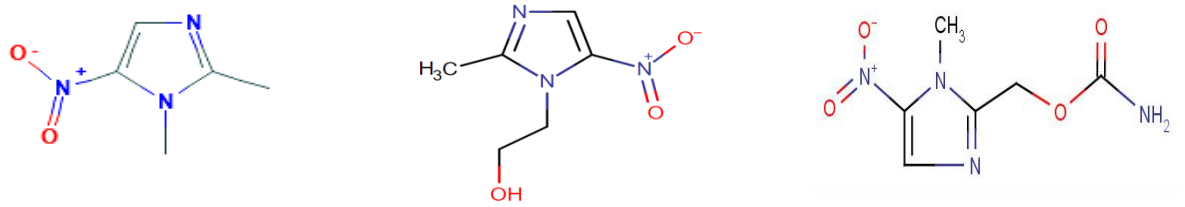
ABSTRACT
Analysis of nitroimidazole antibiotic compounds viz., dimetridazole, metronidazole and ronidazole requires a highly selective and accurate determination in UPLC-MS/MS for which suitability of three different columns (C8, C18 and C18-HILIC) were evaluated based on their retentivity, tailing factor, MS sensitivity and resolution for three different nitroimidazole compounds in chicken meat. Three nitroimidazole compounds of A6 group are individually set at 1µg/kg of chicken meat as MRL by Export Inspection Council of India for export. Residue free chicken meat was spiked with dimetridazole, metronidazole and ronidazole each at 0, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/kg along with metronidazole D4 as internal standard. TIC and MRM for the three nitroimidazole compounds using three columns were obtained. Upon comparison of chromatograms for the three different columns at the selected 0.5 µg/kg which is 50% below the MRL value of nitroimidazole compounds, C18-HILIC showed good peak response, higher MS sensitivity and minimal tailing effect than the C8 and C18 columns. However, the retentivity was found to be similar in all the columns (RT < 2.0 min). Linearity study revealed that the C18-HILIC column showed maximum correlation coefficient of 0.9964, 0.9963 and 0.9983 for dimetridazole, metronidazole and ronidazole respectively in chicken meat samples when compared to C18 (0.9331, 0.9166 and 0.9514) and C8 (0.9793, 0.9859 and 0.9656) elution. A validation study on C18-HILIC column was carried out. It is concluded that C18-HILIC column is suitable for quantitative UPLC-MS/MS estimation of nitroimidazole compounds in chicken meat.

KEYWORDS: LC-MS/MS; Nitroimidazole; C18-HILIC column; chicken meat

INTRODUCTION

Nitroimidazole antibiotics viz., dimetridazole (DMZ), metronidazole (MNZ) and ronidazole (RNZ) are used for the prevention and treatment of various diseases in animals, poultry and fish. Nitroimidazole drugs have been banned in many countries including US, Japan, China, Brazil and Europe for use in food animals due to its in genotoxic, carcinogenic and mutagenic properties [3]. For this reason, the levels of nitroimidazole residues in meat and egg are monitored closely and stringent MRLs are fixed

by Export Inspection Council of India (EIC) for export to other countries, as deliberate use of nitroimidazole compounds as growth promoters could be possible in food animals for increasing the efficiency of meat and egg production. The analysis of banned substances in food matrices generally requires accurate determination with improved instrumentation techniques and to accomplish this, chromatographic techniques like HPLC hyphenated with MS have been the unanimous choice. LC-MS/MS has been proved to be the choice of instrumentation technique and almost the method of greater sensitivity for sub-ppb concentrations in different food matrices including meat, milk, honey, egg and egg products [2]. Nitroimidazole belongs to A6 group under residue monitoring programme of EIC of India and three nitroimidazole compounds viz., dimetridazole, metronidazole and ronidazole are individually set at 1 µg/kg as MRL in chicken meat for export propose. In the LC based technique, column plays a crucial role in improving the separation. Most of the early works on estimation of traces of nitroimidazole [4, 7, 8, 10 and 12] in different food matrices had either used C8 or C18 column chemistry using LC-MS/MS technique. Nitroimidazole compounds (Fig-1), being highly polar in nature, require a column with chemistry of highly polar stationary phase. Our expectation that a C18 HILIC might suit the purpose needs to be validated if found better in recovery of nitroimidazole compounds by comparing certain accepted parameters. Hence, evaluation of three different commonly used columns viz., C8, C18 and C18-HILIC columns in LC-MS/MS for estimation of nitroimidazole compounds in chicken meat is taken up in this work.



Dimetridazole **Metronidazole** **Ronidazole**

Fig. 1: Structures of the nitroimidazole compounds selected for the study

MATERIALS AND METHODS

Chemicals, Reagents and Consumables

MS grade acetonitrile, ammonium formate and formic acid were obtained from *Sigma Aldrich*, India. HPLC grade dihydrogen phosphate, EDTA and citric acid were obtained from Merck, India. Certified reference materials of dimetridazole, metronidazole, ronidazole and metronidazole D_4 were procured from *Clearsynth*, India. Type-I water used in the experiment was purified from an ultrapure water purification system (*Millipore*, Germany). Strata-X cartridges (*Phenomenex*, India) were used for extraction of chicken meat samples.

Solution

Standard solutions of dimetridazole, metronidazole and ronidazole are prepared at a concentration of 1 mg/ml in methanol and stored in refrigerated conditions for preparation of working standard on daily basis. *Mcilvaine* buffer (8 ml of 0.2 M dihydrogen phosphate and 12 ml of 0.1 M citric acid) and 0.1M EDTA were freshly prepared at the time of study.

UPLC- quadruple MS instrument

A Waters Acquity UPLC-H class System (Waters, India) interfaced with a triple quadruple mass spectrometer (*Xevo-TQD*) equipped with both electron spray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources was used for this study. The instrument was operated with *masslynx version 4.1* software program to control the operations of LC and MS. Nitrogen gas was used in the ionization spray chamber and argon gas was used in the collision chamber.

Columns

One each of C8, C18 and C18-HILIC columns is chosen in such a way that the dimensions in terms of length, diameter and particle size were kept identical (Table 1). The columns differed in their chemistry as aim of this work was to evaluate the efficiency of the chemistry in

separating the nitroimidazole compounds in chicken meat.

Table 1: Dimensions and chemistry of types of columns attempted for evaluation in the study

Sl. no	Column type	Dimensions, particle size	pH stability of columns
1	C 8 (Brand A)	100 mm X 4.6 mm, 5µ	1.5 - 9.5
2	C 18 (Brand B)	100 mm X 4.6 mm, 5µ	2 - 8
3	C 18-HILIC (Brand C)	100 mm X 4.6 mm, 5µ	2 - 8

Sample preparation

Two grams of chicken meat were weighed (*Mettler Toledo*, 0.01 mg accuracy) into 50 ml centrifuge tube and 10 ml each of *Mcilvaine* buffer and EDTA was added, vortexed for 2 minutes followed by centrifugation at 4ºC for 20 minutes at 7000 rpm. Supernatant was transferred into 15 ml centrifuge tube. The sample was then subjected to Strata-X Cartridge (3 ml, 60 mg & 30µ) solid phase extract clean up. Cartridges were previously conditioned with 3 ml methanol followed by 2 ml water, repeated 3 times. Sample was passed through the cartridge drop wise (~ 0.3 ml/min) in a manifold using 100% methanol in to injection vial for LC-MS/MS evaluation. Injection volume was 20 µl for standard and sample.

Optimized UPLC conditions

For C8 column, water (A) and acetonitrile (B) mobile phase were used at a flow rate of 0.4 ml/min with a gradient elution as shown below Table-2 [12]. The column was kept at ambient temperature.

Table 2: Gradient followed for column C8

Sl.no	Time (min)	% A	% B
1	0.00	100	0
2	0.30	100	0
3	3.50	0	100 (linear gradient)
4	4.00	100	0

For C18 column, an isocratic solution of 0.1 % formic acid in water (10%) and methanol (90%) at a flow rate of 0.7 ml/min was used with a total run time of 3.50 minutes. The column temperature was maintained at 35º C [10].

For C18-HILIC column, an isocratic solution of 0.1% formic acid in 5 mM ammonium formate (5%) and acetonitrile (95%) at a flow rate 0.7ml/min was followed with a total run time of 3.5 minutes. The column temperature was kept at 40º C [5 and 6].

MS/MS Condition

The three nitroimidazole compounds were detected by Multiple Reaction Monitoring (MRM) mode in the MS/MS analyzer (*IntelliStart XEVO TQD*, Waters, USA). Earlier, the CRM compounds were diluted to 1000 µg/ml with MS grade methanol, further diluted to 500 ng/ml with water and methanol (50:50) and this concentration was used for optimizing the MS using system optimization software facility *(IntelliStart)* in combined UPLC mode with a flow of 0.2 ml/min of 0.1% formic acid in methanol. A comparison of *ESI* and *APCI* modes of ionization was carried out under *IntelliStart* support.

Linearity

Six-point matrix-matched calibration curves for the three analytes were prepared for quantification. Calibration samples were made by spiking prepared blank chicken samples, which were confirmed to be free from any nitroimidazole compounds of our interest. Calibration samples were fortified to end up with final concentrations of 0.0, 0.5, 1.0, 2.0, 5.0, and 10.0µg/ml for each compound. The extraction was done as per sample preparation step mentioned earlier and chromatograms were obtained for each compound using C8, C18 and C18-HILIC columns separately. The linearity study was conducted to compare the chromatogram developed by three different columns on nitroimidazole compounds in terms retentivity (time), tailing factor (deviation from Gaussian chromatogram curve), peak response (by area) and MS sensitivity (S/N).

Method Validation

The column that showed better retentivity, optimal tailing factor and good peak response was selected for further validation through standard validating parameters viz., system suitability, specificity, linearity, repeatability, reproducibility, intermediate precision, recovery, limit of quantification and limit of detection.

RESULTS AND DISCUSSION

Optimization of MS for separation of nitroimidazole compounds

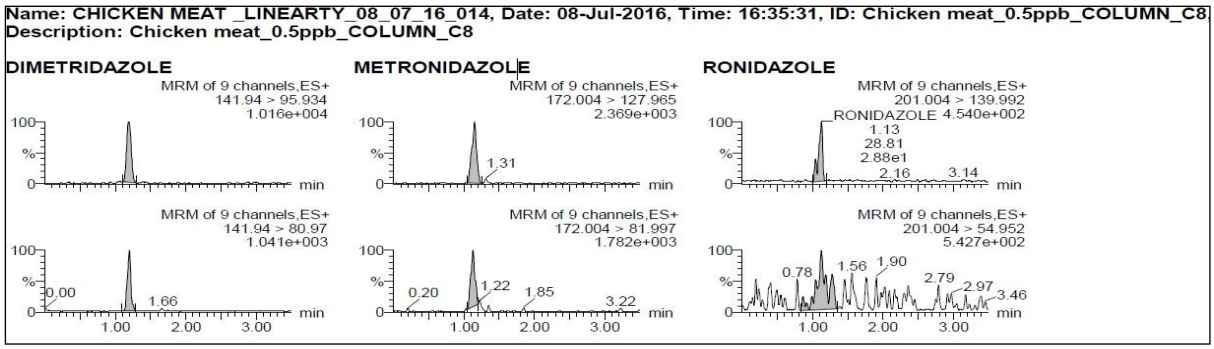
The ion transitions (precursor ions >> product ions) were obtained directly by infusing the three nitroimidazole compounds by *IntelliStart* software into MS by passing LC using flow through needle of mass spectrometer. *ESI* $^{+ve}$ mode was found to be showing better response than the other three modes *(ESI* $^{-ve}$*, APCI* $^{+ve}$ *and APCI* $^{-ve}$*).* The optimized MS parameters under *ESI*$^{+ve}$ for the 3 nitroimidazole compounds were 3.9 V of capillary voltage, 16-28 V of cone voltage, 350°C desolvation temperature, 650L/h of desolvation gas and 12 to 24 V of collision energy. The results of the optimized MS conditions are given in (Table -3).

Table 3: Multiple Reaction Monitor conditions

Sl.no	Name	Precursor ions	Product ions	Dwell (s)	Cone(V)	Collision (V)
1	Dimetridazole	141.94	95.93,80.97	0.025	28	22,15
2	Metronidazole	172.00	127.96,81.99	0.025	22	24,18
3	Ronidazole	201.00	139.99,66.91	0.025	16	16,12

Chromatograms of the columns

Chromatogram obtained for three different columns in the present study for the three nitroimidazole compounds in chicken meat (as a result of 6 point linearity study) showed that all three columns differed in their efficiency of development of chromatogram in terms of sensitivity, tailing factor, peak response and retentivity. The lowest spiking concentration (0.5µg/kg) was chosen for comparison and the chromatograms are depicted in the Fig-2, for all the nitroimidazole compounds. While C8 column incorporates octyl silane as stationary phase, C18 possesses naked silica as packing material and a C18 material as stationary phase. C18-HILIC column incorporates zwitterionic stationary phase without any ion-paring or derivative material to the silica packing material.



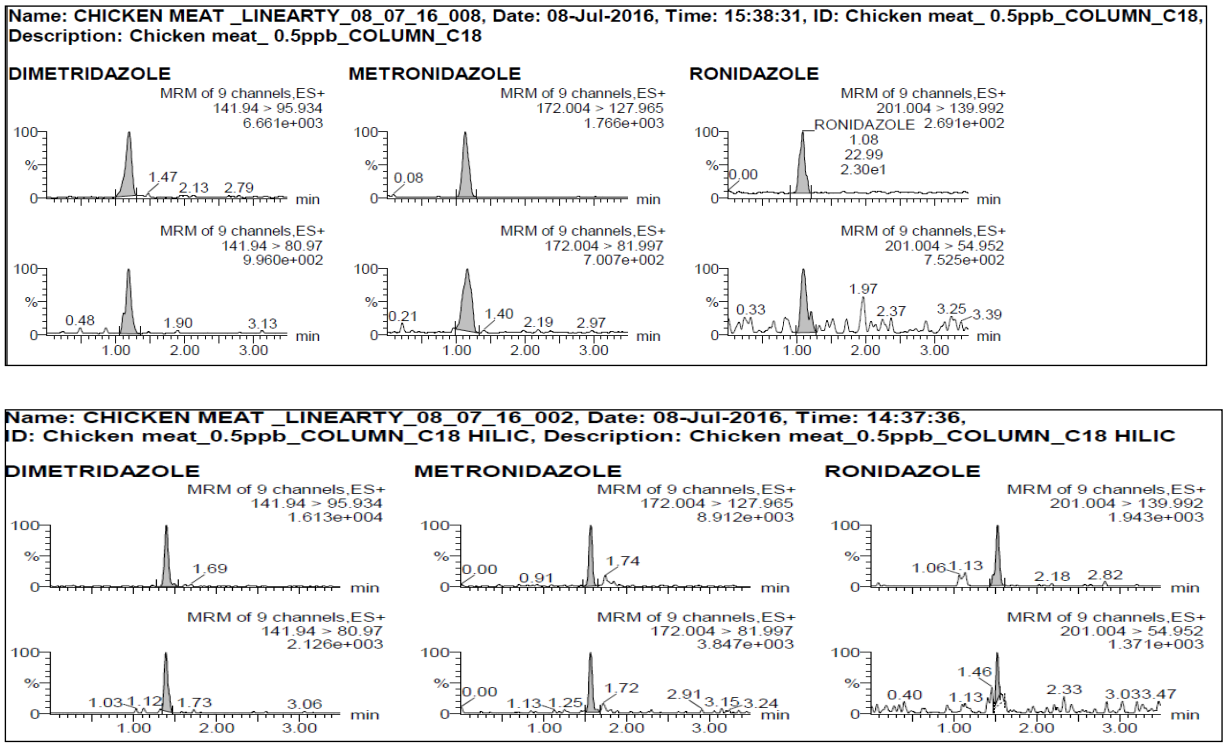


Fig. 2: Chromatograms developed for three Nitroimidazole compounds using three different columns

Though all three types of columns, C8, C18 and C18-HILIC are good in one or other aspects, the effect of their elution on three nitroimidazole compounds is to be seen as point of easy and simple separation efficiency. All the columns used were of similar length, same particle size and diameter (Table-1).

All the columns were claimed to be stable from strong acidic pH to weak acidic pH (as per manufacture specification), but differed in their basic chemistry and from time to time, were studied for their efficiency. It is of great interest that has arisen to develop a suitable application in respect of investigation and examination of chemistry of these three types of columns on the chromatographic behavior of highly polar nitroimidazole compounds.

Retentivity

The retention of nitroimidazole compounds in the columns under evaluation is shown in Table-4. While both C8 and C18 columns had almost similar retention time for all three nitroimidazole compounds, C18-HILIC column showed a longer retention time. However, the order of peaks remained the same for three compounds as Ronidazole > Metronidazole > Dimetridazole. All three columns generally showed retention time of 1-2 minutes. The results clearly showed that dimetridazole was the most polar compound amongst the three nitroimidazole compounds followed by metronidazole and the least polar was ronidazole, Use of isocratic mobile phase normally results in relatively longer analysis time. However, in this experiment, use of isocratic mobile phase had not stretched the retention time in C18 columns but found to stretch a little longer with HILIC column (Table-4).

Table 4: Nitroimidazole retention time (RT) in minutes

Name of the Compounds	Column type		
	C8	C18	C18-HILIC
Dimetridazole	1.19	1.19	1.40
Metronidazole	1.15	1.13	1.57
Ronidazole	1.12	1.08	1.53

Tailing Factor

The tailing factor was measured using the equation $TF = (a+b)/2a$ (USP) where, a is the distance from the leading edge of the peak of the midpoint and b is the distance from the peak midpoint to the tailing edge, both measured at 5% of the peak height. A tailing factor value of 1.0 is considered a normal peak closer to a perfect Gaussian type. C18-HILIC column showed very good tailing factor value for the three nitroimidazole compounds compared to C8 and C18 columns (Fig-3). C8 showed a tailing factor of 1.39 for dimetridazole while C18 showed a value of 0.58, both were unacceptable. C18-HILIC column showed a better tailing factor of 1.22 for dimetridazole. For metronidazole, again C18-HILIC showed a value of 0.99 which is almost a perfect 1.0, while the other two columns were either way behind (0.89) or way ahead (1.22). For ronidazole, both C8 and C18 showed poor tailing factor of 0.61 and 0.66 respectively, while C18-HILIC was better with 0.87.

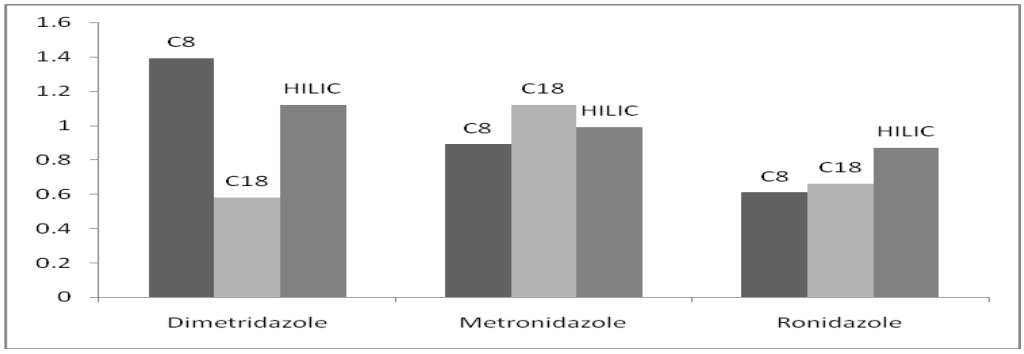


Fig. 3: Tailing factor of the three different columns for nitroimidazole compounds

Peak Response

Peak response was measured by area shown in the chromatograms developed by the columns evaluated for the nitroimidazole compounds. C18-HILIC column showed higher area for all the three nitroimidazole compounds than C8 and C18 (Fig-4).

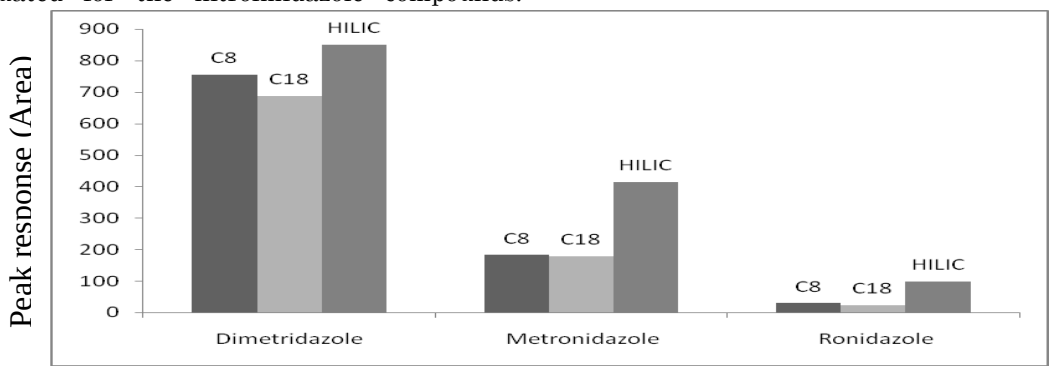


Fig. 4: Peak response of three different columns for three nitroimidazole compounds

MS Sensitivity

Amongst the three columns, for dimetridazole and ronidazole compounds, C18-HILIC afforded the best MS sensitivity (S/N ratio) (Fig-5). While C8 column showed a very good MS sensitivity of 209.56 for metronidazole, C18 and C18-HILIC showed more or less similar MS sensitivity. However, for ronidazole C18-HILIC stayed ahead with a good MS sensitivity of 98.64 while C8 showed a closely better MS sensitivity of

92.39. Though C8 column affored an excellent sensitivity for metronidazole compound, a comparatively poor tailing factor (Fig-3) and poor peak response (Fig-4) reduced its dependance considerably for metronidazole compound in chicken meat samples.

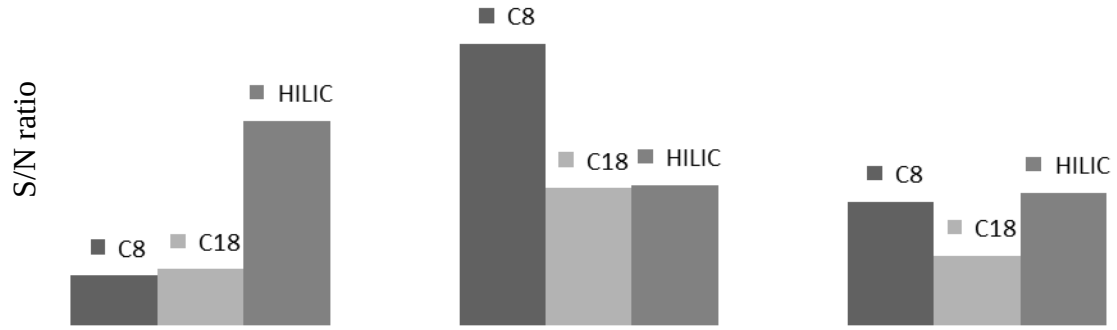


Fig. 5. MS sensitivity of three different columns for three nitroimidazole compounds

Method validation

Based on retentivity, tailing factor, peak response and MS sensitivity values for the three columns attempted, C18-HILIC column afforded a better picture for nitroimidazole compounds analysis in chicken meat. Hence, C18-HILIC was chosen for further validation [1], as usually required [9, 10 and 11]. Validation parameters viz., system suitability, linearity, repeatability, reproducibility, intermediate precision, limit of quantification and limit of detection were performed compared with the recommended acceptance criteria [13].

System suitability (RSD %)

A 2 ppb concentration of the three nitroimidazole compounds were prepared and run through the method developed, for 6 times, concurrently and RSD was worked out. The RSD was found to be within the acceptance criterion of 10% for all three compounds (Table-5). Thus, all the materials and electronic instruments constituted as an integral system for this method passed satisfactorily.

Table 5: System suitability for 2 ppb concentration calculated (RSD%)

S.NO	MNZ	DMZ	RNZ
1	1.938	1.913	2.221
2	1.852	1.858	1.959
3	1.878	1.816	1.961
4	1.880	1.833	1.963
5	1.828	1.832	1.982
6	1.837	1.797	1.968
Avg	1.87	1.84	2.01
SD	0.04	0.04	0.10
RSD	**2.13**	**2.20**	**5.19**

Correlation Coefficient (Linearity r^2)

The r^2 value for a 6 matrix matched calibration for the three nitroimidazole compounds (0, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/g) showed > 0.950 (Table – 6) which is an acceptable criterion (Table 6).

Table 6: Matrix-matched calibration linearity for chicken meat

ppb	MNZ	DMZ	RNZ
0.5	0.500	0.530	0.536
1.0	0.900	0.886	0.844
2.0	2.000	1.921	1.984
5.0	5.100	5.187	5.295
10.0	10.400	10.555	10.323
r^2 value	**0.993**	**0.990**	**0.985**

Recovery (%)

Recovery of analyte of interest was carried out by choosing the middle level concentration (2.0 ppb) attempted for 6 times, expressed in percentage, which showed values within accepted range (Table 7).

Table 7: Recovery of Nitroimidazole compounds in chicken meat samples

Sl.No.	Area					
	MNZ(AQS)	MNZ (SPIKED)	DMZ (AQS)	DMZ (SPIKED)	RNZ (AQS)	RNZ (SPIKED)
1	4690	3691	8178	6089	1567	1144
2	4591	2958	8668	4776	1620	1004
3	4402	4077	8685	6429	1633	1308
4	4244	4001	8692	6262	1504	1134
5	4400	3573	8412	5725	1515	1023
6	4428	3847	8420	6395	1622	1242
Mean	4459.2	3691.2	8509.2	5946.0	1576.8	1142.5
Recovery %	**82.78**		**69.88**		**72.46**	

Repeatability (accuracy)

50, 100 and 150 % of MRL value of 1.0 ppb (of EIC, India) for all three nitroimidazole compounds were run repeatedly for two times within-day (accuracy, Table-8) and the repeat values for all the three compounds were found to be within the accepted values.

Table 8: Repeatability values of spiked chicken meat samples (RSD%)

MRL	50%			100%			150%		
Sl.no	**MNZ**	**DMZ**	**RNZ**	**MNZ**	**DMZ**	**RNZ**	**MNZ**	**DMZ**	**RNZ**
1	0.449	0.467	0.397	0.851	0.897	0.818	1.254	1.342	1.140
2	0.428	0.488	0.467	0.825	0.883	0.768	1.241	1.343	1.131
3	0.434	0.475	0.406	0.850	0.893	0.785	1.235	1.351	1.146
4	0.470	0.455	0.447	0.809	0.891	0.752	1.219	1.391	1.235
5	0.430	0.465	0.354	0.865	0.844	0.759	1.232	1.382	1.194
6	0.449	0.468	0.405	0.846	0.923	0.869	1.221	1.341	1.128
Avg	0.443	0.470	0.413	0.84	0.89	0.79	1.23	1.36	1.16
SD	0.02	0.01	0.04	0.02	0.03	0.04	0.01	0.02	0.04
RSD	**3.60**	**2.35**	**9.65**	**2.41**	**2.89**	**5.62**	**1.06**	**1.64**	**3.69**

Reproducibility (precision)

MRL value of 1.0 ppb (of EIC, India) for all three nitroimidazole compounds was run between days for two times (precision, Table-9) and the repeat values for all the three compounds were found to be within the accepted values.

Table 9: Reproducibility values of spiked chicken meat samples (RSD%)

Sl.No.	Day -1			Day-2		
	MNZ	**DMZ**	**RNZ**	**MNZ**	**DMZ**	**RNZ**
1	0.851	0.897	0.818	1.035	1.086	1.181
2	0.825	0.883	0.768	0.814	0.849	1.055
3	0.85	0.893	0.785	1.151	1.147	1.329
4	0.809	0.891	0.752	1.128	1.117	1.172
5	0.865	0.844	0.759	0.999	1.020	1.073
6	0.846	0.923	0.869	1.082	1.141	1.269
AVG	0.84	0.89	0.79	1.03	1.06	1.18
SD	0.02	0.03	0.04	0.12	0.11	0.11
RSD	**2.41**	**2.89**	**5.62**	**11.79**	**10.69**	**9.07**

Limit of quantification (LOQ)

A concentration of 50 % (0.5 ppb) of MRL value of 1ppb for the 3 nitroimidazole compounds was prepared and analyzed for 3 times. The RSD % for all the 3 nitroimidazole compounds showed an acceptable estimation concentration of < 10% (Table 10)

Limit of detection (LOD)

The capability of the selected method to detect the lowest concentration was attempted amongst four diluted concentration from the LLOQ (0.1, 0.2, 0.3, 0.4 and 0.5 ppb). The LOD was 0.2, 0.1 and 0.4 ppb for dimetridazole, metronidazole and ronidazole respectively (Table 10)

Table-10 Limit of Quantification and Limit of Detection of Nitroimidazole compounds

	MNZ					**DMZ**					**RNZ**				
Conc (ppb)	**0.5**	**0.4**	**0.3**	**0.2**	**0.1**	**0.5**	**0.4**	**0.3**	**0.2**	**0.1**	**0.5**	**0.4**	**0.3**	**0.2**	**0.1**
S.NO	**LLOQ**	**LOD**	**LOD**	**LOD**	**LOD**	**LLOQ**	**LOD**	**LOD**	**LOD**	**LOD**	**LLOQ**	**LOD**	**LOD**	**LOD**	**LOD**
1	1012	718	554	438	189	2216	1526	1041	833	457	336	247	169	147	31
2	917	804	508	451	167	2114	1602	1129	790	327	280	238	202	147	28
3	897	833	568	436	182	1911	1645	1170	951	449	316	223	172	130	40
Avg	942.00	785.00	543.33	441.67	179.33	2080.33	1591.00	1113.33	858.00	411.00	310.67	236.00	181.0	141.33	33.0
SD	61.44	59.81	31.39	8.14	11.24	155.26	60.26	65.91	83.36	72.86	28.38	12.12	18.25	9.81	6.24
RSD	**6.52**	**7.62**	**5.78**	**1.84**	**6.27**	**7.46**	**3.79**	**5.92**	**9.72**	**17.73**	**9.13**	**5.14**	**10.08**	**6.94**	**18.92**
Result	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Fail	Pass	Pass	Fail	Pass	Fail

CONCLUSION

Among the three columns with different generic material make-up compared for quantification of nitroimidazole compounds, C18-HILIC column showed better retentivity, least tailing, good MS sensitivity and comparable peak response than C8 and C18 columns. Further validation of the method using the C-18 HILIC column showed all the parameters within the satisfactory values. Hence, it is concluded that for estimating nitroimidazole compounds viz., dimetridazole, metronidazole and ronidazole in chicken meat by LC-MS/MS instrumental analysis, C18-HILIC column was found to be more suitable than C8 and C18.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this research article.

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