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Method development for simultaneous estimation of acetaminophen and its related substance by liquid chromatography-mass spectrometry (LC-MS)

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Abstract
Development and Analysis of drugs involve the most stimulating work in the medicinal field in that way, in that way, there emergence of new drug in day to day. Acetaminophen is a common Over the counter drug used by worldwide as the use of this drug increased globally requirement for detecting its impurities is mandatory and its determination of related substance were evaluated by using Liquid Chromatography Mass spectrometry, this method has emerged as a capable and multipurpose tool for analysing various types of drug product and its impurities. It has the capability of detecting small molecule to high molecule with high sensitivity. The optimized method was developed for estimation of related substance of acetaminophen using a gradient program mobile phase of formic acid and acetonitrile with 0.6 ml/min flow at 210 and 254 nm wavelength with optimized mass condition. In this paper, briefly discussed about method development for determination of related substance of acetaminophen along with its active pharmaceutical ingredient in a finished marketed product.

Keywords: Liquid chromatography mass spectrometry, acetaminophen, related substance, method development, identification

1. Introduction

Pharmaceutical analysis is one of most interesting branches in the medicinal field because it involves the qualitative and quantitative analysis of compound or drug product using various analytical techniques. The analysis of drugs reveals about the strength, purity and its nature There are various types of analytical techniques like chromatographic technique, spectroscopic technique, elemental analysis, thermal analysis etc., Chromatographic techniques are the techniques in which separation of compounds from a mixture using mobile phase and stationary phase, whereas Spectroscopic techniques are used to study nature of components by using Electromagnetic radiation.

Hyphenated techniques are the analytical method in which chromatographic technique coupled with spectroscopic technique Chromatographic techniques are used to separation of compound whereas spectrometric technique is used to identification of compound [for e g. LC-MS, MS-MS, GC-MS, LC-NMR, CE-MS]. LCMS {Liquid Chromatography Mass Spectrometry}is one of the hyphenated techniques used to separation of compound, followed by identification. It also used for determination of molecular weight, purity etc.,

Acetaminophen or commonly known as paracetamol (structure as shown fig 1), its most popular OTC drug used as an antipyretic, analgesic etc... acetaminophen is a quickly and completely absorbed in systemic circulation after oral administration (bioavailability 90%). As the use of paracetamol drug increased globally requirement for detecting its impurities is globally important. Impurities are the unwanted chemicals that present in drug product, that may be classified into organic impurity, inorganic impurity and residual solvents. Related substances are the organic impurities present in the drug that may arise from the raw materials, process related by-products, degradation products. Several methods had been development for determination of its related substance by HPLC. In this article we discussed about the a simple, and a single a novel method is used for determination of impurities present in acetaminophen tablets by LCMS method.
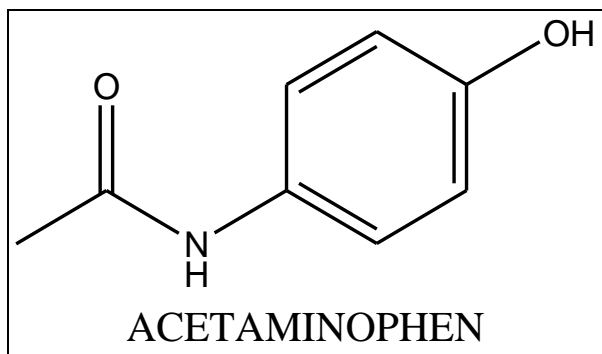
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Fig 1: Structure of Acetaminophen

Analytical method development is the process of selecting and optimizing analytical methods to measure the specific attributes of a drug substance or product. Analytical method development are critical tools for ensuring the quality, safety and efficacy of the drug product. The goal of the method development is to ensure that the methods are used to identity, purity and reliable. Analytical method development is the creation of a set of experimental condition to perform analytical procedures in chemical samples. Developed analytical methods can be used to identify, separate and used to learn more about the chemical components in drug components. The steps involved in the method development are

- Define a objectives of method development
- Collect the literature survey
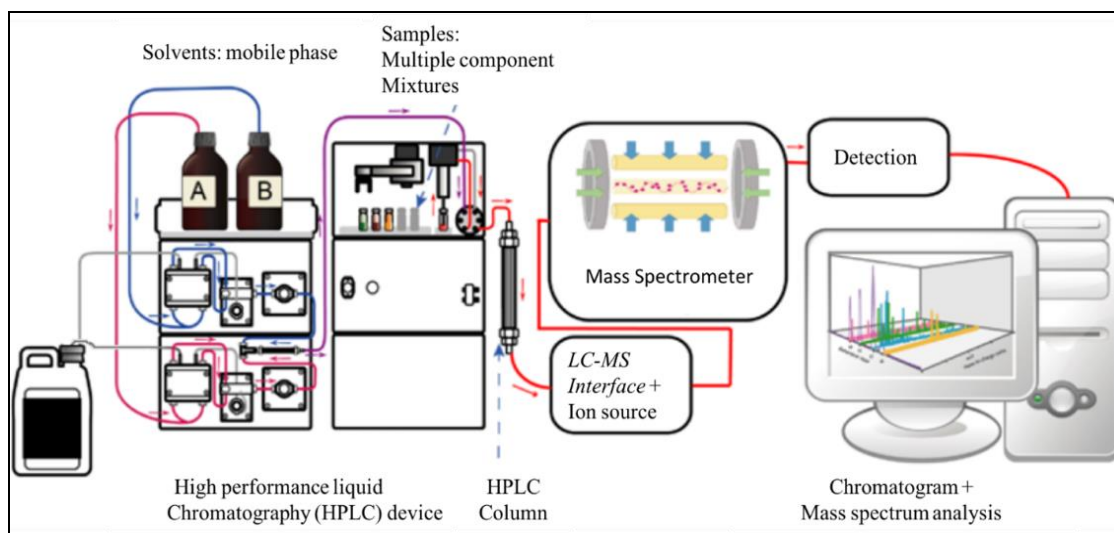- Develop the method plan.
- Optimize the method

- Validate the method
- Sample analysis

2. Experimental design
2.1 Chemical reagents
Acetaminophen tablets 500 mg tablets of (Batch no: APCI 0720 02) purchased from market, Formic acid purchased from Honeywell having MS grade, Acetonitrile purchased from MERCK having MS grade and Methanol from MERCK having MS grade and Deionised water were purified using milli Q water used for this project.

2.2 LC-MS instrumentation and condition
For a LCMS system the instrumentation comprises of:
- a LC unit,
- an interface between the LC and MS,
- an ion source that ionizes samples (e.g. API unit),
- an ion guide (an electrostatic lens that efficiently introduces the generated ions into the MS
- a mass analyser unit that separates the ions based on their mass-to-charge (m/z),
- a detector unit that detects the separated ions.

The basic components of a LC unit consist of:
- Pump − delivers the mobile phase at a required flow rate,
- Autosampler − injects the samples,
- Column − for separation of sample,
- Detector − for the analysis of the separated components in a sample.

Fig 2: Instrumentation of LC-MS unit

The liquid chromatographic techniques and mass spectrometry were achieved by coupling waters made HPLC with mass spectrometry (waters USA). LC having quaternary pump of automated sample injection with thermostat column temperature of STATIONARY PHASE Column – waters, C18, 100ˣ 4.6mm, 5μ with a dual wavelength 210 nm and 254 nm of 0.6 ml/min flow rate with gradient mobile phase programme (shown in table 1) with injection volume of 20μl. Mass spectrometry electron spray ionization technique with condition being achieved by proper selection of desolvation temperature 400 °C, source temperature 130 °C with optimum gas flow (Nitrogen) 800 L/HR., cone voltage 30V.

Table 1: Gradient programme in LC unit

Time in min	Flow rate per min	Mobile phase A	Mobile phase B
Initial	0.600	100.0	0.0
10.00	0.600	100.0	0.0
15.00	0.600	90.0	10.0
40.00	0.600	80.0	20.0
60.00	0.600	70.0	30.0
90.00	0.600	40.0	60.0
91.00	0.600	100.0	0.0
110.00	0.600	100.0	0.0

Table 2: LCMS optimized method

LC Condition		MS Condition	
Make	Waters, USA	Make	Waters, USA
Model	Acquity	Model	Xevo G2-XS Q-tof
Stationary phase	C18, 100×4.6mm, 5μ	Ionization technique	ESI
Mobile phase	Gradient elution		30V both in + and – mode
M.P. A-	100% Acetonitrile	Cone voltage	
M.P. B-	0.1% Formic acid in water		
Flow rate	0.6 ml/min	Desolvation temperature	400 °C
Temperature	Ambient temperature	Source temperature	130 °C
Wavelength	210 nm and 254 nm	Gas flow	800L/Hr

2.3 Preparation of solution
2.3.1 Preparation of mobile phases
Mobile phase A: Acetonitrile (100%)
Mobile phase B: 0.1% Formic acid: Transferred 1ml of formic acid in 1lit of water and mixed well

2.3.2 Preparation of diluent
Mixed methanol and water in equal volumes (50:50)

2.3.3 Preparation of Sample Solution
Weighed 20 tablets and crushed into fine powder. Weighed 10 mg of powdered sample and transferred into 10 ml flask (concentration having 1mg/ml) and diluted with diluent and filtered and injected.

2.4 Validation of Method
2.4.1 System suitability test
The system suitability of the method was performed by injecting blank once and six times of standard solution of acetaminophen solution and determined its % RSD

2.4.2 Linearity
The linearity of method represents the direct proportional relationship between concentration and result. It was done by injecting solution of concentration having 25%, 50%, 75%, and 100%. And result observed

2.4.3 Robustness
The robustness of the method was verified by investigating the effects caused by deliberate minor changes in experimental conditions to analyse results.
- Change in flow (+ or – 0.5 ml per min)
- Change in temperature (+ or – 5 °C)
- Change in gradient programme

2.4.4 Precision
The precision of the method validated by two condition method precision and system precision. System precision involves the injecting six times of the standard solution and determined its % RSD. Method precision involves repeatability and intermediate precision.

3. Results and Discussion
By above mentioned parameters method was developed and. Successfully determined the related substance of acetaminophen in a finished product as complies with its molecular weight as shown in table
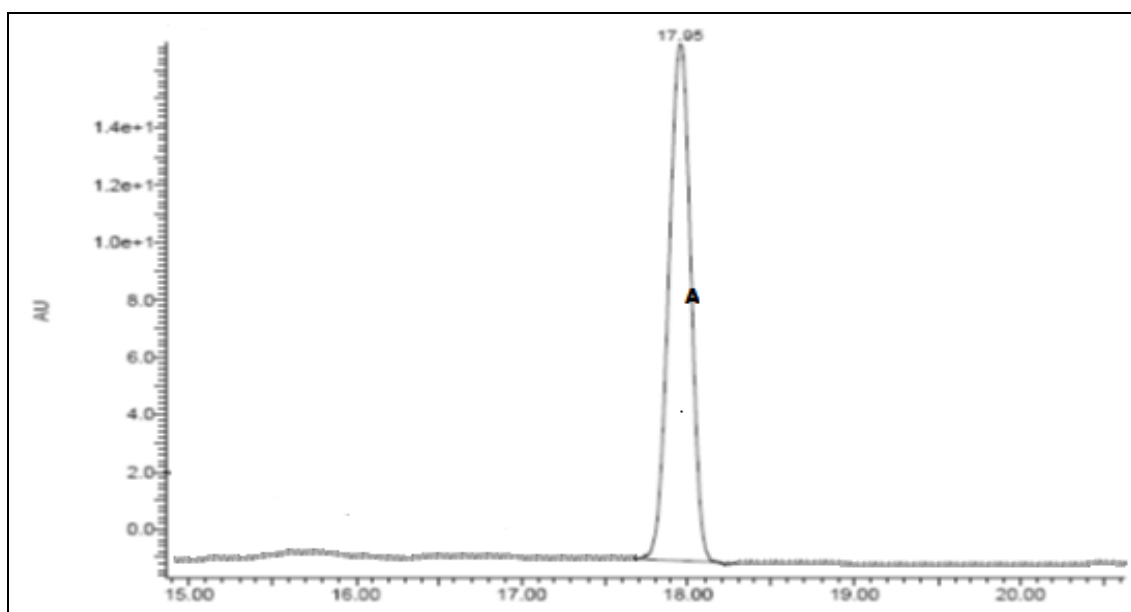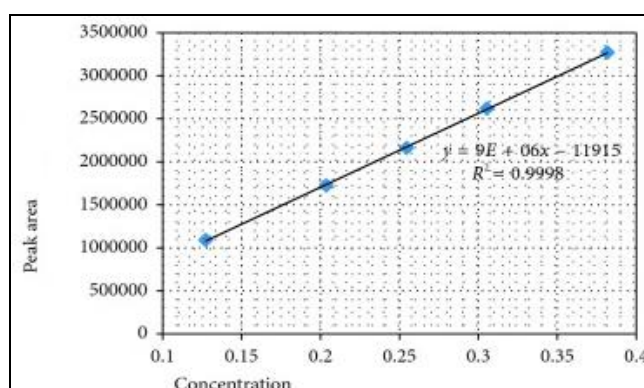
3.1 System suitability

Fig 3: Acetaminophen standard solution peak

Table 3: System suitability parameters

System Suitability	Limit	Observed
Tailing factor	NMT 2	1.1
Plate count	NLT 2000	4534

3.2 Linearity

Fig 4: Linearity cure

Analytical method linearity is defined as the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a specific range. The mean peak area obtained from the HPLC was the was plotted against corresponding concentrations to obtain the calibration graph. From the regression analysis, the linear equation was obtained: $y = 9587106x - 11915$, and the coefficient of determination R^2 was 0.999, indicating a linear relationship between the concentration of analyte and area under the peak.

3.3 Precision

The precision of the method is defined as "the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions," and it is normally expressed as the relative standard deviation. In terms of system precision, the RSD of retention time, peak area, and performance of chromatographic system, represented by the number of theoretical plates and tailing factors, were all less than 2.0%

Table 4: Method precision

	AREA
1	2753876
2	2756345
3	2755643
4	2751245
5	2751980
6	2755435
Area	2754087
SD	2092.74
RSD	0.08%

% RSD IS NOT MOTE THAN 2

3.4 Robustness

The analytical method robustness was tested by evaluating the influence of minor modifications in HPLC conditions on system suitability parameters of the proposed method

Table 5: Robustness Parameters

	Robustness Parameters		RT IN MIN
1	Change In Flow	-0.5	17.5
		0.5	16.5
2	Change In Temperature	-5	16.7
		5	16.8
3	Change In Wavelength	-1	16.8
		1	16.4

The following figures of peak observed in LC and TIC of individual impurity (zoomed) and mass spectrum of each related substance as shown below.
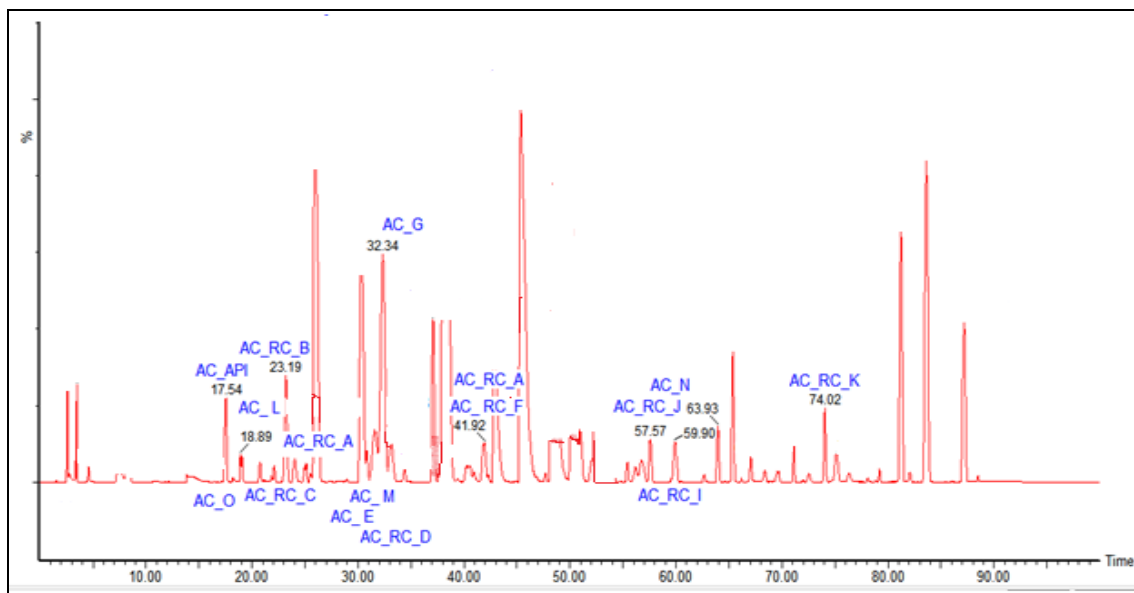
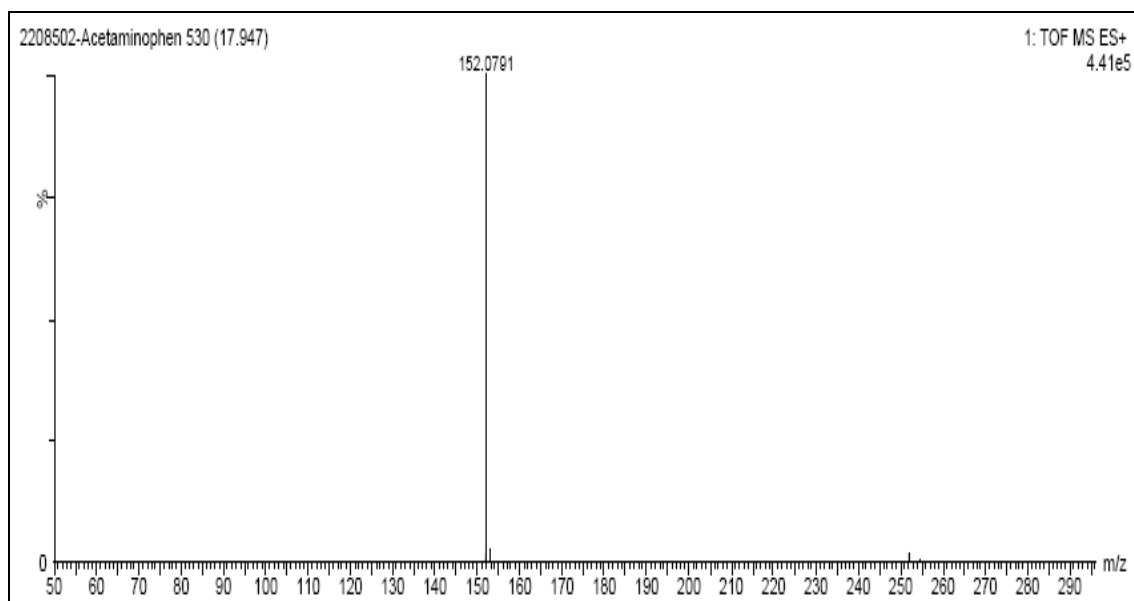**Fig 5:** TIC of all related substance of acetaminophen

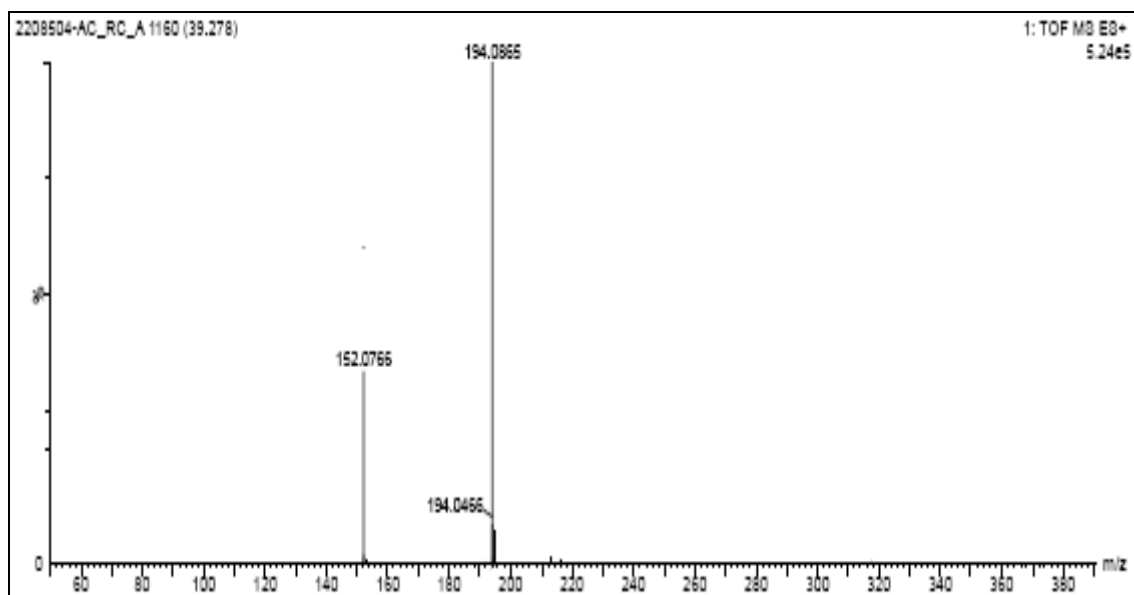**Fig 6:** Mass spectrum of Acetaminophen API

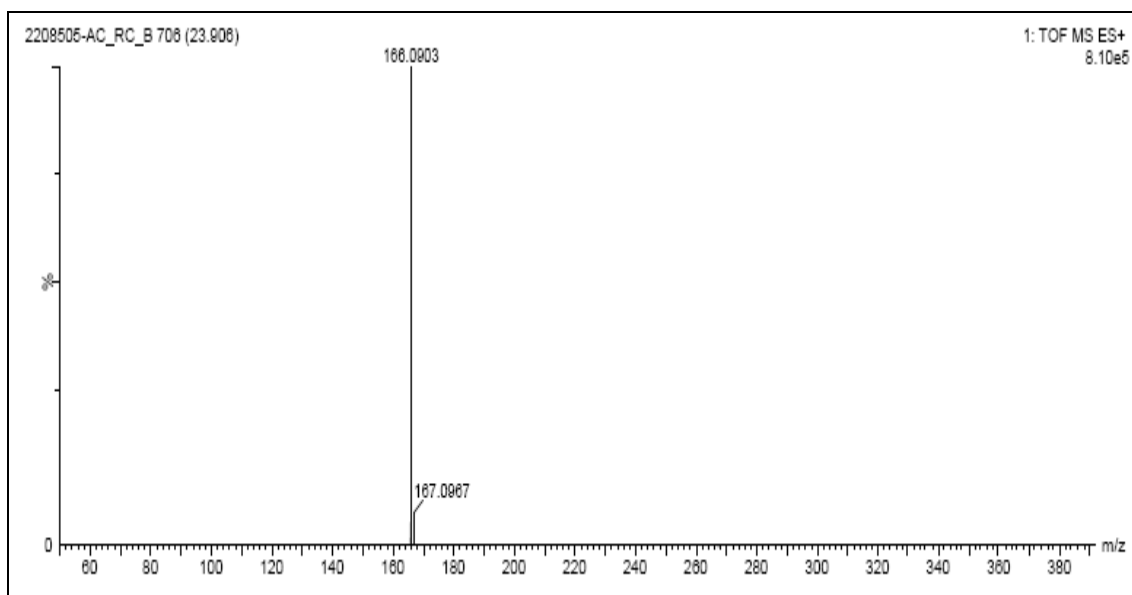**Fig 7:** Mass spectrum of Acetaminophen related compound A

Fig 8: Mass spectrum of Acetaminophen related compound B
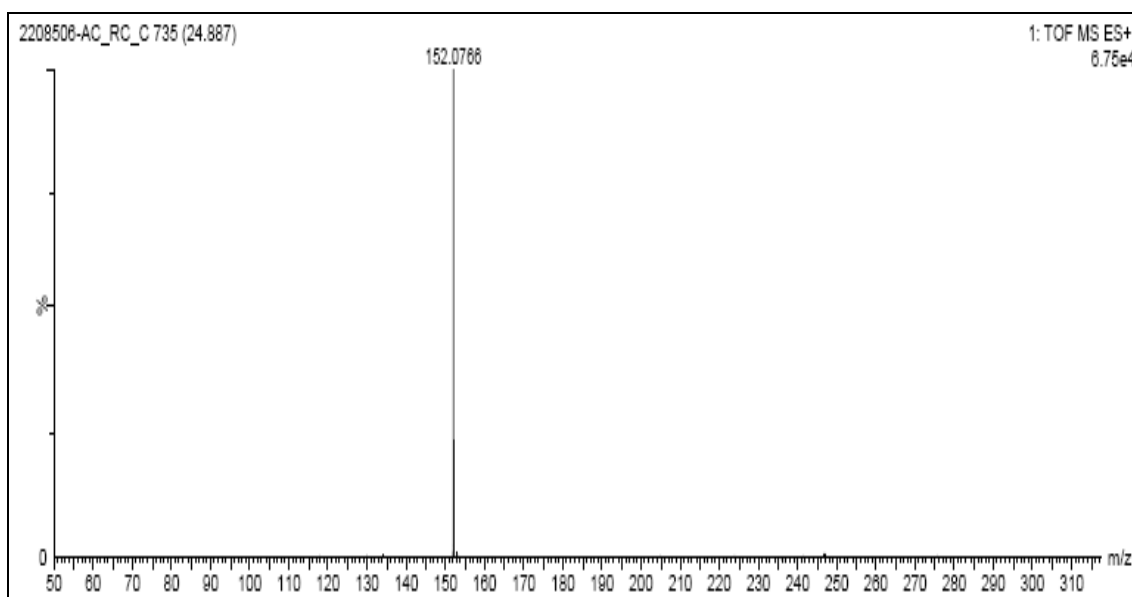

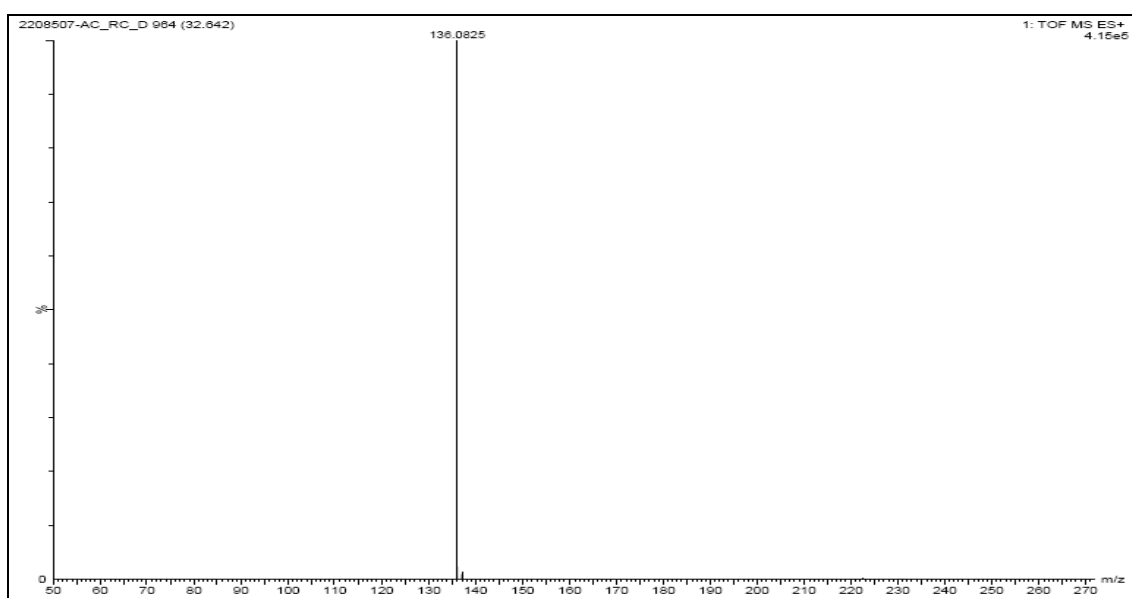**Fig 9:** Mass spectrum of Acetaminophen related compound C

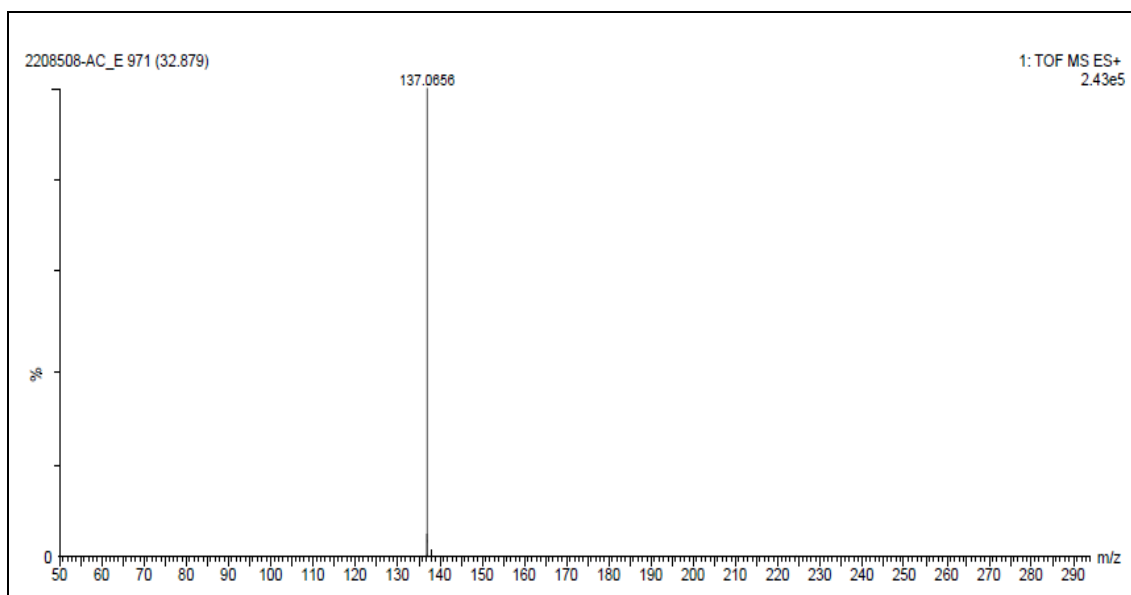**Fig 10:** Mass spectrum of Acetaminophen related compound D

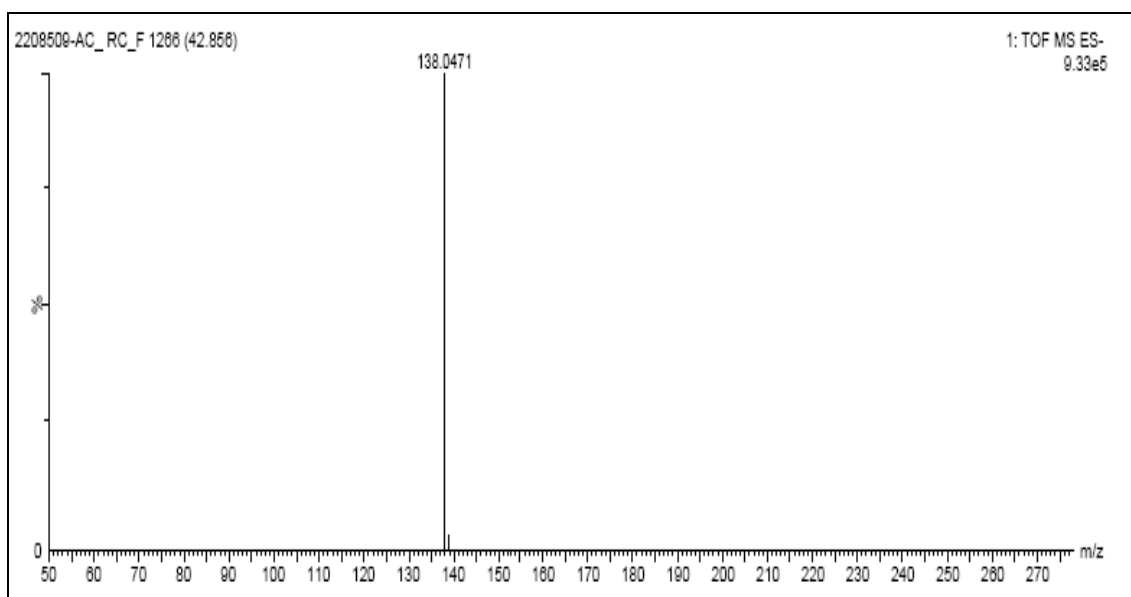**Fig 11:** Mass spectrum of Acetaminophen Impurity E
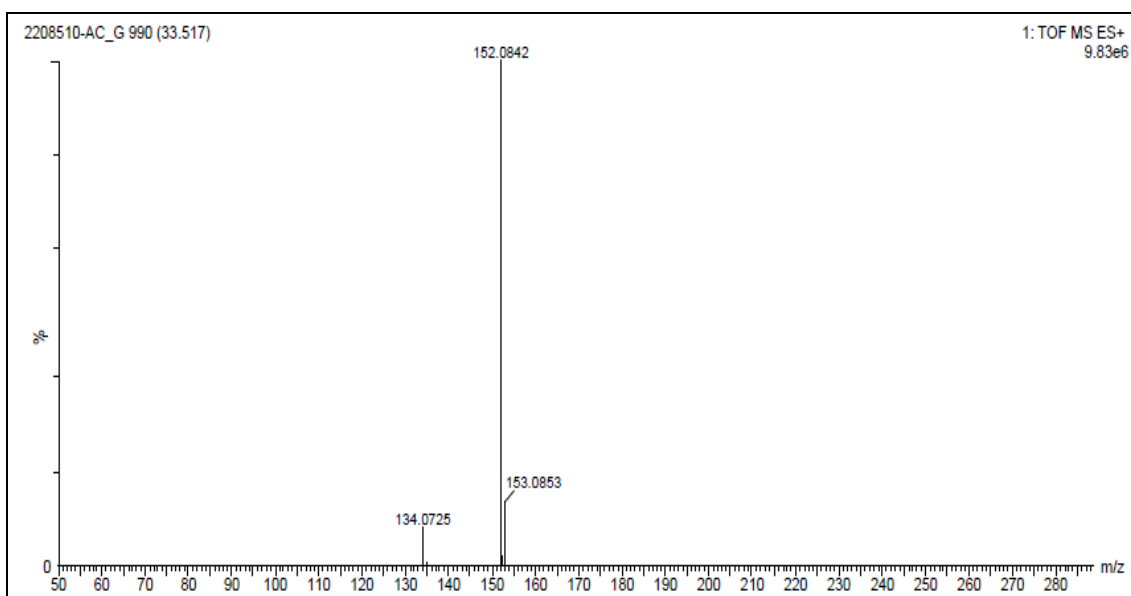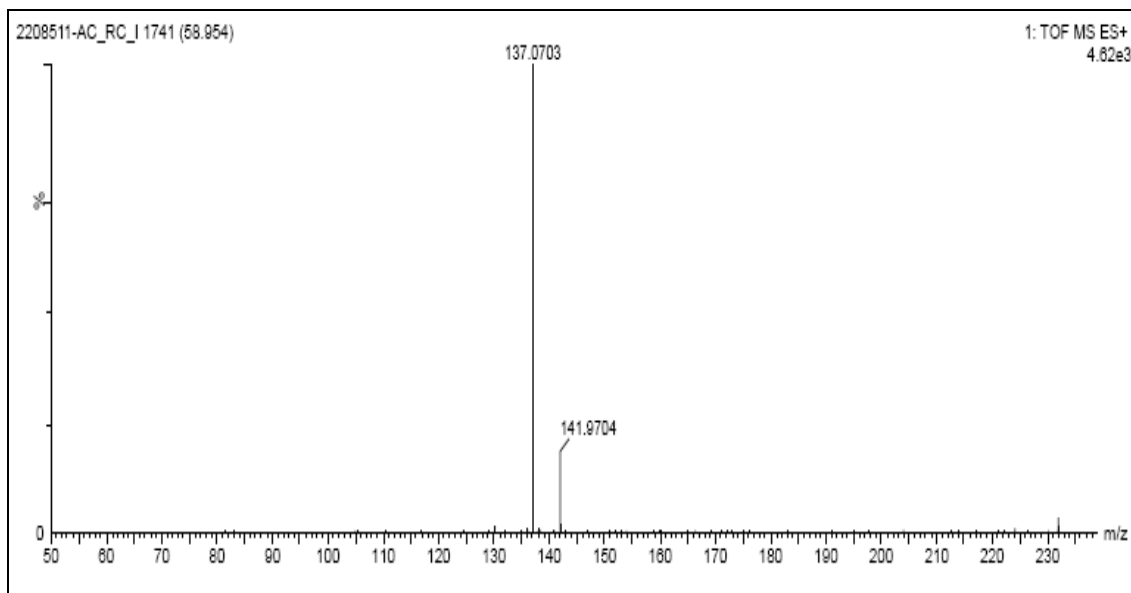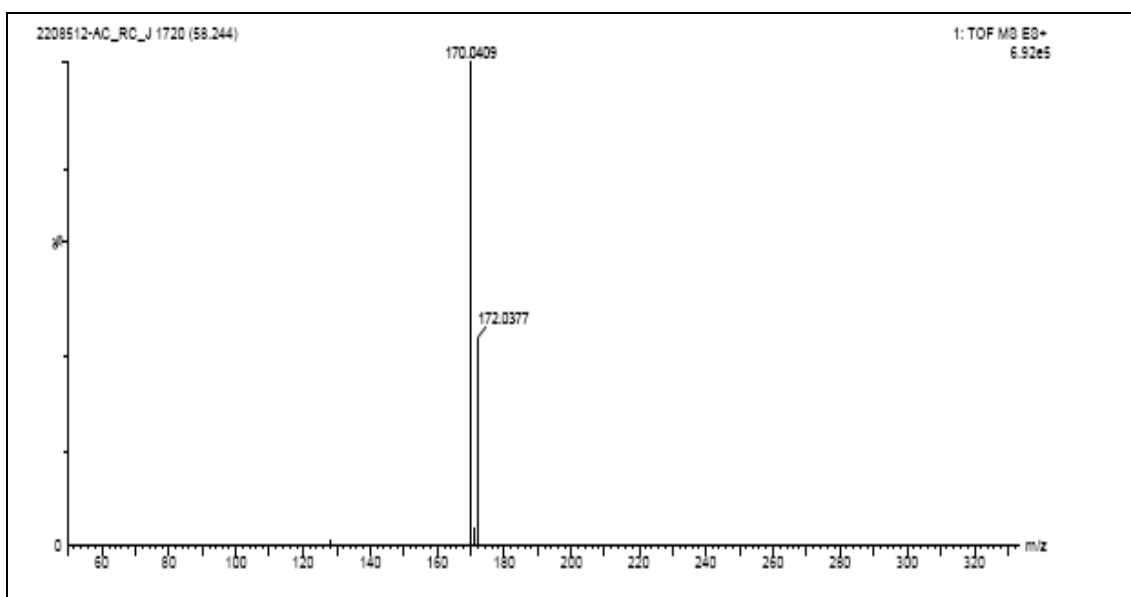

Fig 12: Mass spectrum of Acetaminophen related compound F

Fig 13: Mass spectrum of Acetaminophen Impurity G

Fig 14: Mass spectrum of Acetaminophen related compound I

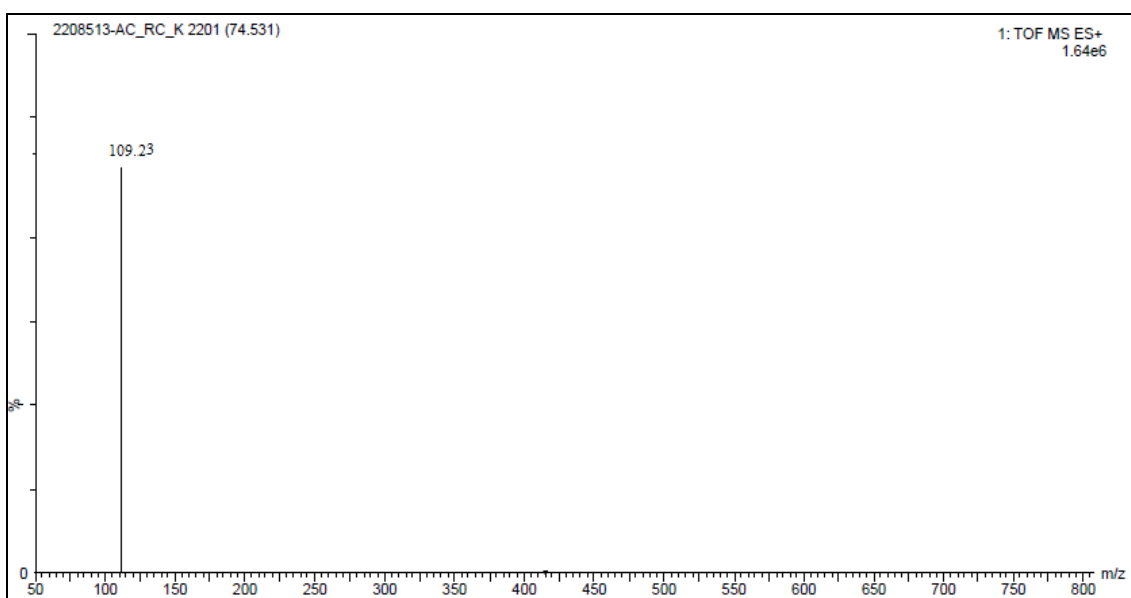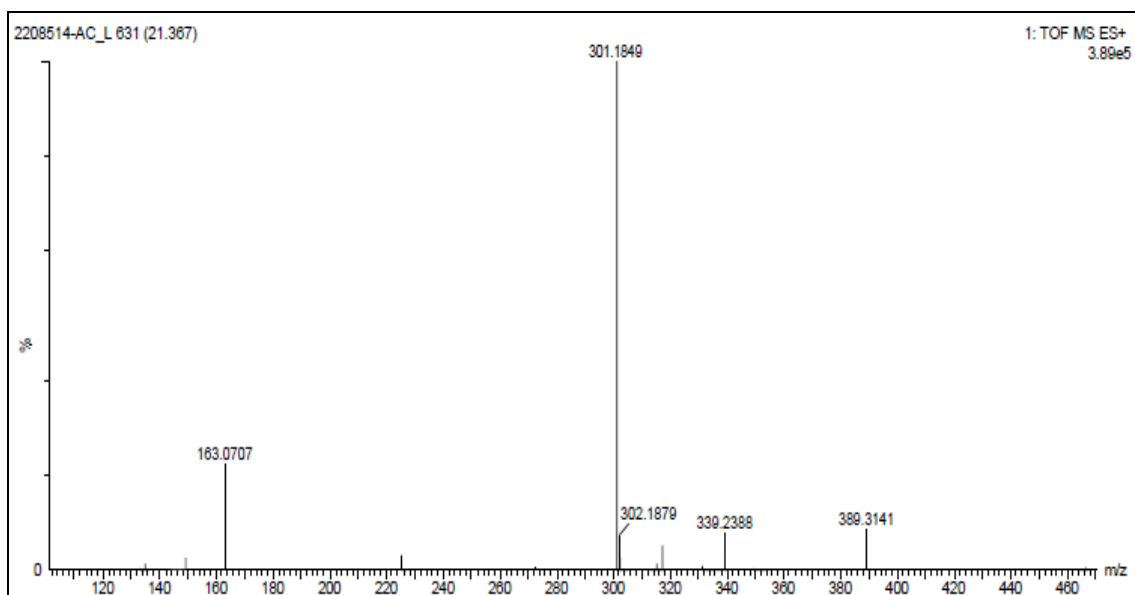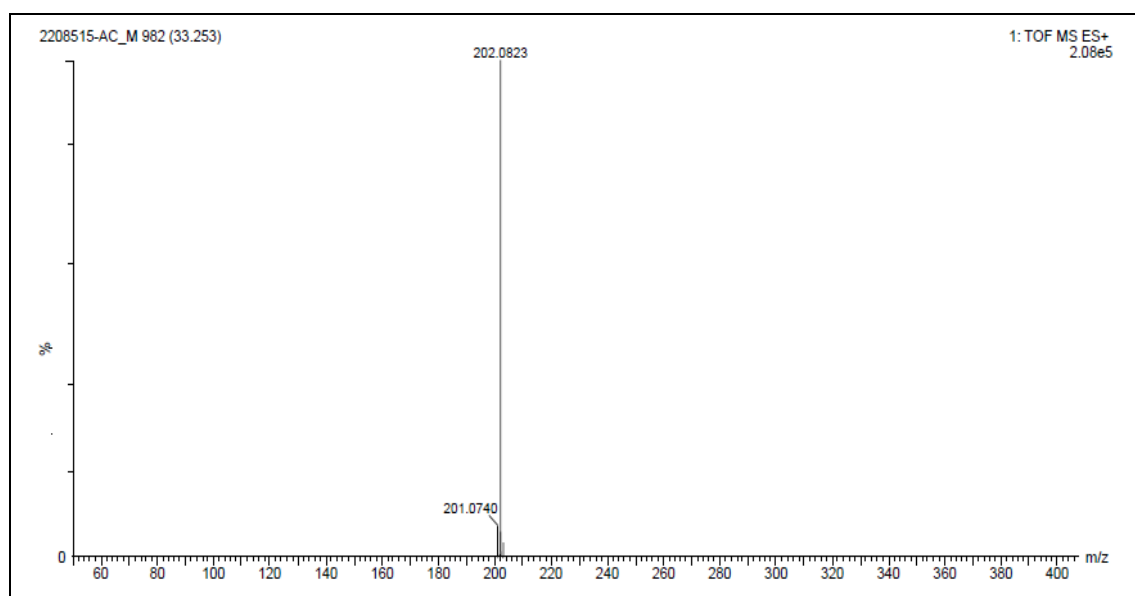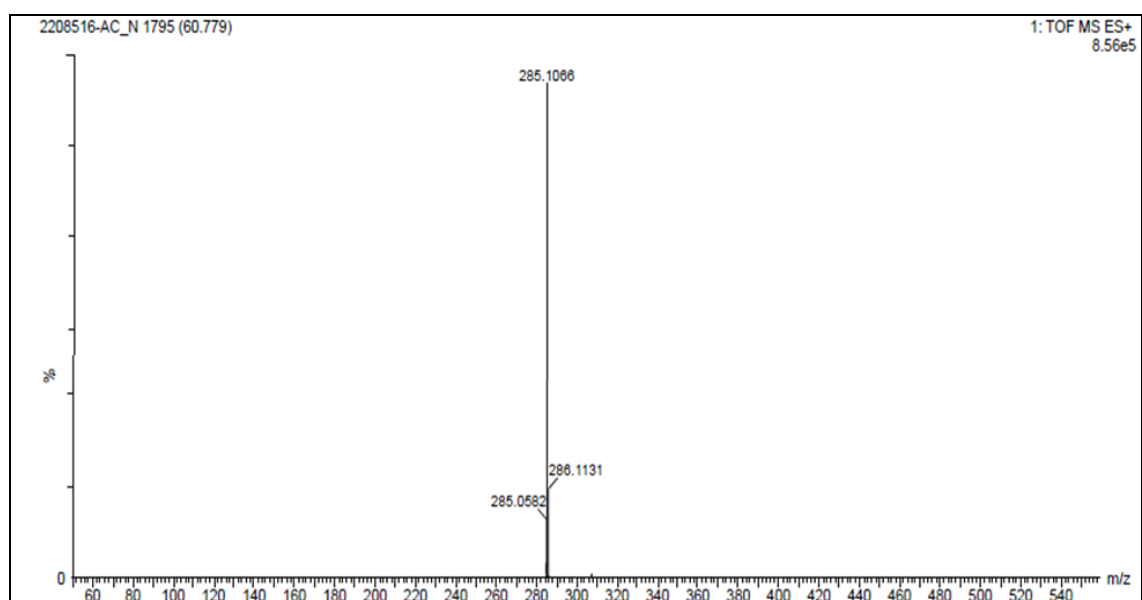**Fig 15:** Mass spectrum of Acetaminophen related compound J

Fig 16: Mass spectrum of Acetaminophen related compound K

Fig 17: Mass spectrum of Acetaminophen Impurity L

Fig 18: Mass spectrum of Acetaminophen Impurity M

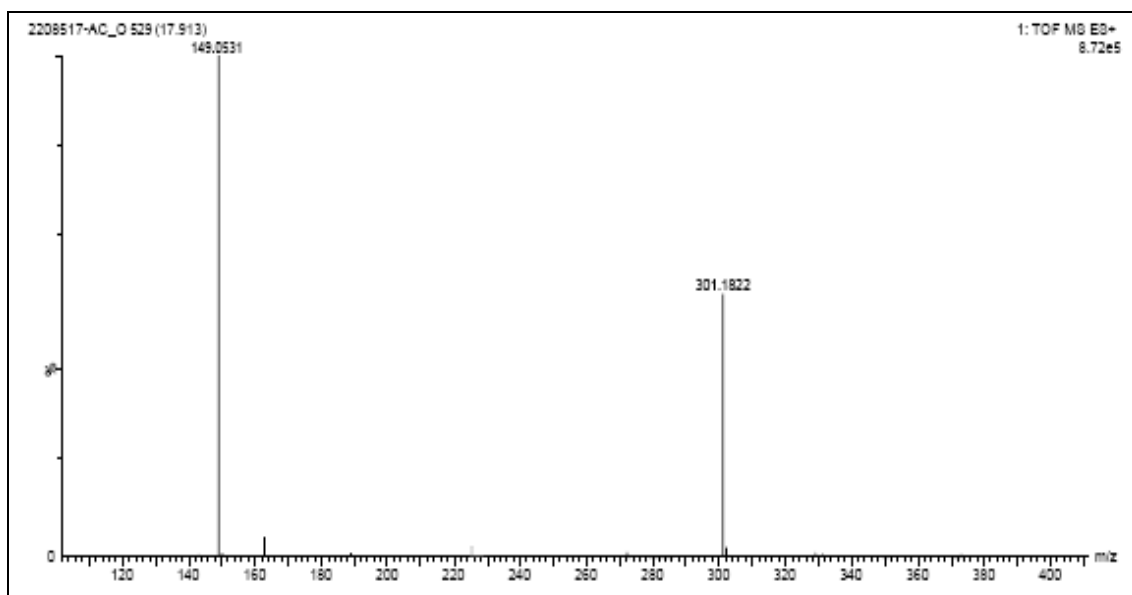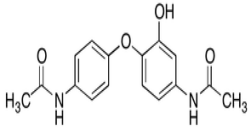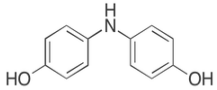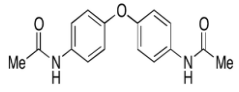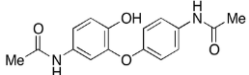**Fig 19:** Mass spectrum of Acetaminophen Impurity N

Fig 20: Mass spectrum of Acetaminophen Impurity O

Table 6: List of Acetaminophen Impurities

Sl. No.	Name of the compound	Code	IUPAC name	Structure of the molecule	Molecular weight
1	Acetaminophen API	AC_API	4'-hydroxy acetanilide		151.2
2	Acetaminophen related compound A	AC_RC_A	4-(Acetylamino) phenylacetate		193.2
3	Acetaminophen related compound B	AC_RC_B	N-(4-hydroxy phenyl)propan amide		165.2
4	Acetaminophen related compound C	AC_RC_C	N-(2-Phydroxy phenyl)acetamide		151.2
5	Acetaminophen related compound D	AC_RC_D	**N-Phenyl-acetamide**		135.2
6	Acetaminophen Impurity E	AC_E	4′-Hydroxy acetophenone		136.2
7	Acetaminophen related compound F	AC_RC_F	4-Nitrophenol		139.2
8	Acetaminophen Impurity G	AC_G	1-(4-Hydroxyphenyl) ethanone oxime		151.2
9	Acetaminophen related compound I	AC_RC_I	1-(2-Hydroxy phenyl)ethanone		136.2
10	Acetaminophen related compound J	AC_RC_J	**N-(4-Chlorophenyl) acetamide**		169.6
11	Acetaminophen related compound K	AC_RC_K	4-Aminophenol		109.1

12	Acetaminophen Impurity L	AC_L	N-[4-[4-Acetamido -2-hydroxyphenoxy) phenyl]acetamide		300.3
131	Acetaminophen Impurity M	AC_M	4-(4-hydroxy phenylamino) phenol		201.2
114	Acetaminophen Impurity N	AC_N	Bis(p-acetylamino phenyl) ether		284.3
15	Acetaminophen Impurity O	AC_O	2-Hydroxy-4',5-diacetamido-diphenyl ether		300.3

4. Conclusion

A single and simple method is developed for determination of impurities of acetaminophen along its Active ingredient in a marketed acetaminophen drug. Thereby I concluded that in a single injection it's possible to identify acetaminophen and its related substance by LCMS (as shown in figure 5). Hence, this paper reveals that by a using simple method it is possible to identify all the impurities of acetaminophen in a finished product by a single injection.

5. Acknowledgement

6. Abbreviations

- LCMS- Liquid Chromatography – Mass Spectrometry
- OTC – Over the Counter
- HPLC – High performance liquid chromatography
- ESI – electron spray ionisation
- LC – Liquid chromatography
- TIC – Total ionic current
- V- voltage
- GC- Gas Chromatography
- NMR- Nuclear Magnetic Resonance
- CE- capillary Electrophoresis
- MS- Mass spectrometry
- RSD- Relative standard deviation

7. References

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