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RESEARCH ARTICLE

USING LACTIC ACID AS AN ANTIMICROBIAL AGAINST *PSEUDOMONAS AEROGINOSA* AND VALIDATION OF ITS TEST METHOD BY LC-MS/MS.

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Abstract

A liquid chromatography-tandem mass spectrometry based method was validated to be used in the analysis of Lactic acid in different food/feed substrates. Validation results revealed that, the Limit of detection and Limit of quantitation values were 50 ng/ml. The Accuracy percentage values ranged from 99.3% to 130% and the linearity regression values were 0.9996 for Lactic acid. It can be concluded that, LC-MS/MS technique is a reliable, specific and accurate technique which can be used for qualitative and quantitative analysis of Lactic acid in different food/feed categories.

Introduction:-

Organic acids are "Generally Regarded as Safe" compounds often containing one or more carboxyl groups (–COOH) with antimicrobial properties[1]. Some of the most common are those with short chains (C1–C6) including, formic, lactic, propionic and citric acids and their salts. Many of these are known to inhibit various pathogenic strains in vitro but are highly dependent on the type and level[2, 3, 4] when tested in vivo with various aquatic animal species. It is believed that the primary antimicrobial action of organic acids is by altering the cell cytoplasm pH of bacteria and those that are sensitive to such changes are inhibited, thus reducing harmful bacteria within the gastrointestinal tract of the host animal[5]. Therefore a reduction of gastrointestinal bacteria, that often includes pathogenic species, may improve growth and disease resistance in the host animal. Meanwhile, organic acids can also lower dietary and gastrointestinal pH, which may chelate or solubilize minerals to make these more available for absorption, as well as break up anti-nutritional compounds[6]. The inclusion of organic acids in the diets of aquatic animal has been reported to increase the activity of several digestive enzymes[7]. Generally, reports on organic acids improving growth performance and nutrient utilization in aquaculture animals have been positive, but appear to be dependent on the type of organic acids used and host species[4].

Lactic acid (2-hydroxypropanoic acid, pKa 3.86) is a small organic acid of biological importance that was first isolated from sour milk[8].

Lactate has two optical isomers, L-lactate and D-lactate. L-lactate is the most abundant enantiomer of lactate. It is formed mainly during anaerobic glycolysis by conversion of pyruvate to L-lactate by lactate dehydrogenase[9]. D-lactate is often considered as the non-physiological counterpart of L-lactate[8]. Under physiologic conditions, the concentration of D-lactate is a 100-fold lower when compared to L-lactate[10].

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So far, D- and L-Lactate have been analyzed by several different techniques ranging between chiral stationary phase liquid chromatography using UV or fluorescence detection[11, 12, 13, 14, 10, 15] enzymatic assays[16, 17, 18, 19, 20, 21, 22] gas chromatography-mass spectrometry (GC/MS) methods[23, 24] liquid chromatography-mass spectrometry (LC/MS) methods[8, 25] and reversed phase liquid chromatography using fluorescence detection[26] However, these techniques have several shortcomings such as low sensitivity[27, 15, 25] large sample volume[19, 23] complex chromatographic systems[28, 14, 10] and long run times[14, 10, 27, 26].

High-performance liquid chromatography (HPLC) method has gained importance in an organic acid analysis. Because of the speed, sensitivity, reliability, and simple sample preparation methods involved in HPLC[29].

Advantages of LC/MS/MS provides an exceptionally clean product (fragment) ion chromatograms for quantification; useful for the rapid screening of complex samples where analytes of interest are known; compound identity confirmation can be achieved by MS/MS using the product ion scan mode; detecting a specific product ion (precursor ion mode) or charged fragments resulting from a neutral loss (neutral loss mode) and a compound of interest can be classified[30].

The aim of this work is to find out a safe, rapid, accurate, precise and reliable test method for Lactic acid analysis with high sensitivity and to validate and optimize this method to be used for qualitative and quantitative analysis of lactic acid. Also, to estimate the Limit of Detection (LOD), Limit of Quantitation (LOQ), linearity and accuracy of the used technique.

Materials and Methods:-

Calibration, tuning, optimization, method elaboration and batch submission of lactic acid:

Instrument calibration has been done following the user's manual of the used instrument (Applied Biosystems sciex 4000Q Trap) for positive and negative ion modes.

Compound-specific parameters setup was performed manually following procedure described in the user manual by determining a range from 40 to 140 Da convenient to the expected masses (89.1 m/z) using the specific software (Analyst®).

To set up compound optimization procedure, steps described in the users' manual were followed including engagement of compound optimization on negative ion modes and setup of the molecular weight (90.1).

In order to submit a batch and obtain data, an acquisition method was created for both LC and MS following procedures described in the user manual including parent masses, daughter masses, DP, CE and CXP for lactic acid (Tables 1, 2 and 3) using isocratic mobile phase solutions (15% (v/v) H_2O and 85%(w/w) acetonitrile, 0.1% Formic acid).

- **Determination of Signal to Noise ratio, LOD and LOQ for lactic acid compound (according to Finete *et al.*, 2015)[37]:**

1- A standard solution of lactic acid 50 ng/ml were prepared and were introduced to be analyzed by LC-MS/MS using all related parameters.
2- Signal to Noise (S/N) ratio was measured as described in the users' manual and serial dilution of the used standard solution was performed to reach S/N ratio of 3 to be considered as LOD and S/N ratio of 10 to be considered as limit of quantitation.

- **Building calibration curves for lactic acid:**

Different concentrations of both lactic acid (50ng/ml, 500ng/ml, and 850ng/ml) were injected into MS/MS with passing through LC (separating column) for obtaining standard curves for lactic acid from which linearity and accuracy were estimated.

- **Column used:** Phenomenex Luna 3µm C18 (2) LC column 150X3 mm.

- **Determination of Minimum Inhibitory concentration of lactic acid against pseudomonas aeroginosa:**

The minimum inhibitiory concentration (MIC) of Lactic acid against *Pseudomonas aeruginosa* was determined by broth dilution method (according to **Karunanidhi *et al.*, 2012**)[38]. For MIC testing , lactic acid at a concentration of (0.05 , 0.1 , 0.2 , 0.3 , 0.4 , 0.5 ,0.6 , 0.7 ,0.8 , 0.9 , 1 %) are prepared. Briefly, 11 sterile test tubes 1 through 11 were loaded with (5µL : 10µL : 20µL : 30 µL :40 µL :50 µL :60 µL : 70µL : 80µL : 90 µL :100 µL) of lactic acid. To avoid carryover pipette tips were changed between each tube. All tubes were inoculated with 500 µL of bacterial inoculum (10^6 CFU mL^{-1}) and incubated at 37^o C for 24 h. After incubation, the lowest concentration of the lactic acid at which invisible growth occurred was considered as MIC.

Results and Discussion:-

Many researchers outline various analyses for lactic acid detection[28, 12, 13, 14, 10, 15] among which Liquid chromatography-mass spectrometry (LC-MS) is considered as a widely available and reliable tool for identifying of Lactic acid which has been a method recommended[11, 14, 10].

Lactic acid has been analyzed using ESI in negative ion (ESI-) mode. The protonated molecular ion of in ESI- mode is m/z 89.1. The fragments of m/z 89.1 are m/z 89.1>43.1 where m/z 43.1 is the dominant ion, as shown in Figure 1 in product ion scan mode.

It is clear from the obtained figure that, lactic acid gave the distinctive signal at 89.1 *m/z* in negative ion mode[8, 29, 31, 32, 33]who reported that, parent ion of Lactic acid could be produced at 89.1 m/z.
Figure (2) showed the obtained daughter ion transitions of Lactic acid which was 89.1/43.1[8, 29, 31, 32, and 33] who stated that the daughter ion of Lactic acid after fragmentation was 43 m/z.

It is clear from the obtained data that, Lactic acid optimization showed the presence of a specific signal (peak) at 89.1 m/z[8, 29, 31, 32, 33] who could use ESI- mode for Lactic acid optimization process and obtained the same mass to change signal.

The result of LC separation in building acquisition method for Lactic acid analysis using a standard solution with a concentration of 500 ng/ml is illustrated in Figure (3). It is clear from the obtained results that, using Phenomenex separation column with the prescribed gradient protocol of the used isocratic mobile phase solution (Table 2) with the parameters obtained from the compound optimization procedure could result in a reliable peak at constant retention time (5.02 min) which was accepted following the guidelines[35] who stated that, the critical ions must be present and exceed a signal-to-noise ratio > 5:1(Figure 4)

Blank solutions were analyzed by LC-MS/MS technique to exclude the presence of the compounds under study in the used solvents (solvent's carry over). It is clear from figures 5 that, the used solvents were free from being contaminated with the compound under investigation.

Many authors could separate Lactic acid at retention times similar to that obtained in this study like[36] who get retention time of lactic acid at 5.02 min. Different retention time values were obtained by others studies like[34] who get retention time at 4 min. This variation may be due to differences in the composition of the mobile phase solution or the gradient protocol and may be also due to differences in the analytical parameters including the instrument itself. However, all results united in one condition that is; the compounds under test have been released from the used column in a situation at which the aqueous concentration is greater than the solvent concentration confirming the polar nature of these compounds.

Figure (6) showed the obtained standard curve after injection of four different concentrations of the standard solution of lactic acid compound (0, 50, 500, 850 ng/ml).

Data obtained in this figure showed excellent linearity with regression value of 0.9996 demonstrating a good adherence to the linear model.

From the same figure, accuracy % was estimated for lactic acid analysis as it was 130%, 99.3% and 100% at concentrations of 50, 500, and850 ng/ml, respectively.

The calculation of the LOD and LOQ as 3 S/N and 10 S/N ratios was determined by the used software (Analyst®). The LOD and LOQ obtained in the analytical curve were 50 ng/ml, 500 ng/ml and 850 ng/ml.

The obtained LOD and LOQ values were lower than that obtained in previous studies; these differences may be due to different analytical techniques and different measuring instruments.

- MIC determination: the results of the MIC indicated that lactic acid inhibated the tested organism**.**

MIC of lactic acid against *Pseudomonas aeruginosa*:-

Results obtained from the MIC testing procedure illustrated that, using lactic acid 85% of aconcentration of 0.2 % had the bacteristatic effect on bacterial species under study which was clear from the absence of any visited growth in the test tube containing the above mentioned concentration. This result was supported by that obtained by **Stanojevic´ -nikolic´et al. (2016)**[39] who reported that Lactic acid minimal inhibitory concentration against Gram negative bacteria was ≥0.125%, while minimal biocide concentration was ≥0.25 %. Lactic acid could be used as an efficient natural antimicrobial agent improving the safety of all-natural foods. However **Hsiao and Siebert (1999)**[40] who showed that minimal inhibitory concentration against Gram negative bacteria was 0.34% lactic acid, while minimal inhibitory concentration was 0.37% which disagreed with results obtained in this study. This difference may be attributed to difference of concentration of the used acid or different behavior related to the tested strains which may be related to the structure of its cell wall or any other physiological charecteristic.

Table (1):- MS parameters required for analysis of lactic acid compound:

Used instrument	Applied Biosystems sciex 4000Q Trap
Ionization type	Electrospray (ESI)
Polarity	Negative
Injection volume	2 µl
Column temperature	40 ˚C
Temperature	400˚C
Gas	Curtain gas (20 psi) Gas1: (40 psi) Gas2: (40 psi)
Declustering potential (DP)	-55 V
Entrance potential (EP)	-10 V
Collision energy (CE)	-20 V
Collision exit potential (CXP)	-5 V
Scan mode	MRM
Resolution	UNIT
Scan time for each transition	300 ms

Table (2):- LC parameters required for analysis of lactic acid compounds:

Step	**Time (min)**	**Flow rate (µl/min)**	**Mobile phase A%**	**Mobile phase B %**
0	0.10	200	0	100
1	5.00	200	0	100

Table (3):- MS/MS Parameters for Lactic acid

Parameters Analyte	**Molecular Weight**	**Ion Mode**	**Precursor ions (m/z)**	**Daughter ions (m/z)**	**Declustering Potential (DP)**	**Collision Energy (CE)**	**Cell Exit Potential (CXP)**
Lactic acid	90.1	Negative	89.1	43.1	-55	-20	-5

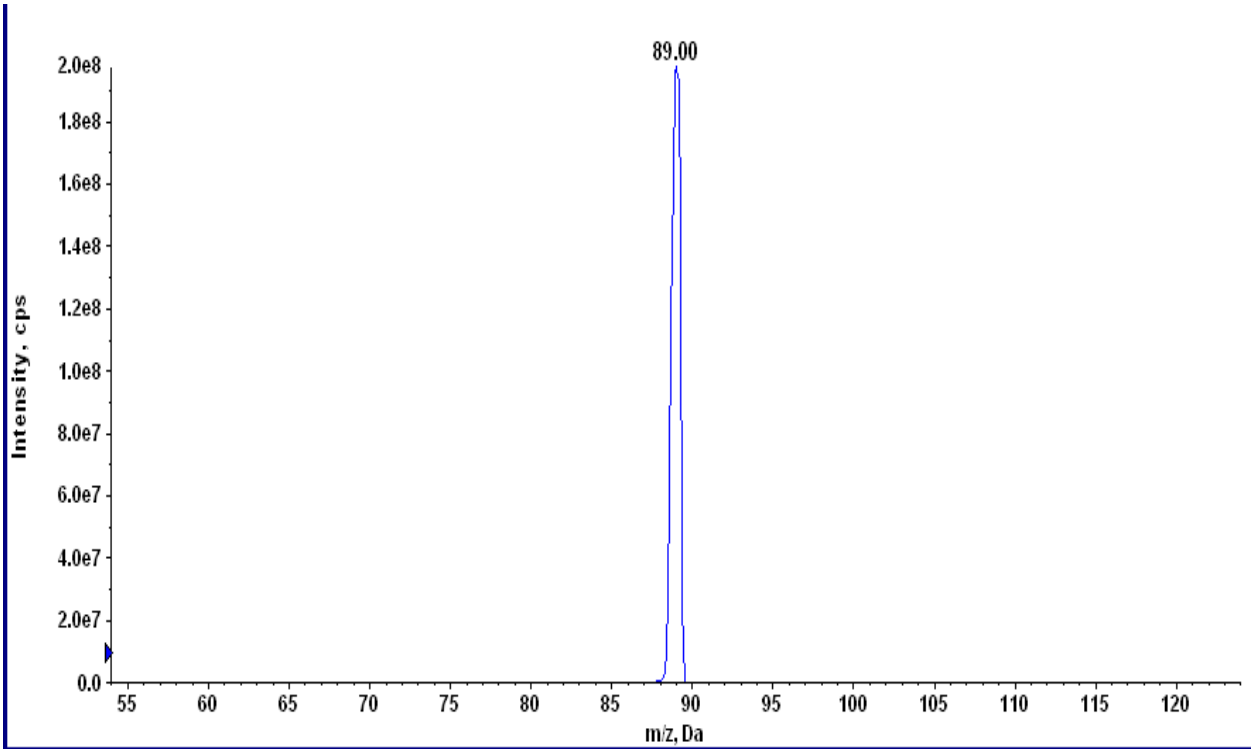


Figure (1):- Result of manual tuning of Lactic acid compound using AB Sciex 4000 Q trap mass spectrometry and its related software (Analyte®).

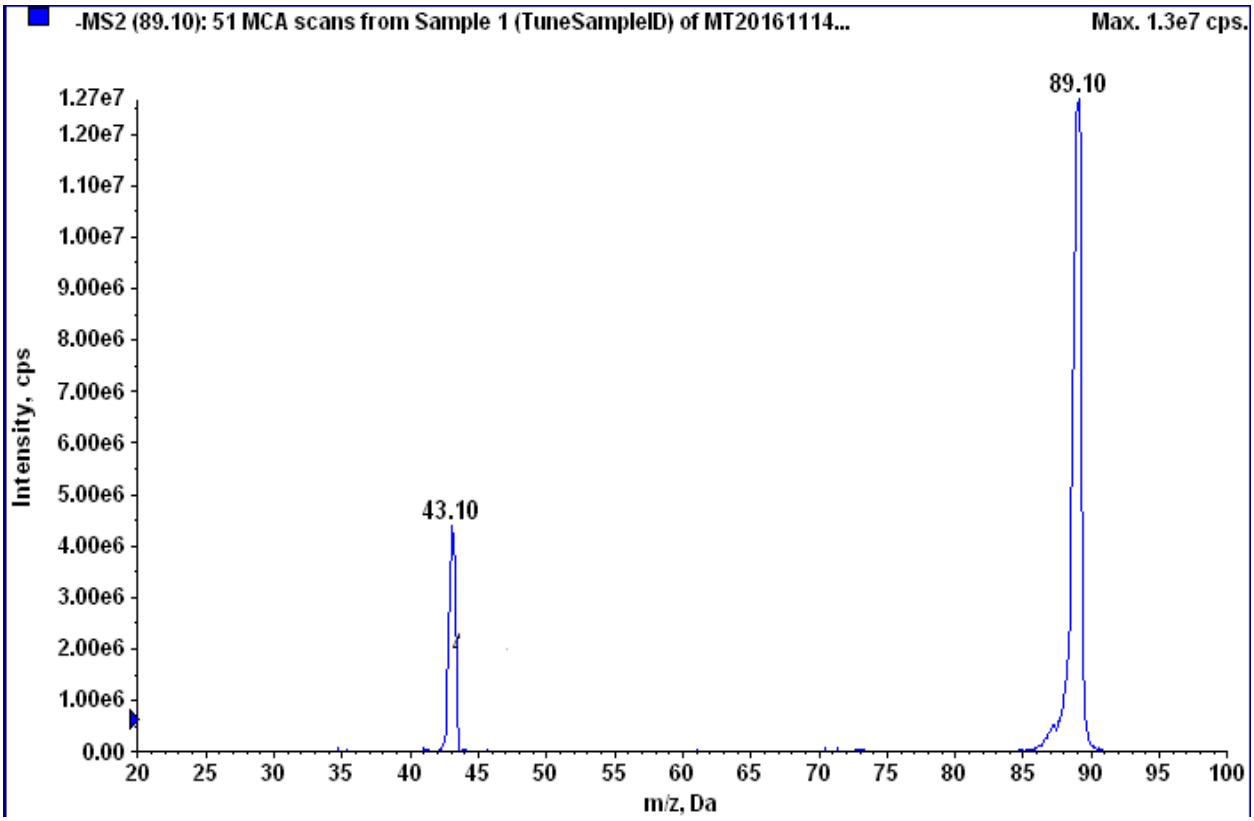


Figure (2):- Transitions of Lactic acids daughter ion mass.

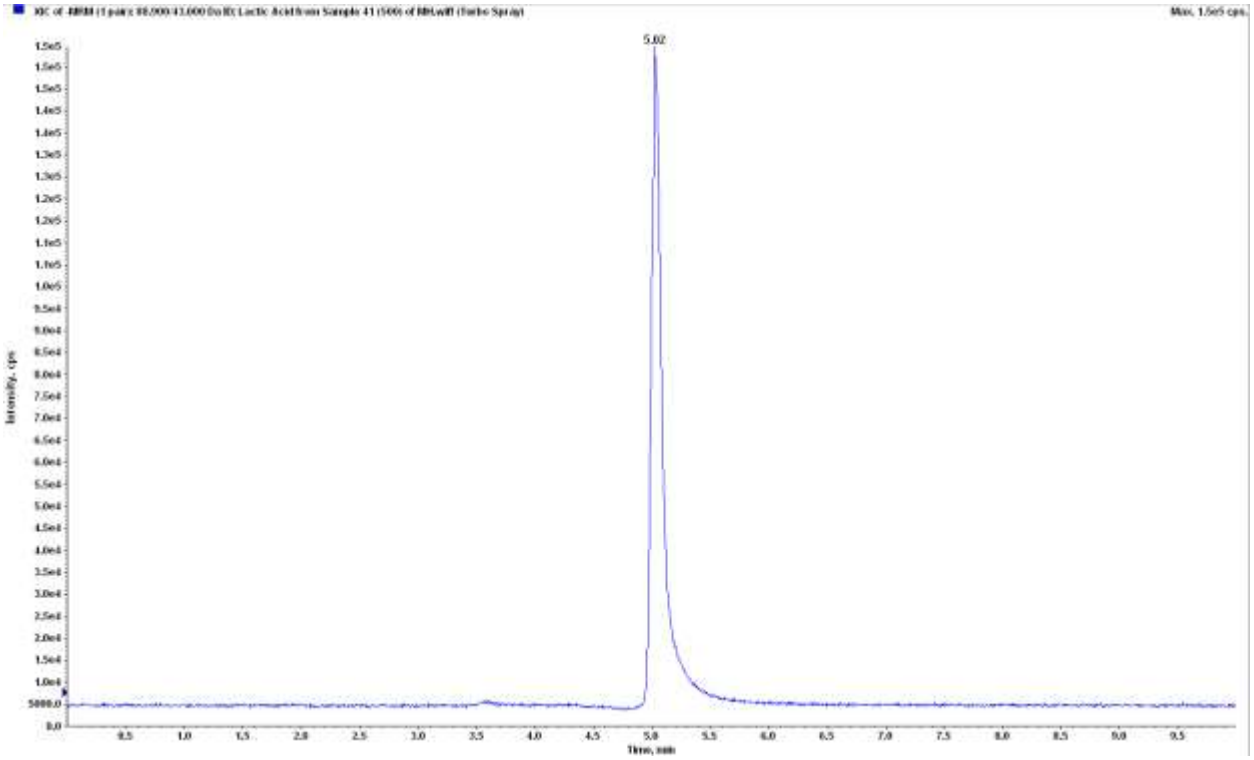


Figure (3):- LC separation Retention Time (RT) of Lactic acid in negative Electrospray Ionization (ESI-).

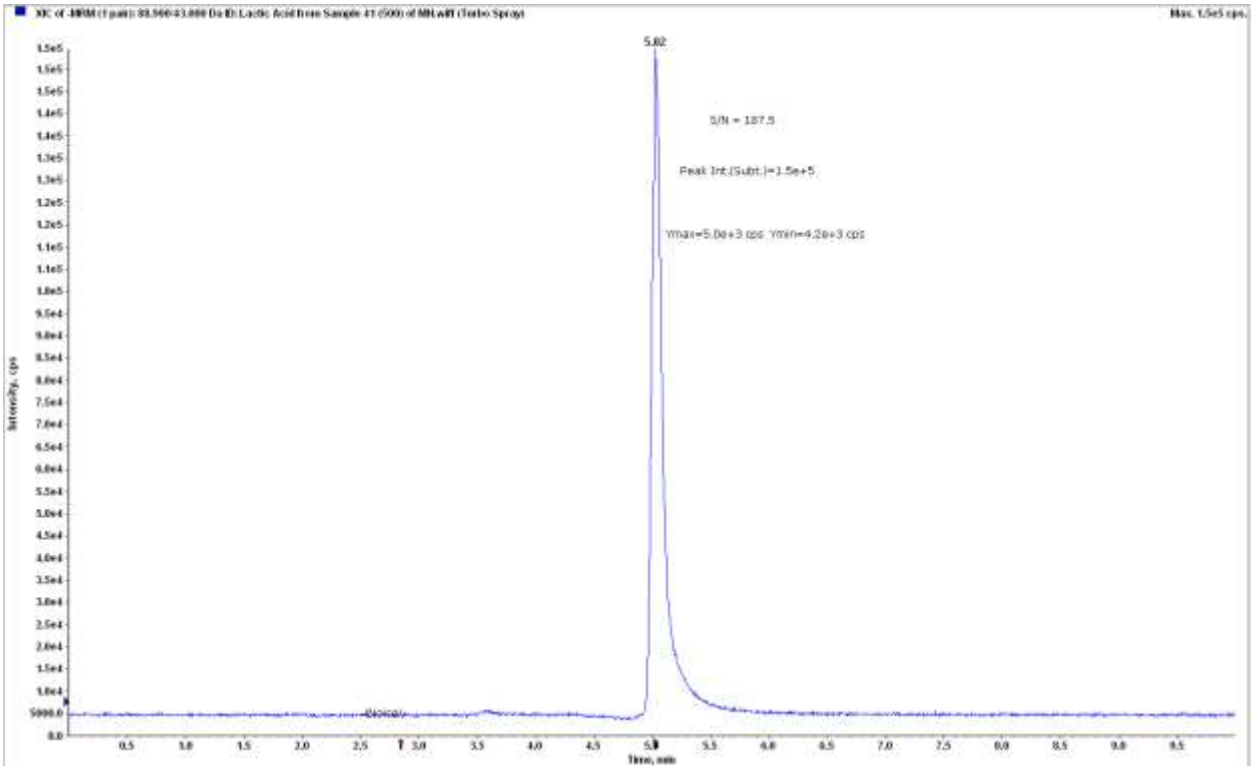


Figure (4):- Signal to Noise (S/N) ratio of Lactic acid at its specified retention time.

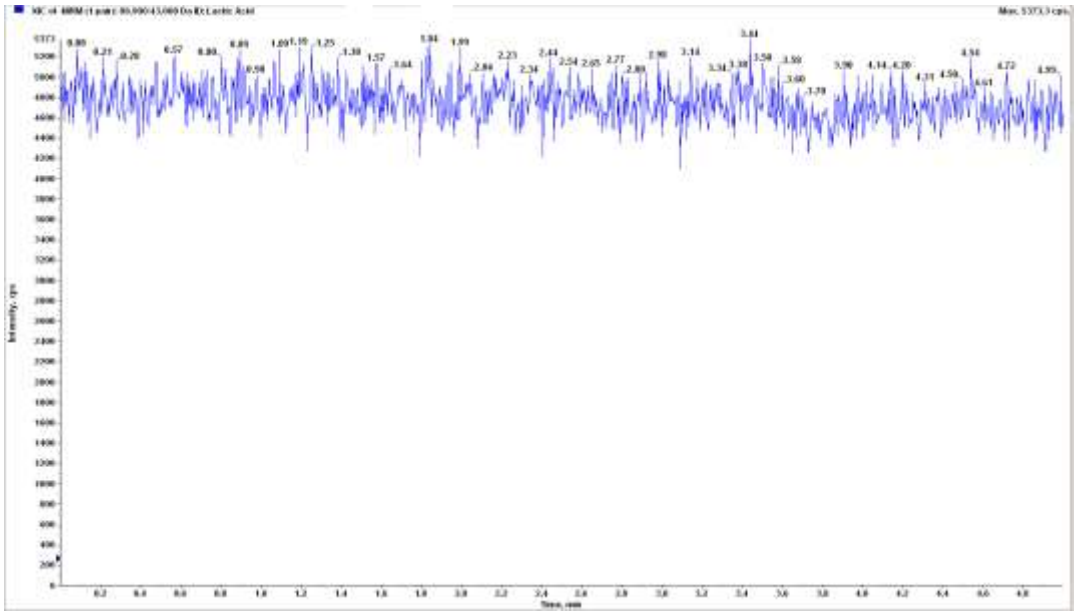
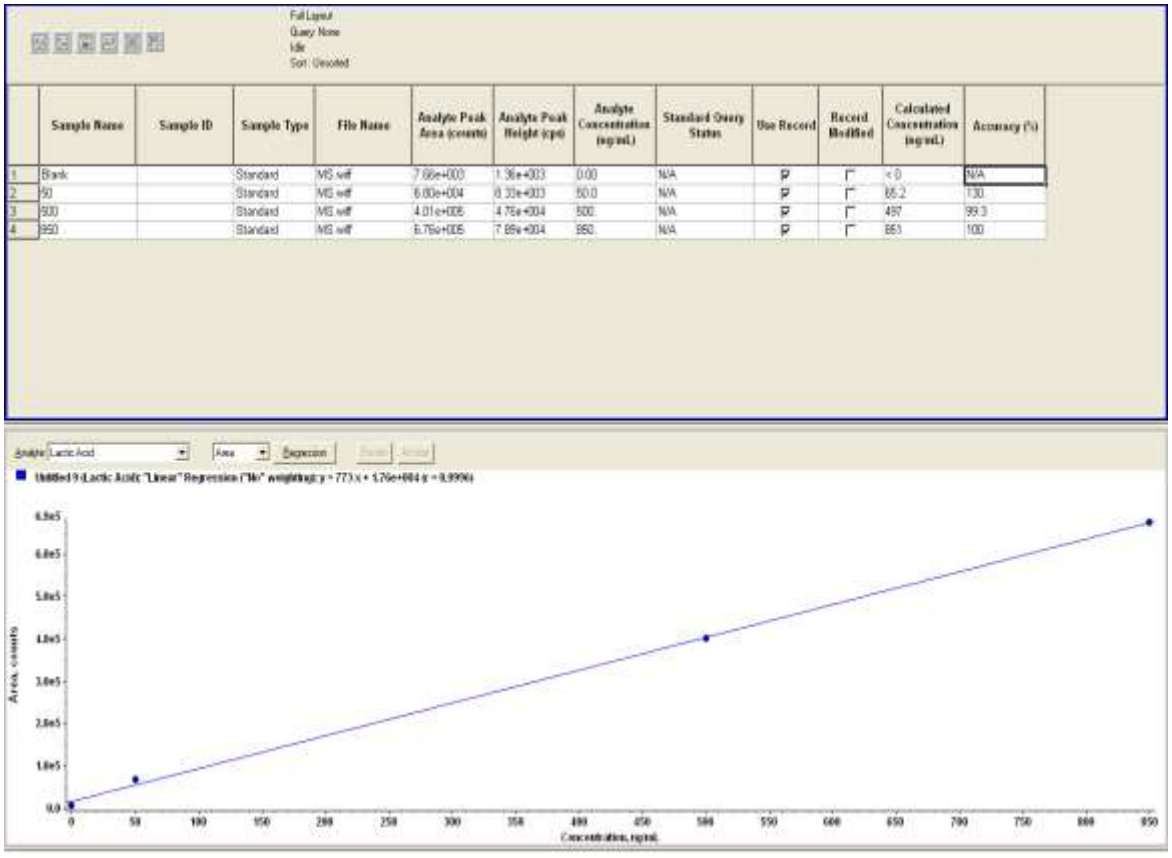


Figure (5):- Result of LC analysis of Blank solutions at ESI-



	Sample Name	Sample ID	Sample Type	File Name	Analyte Peak Area (counts)	Analyte Peak Height (cps)	Analyte Concentration (ng/mL)	Standard Query Status	Use Record	Record Modified	Calculated Concentration (ng/mL)	Accuracy (%)
1	Blank		Standard	MS.wiff	7.66e+003	1.36e+003	0.00	N/A	☑	☐	< 0	N/A
2	50		Standard	MS.wiff	6.80e+004	8.33e+003	50.0	N/A	☑	☐	65.2	130
3	500		Standard	MS.wiff	4.01e+005	4.76e+004	500	N/A	☑	☐	497	99.3
4	950		Standard	MS.wiff	6.75e+005	7.89e+004	950	N/A	☑	☐	951	100

Figure (6):- Control chart, linearity and accuracy of lactic acid standard solution.

Conclusions:-

From this study, it can be concluded that, LC-MS/MS technique can be used reliably in the determination of lactic acid concentration in different samples after validation process; LC-MS/MS technique can be used to quantify concentration of lactic acids in different feed additives and other substrates used in animal nutrition in order to study

its effect on performance and nutrigenomics parameters. Further studies using this approach can be applied on other organic acids (e.g. lactic acid, propionic and acetic in fish nutrition) to widen the scope of research in this area. Results obtained from this research work illustratel that lactic acid 85% can be used as bacteristatic substance against pseudomonase at acouc of 0.2%.

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