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Original Research Article

Using MS Detector for Quantitative Analysis Pharmaceutical Products with HPLC

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Abstract: This work is devoted to the quantitative analysis of pharmaceuticals using MS detector connected to HPLC. A theoretical model for constructing a calibration curve for MS quantitative analysis using electrospray ionization has been developed. The model's practical applicability is illustrated with examples. The applicability of the proposed model was tested for both cations and anions. The influence of the composition of the mobile phase, which consisted of water-methanol solutions of three modifications, was considered: neutral, acidified with formic acid, and alkalized with ammonium hydroxide. The test results are summarized in tables. The practical application of quantitative MS analysis is demonstrated using the example of determining inositol in pharmaceutical products.

Keywords: MS Quantitative Analysis, HPLC/MS, ESI, Electrospray Ionization.

INTRODUCTION

Mass spectrometry (MS) identifies compounds by measuring the mass-to-charge ratio (m/z) of their ions. This technique involves ionizing samples, ionizing molecules, separating the ions by mass, and then detecting them to produce a spectrum (a plot of intensity versus m/z). Traditionally, Electrospray Ionization (ESI) (Wilm M, 2011 and Ho C *et al.,* 2018) based MS detectors (MSDs) are used in tandem with an ultraviolet (UV) or diode array detector (DAD) for the qualitative analysis of the substances under study (Urban P.L, 2016). But in many cases MSD may be used for quantitative analysis, when DAD detectors do not detect substances, glutathione (Yefimov S.V, 2021) and inositol are of such substances. Typically, a calibration curve is used for quantitative assessment, assuming a linear relationship between the MS signal and the concentration of the substance (Urban P.L, 2016), (Gale P. J *et al.,* 2015). Article (Lavagnini I *et al.,* 2006) describes a method for approximating calibration points both by a line and a quadratic parabola using the weighted least squares method, where the inverse of the variance is used as the weighting factor. In this work, we used a different approach, which consisted in that we did not look for the best approximation of the experimental data, but derived the equation of the calibration curve from the model of the formation of molecular ions in electrospray ionization.

MATERIALS AND METHODS

Chemicals**.** All liquid reagents including water, acetonitrile, and methanol were HPLC grade. Formic acid, 98–100% analytical grade, was from Merck. 30% aqueous NH_3 solution was from Sigma. Ascorbic acid, atenolol, glycine, and inositol were USP analytical standards.

Agilent LC/MS instrument (Agilent Single Quadrupole LC/MS instrument, 2019) consists of the following components: Single quadrupole (SQ) mass selective detector (MSD) with electrospray ionization (ESI) and 150 V fragmentor, plus Diode Array Detector (DAD). Gas temperature is 300°C, capillary voltage 4000 V and nebulizer 15psi. Column was Ascentis Express 90A OH5 150x4.6 mm, 2.5µm particles. Quaternary pump with flow: 1.0 mL/min, high pressure limit - 600 bar. Isocratic elution was performed using a 50/50 methanol/water mobile phase with or without modifications (0.1% formic acid or 0.1% NH_4OH).

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Preparation of standard stock solution. An accurately weighed 10 mg of USP calibration was dissolved in 10 mL of water. A 0.45µm cellulose acetate membrane filter was used to filtrate the solution. A set of standard solutions with various concentrations were prepared from the stock by dilution with water.

Samples**.** Inositol was determined in a 50 g/L injection solution containing methionine, choline chloride, CN-cobalamin, and benzyl alcohol. The injection solution was diluted 100-fold with the mobile phase to the inositol concentration of approximately 0.5 g/L and filtered through a 0.45 µm filter.

Calibration curve and coefficient of correlation. The sets of dilutions of standard stock solutions in range of from 0.1g/L to 1.0g/L were tested. Calculation of the calibration curve was performed automatically by OpenLAB CDS (Agilent Single Quadrupole LC/MS instrument, 2019).

Limits of detection (LOD) and quantitation (LOQ). LOD characterizes the sensitivity of a method; it is the minimum amount of a substance that can be measured by a given method, whereas the LOQ is the lowest concentration with acceptable linearity, accuracy, and precision. If the equation of the calibration curve is an equation of the first degree (straight line) then LOD is calculated by formula (1) (European Medicines Agency. 2006) where (σ) is the standard deviation of the response, and (a) is slope of the line.

$$LOD = 3.3 \times \frac{\sigma}{a} \quad (1)$$

Similarly, if the equation of the calibration curve is an equation of the second degree, parabola ($y=ax^2+bx+c$), then the LOD is the root of the quadratic equation, which is calculated by formula (2) (Yefimov S.V, 2021).

$$LOD = \frac{\left(-b+\sqrt{b^2-4\times a\times(c-3.3\times\sigma)}\right)}{2\times a} \quad (2)$$

Where the (σ) is the residual standard deviation of the regression line. LOQ is 3 times LOD.

Statistical analysis included calculating mean, standard deviation (S.D.), relative standard deviation (RSD), and correlation coefficient (r). Results $p < 0.05$ were considered statistically significant. Least Squares regression analysis was used. In most cases, the calculation was performed automatically by the OpenLAB CDS program.

RESULTS AND DISCUSSION

Ions observed by mass spectrometry may be quasimolecular ones (https://en.wikipedia.org/wiki/Electrospray_ionization#cite_note-23). If the injector is anode and detector is a cathode Figure 1, quasimolecular cations such as $[A+p]^+$ or $[A+Na]^+$, where A – analyte, p – proton, Na – sodium ion, are registered. If the injector is cathode and detector is anode Figure 1, quasimolecular anions such as $[A-p]^-$ are registered. The molecular mass of quasi-molecular cations exceeds the molecular mass of the corresponding quasi-molecular anions.

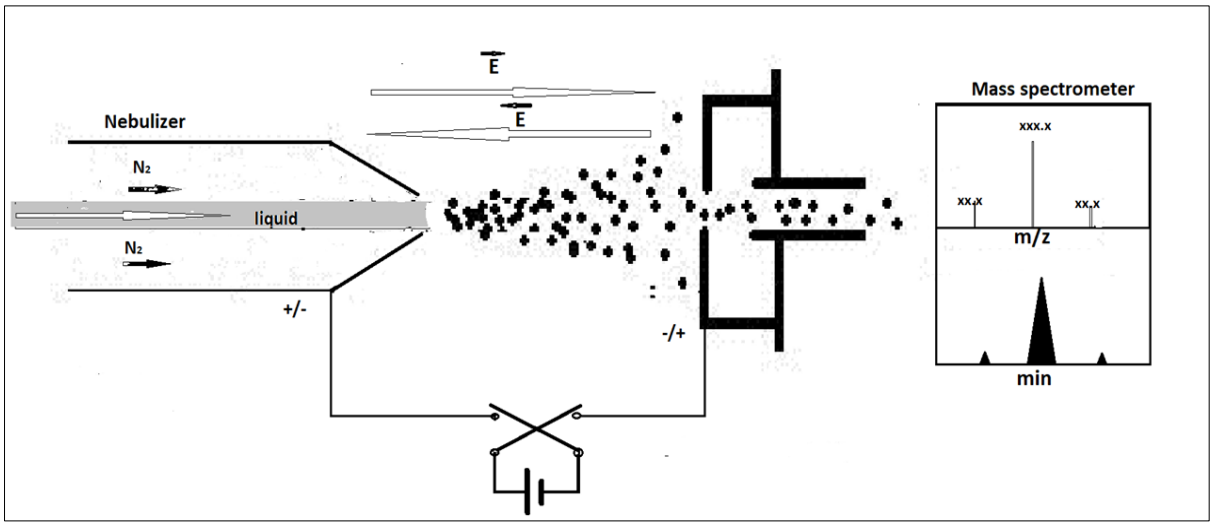


Figure 1: Electrospray Ionization in LC-MS. Schematic diagram.

Let us model the process of formation and annihilation of ions and derive the dependence of the MS signal magnitude on the concentration of the analyte in the solution.

The mechanism of formation and evolution of quasimolecular ions.
Cation Formation: A → $[A+p]^+$, the concentration of newly formed ions $[[A+p]^+]=C[A]$, where C are the ionization constants.
Similarly for Anions: $[[A-p]^-]=C[A]$, where C are the ionization constants.
Electroneutrality Equations: $[[A+p]^+] + [e^-] = 0$, and $[[A-p]^-] + [p^+]=0$
Cation Annihilation: $[A+p]^+ + e^- \rightarrow A$, $[A]= C[A+p]^+[e^-]$, Considering electroneutrality, we obtain: $A= C([A])^2$, and for anions: $[A-p]^- + p^+ \rightarrow A$, $[A]=C[A-p]^- [p^+]$, Considering electroneutrality, we obtain: $A= C([A])^2$

The signal registration level (D) is proportional to the difference in concentrations of newborn and annihilated ions.
$D= C[A] - C([A])^2$ or $D(A) = -a([A])^2 + b([A])$, where **"a"** and "**b**" – constants, [A] – the analyte concentration in solution.

Thus, the calibration curve for MSD should resemble an inverted quadratic parabola $y=-ax^2+bx$ with its vertex at x=b/2a. The acceptable measurement range is from 0.0 to b/2a µg, Figure 2.

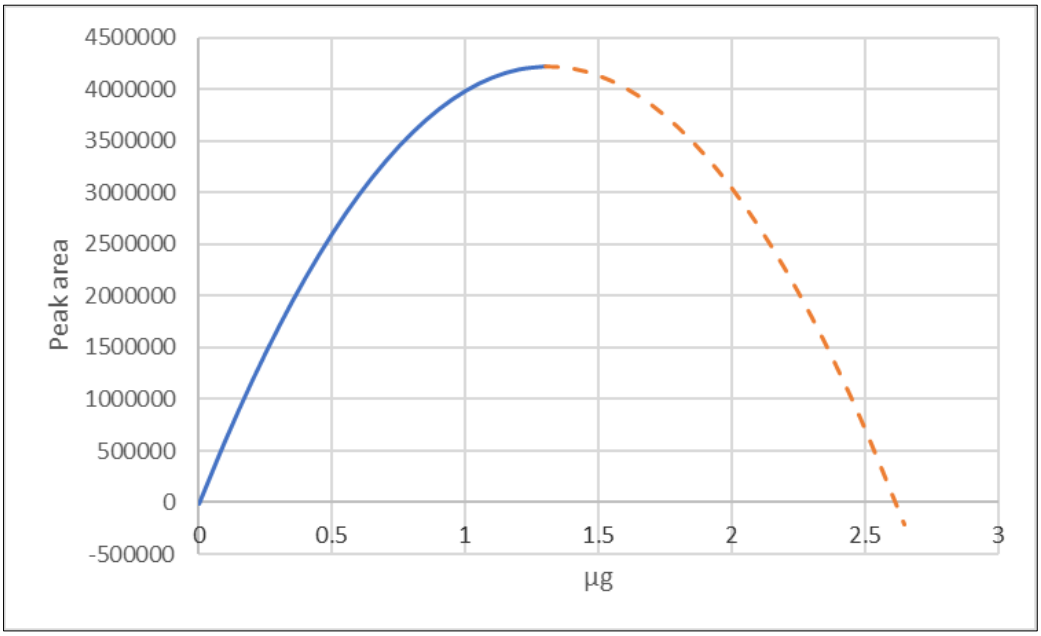


Figure 2: Calibration curve for determining analyte concentration using ESI MSD. The curve was constructed using data from (Yefimov S.V, 2021) for glutathione

As an illustration, we have taken three types of analytes: a weak acid (pKa=4.17), a weak base (pKa=9.6), and a zwitterionic substance (pKa=2.34, and pKa=9.6). The mobile phase used was a mixture of methanol and water, either neutral, acidified with formic acid (0.1%), or alkalized with ammonia (0.1%). Measurements were performed in two modes: positive, where the injector was cathode and detector the anode, and negative, with the opposite polarity. The test results are presented below in tables and figures.

Table 1: Results of ascorbic acid analysis by HPLC/MS

Ascorbic acid, MW=176.1, pKa=4.17. Column: poroshell 120 hilic 50 x 4.6 mm 2.7µm. Mobile phase: Methanol/water 50/50 ± additive acid or base. r – correlation coefficient.		
Additive	**Positive mode**	**Negative mode**
No additive	RT=0.77 min r= N.A. m/z^+= 199	RT= 0.8 r= 0.995 m/z^-=175; 351
0.1% formic acid	RT= 0.82 min r=0.996 m/z^+= 177; 199	RT=0.84 r=0.999 m/z^-= 175; 351
0.1% NH_4OH	RT= 0.54 min r=0.995 m/z^+= 177	RT=0.55 r=0.998 m/z^-=175

Ascorbic acid can be quantified in both acidified and alkaline mobile phases. Unlike other analytes, it has a slightly better correlation coefficient for the anionic form Table 1 and Figure 3.

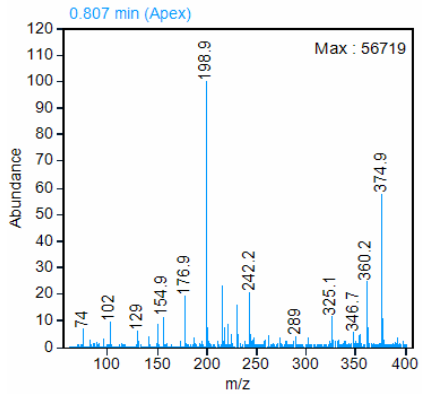
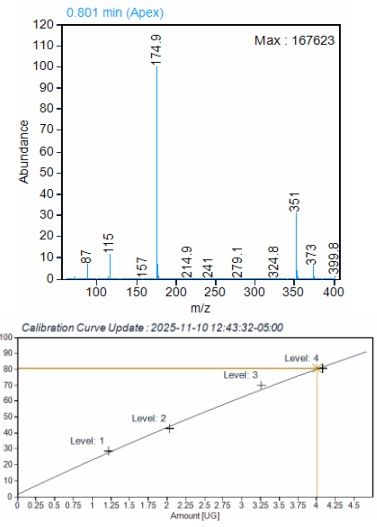
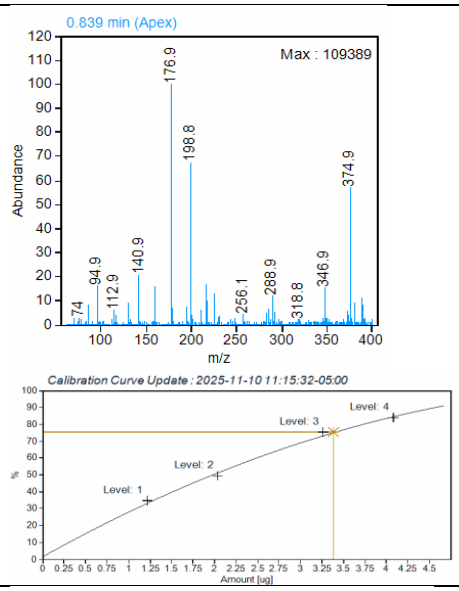
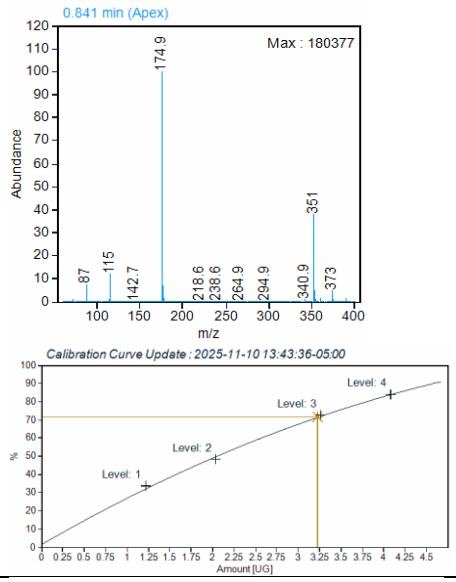
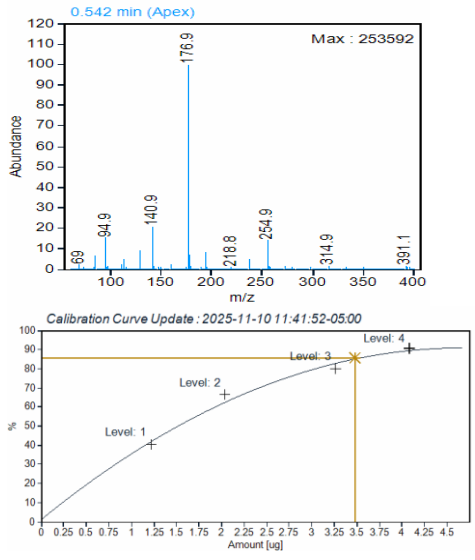
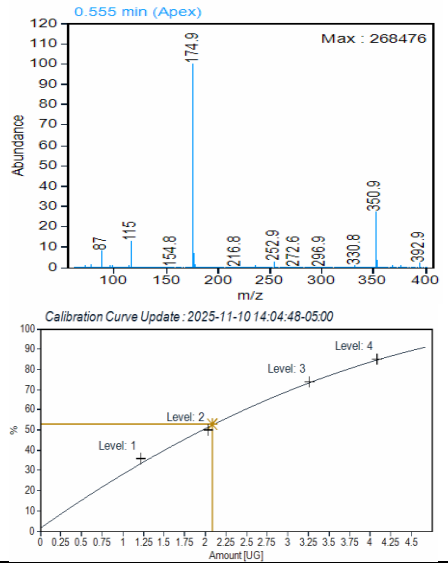
Ascorbic acid, MW=176.1, pKa=4.17. Column: poroshell 120 hilic 50 x 4.6 mm 2.7µm. Mobile phase: Methanol/water 50/50 ± additive acid or base. Calibration curve: $y = ax^2 + bx + c$		
Additive	**Positive mode (cations)**	**Negative mode (anions)**
No additive		
0.1% formic acid		
0.1% NH_4OH		

Figure 3: MS spectra and calibration curves of ascorbic acid under different conditions

Atenolol is only well detected in the cationic form in the acidified mobile phase Table 2 and Figure 4.

Table 2: Results of atenolol analysis by HPLC/MS

Atenolol, MW=266.3, pKa=9.6. Column: poroshell 120 hilic 50 x 4.6 mm 2.7µm. Mobile phase: Methanol/water 50/50 ± additive acid or base. r – correlation coefficient.		
Additive	**Positive mode**	**Negative mode**
No additive	RT=0.97 min r=N.A. m/z^+= not detected	RT= 0.97 r= N.A. m/z^-= not detected
0.1% formic acid	RT= 0.91 min r=0.999 m/z^+= 267	RT=0.93 r= N.A. m/z^-= not detected
0.1% NH_4OH	RT= 0.94 min r= N.A. m/z^+= not detected	RT=0.93 r= N.A. m/z^-= not detected

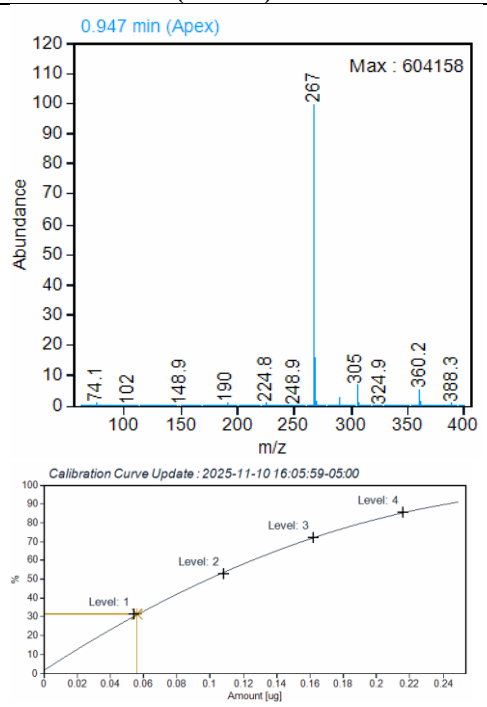
Atenolol, MW=266.3, pKa=9.6. Column: poroshell 120 hilic 50 x 4.6 mm 2.7µm. Mobile phase: Methanol/water 50/50 + additive (acid). Calibration curve: $y = ax^2 + bx + c$	
Additive	**Positive mode (cations)**
0.1% formic acid	

Figure 4: MS spectra and calibration curve of atenolol.

A good correlation coefficient (r=0.999) for glycine was obtained in the acidified mobile phase for cations. Quantitative analysis is also possible in negative mode in an acidified environment, but with less accuracy (Table 3 and Figure 5).

Table 3: Results of glycine analysis by HPLC/MS

Glycine, MW=75.07, pKa=2.34; 9.6; Column: poroshell 120 hilic 50 x 4.6 mm 2.7µm. Mobile phase: Methanol/water 50/50 ± additive acid or base. r – correlation coefficient.		
Additive	**Positive mode**	**Negative mode**
No additive	RT= not detected r=N.A. m/z^+= not detected	RT= 0.93 r=N.A. m/z^-=74
0.1% formic acid	RT^+= 0.93 r=0.999 m/z^+= 76	RT= 0.90 r=0.985 m/z^-= 74
0.1% NH_4OH	RT= not detected r=N.A. m/z^+= not detected	RT=0.64 r=N.A. m/z^-=74

<table>
<tr><td colspan="3">Glycine, MW=75.07, pKa=2.34 and 9.6; Column: poroshell 120 hilic 50 x 4.6 mm 2.7µm. Mobile phase: Methanol/water 50/50 ± additive acid or base; Calibration curve: y = ax² + bx + c</td></tr>
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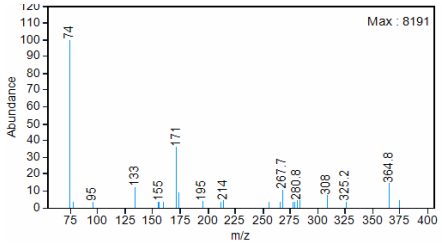
<tr><td>No additive</td><td></td><td> 
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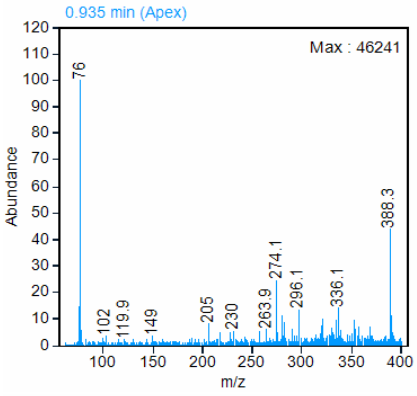
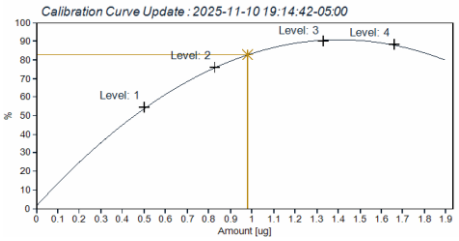
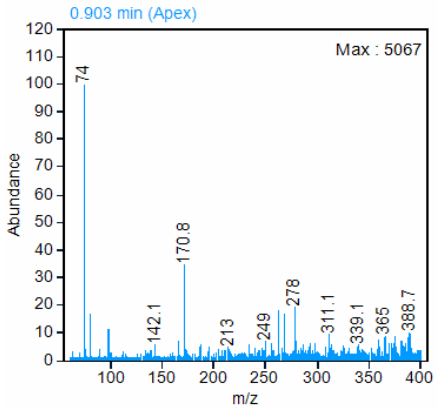
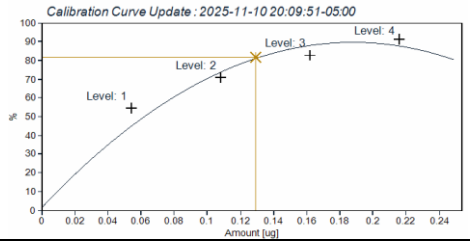
<tr><td>0.1% formic acid</td><td> 


</td><td> 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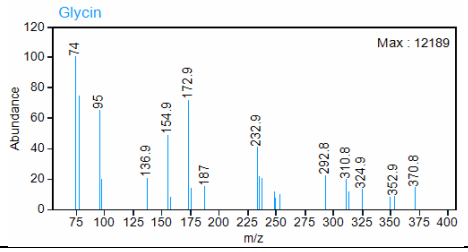
<tr><td>0.1% NH_4OH</td><td></td><td> 
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Figure 5: MS spectra and calibration curves of glycine under different conditions.

Analysis of inositol in a multicomponent injection solution. When analyzing a multicomponent solution containing inositol (Figure 6), we used MSD to quantify the latter, since it is not visible for DAD.

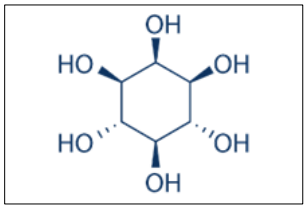

Figure 6: Inositol (Cyclohexane-1,2,3,4,5,6-hexol) MW= 180.16 g/mol

We used NP column Ascentis Express 90A OH5 150 x 4.6, 2.5µg and mobile phase - Acetonitrile/H_2O 3/7 +0.1% formic acid. The chromatogram of the mixture, MS spectrum and calibration curve are shown in the screenshot Figure. 7.

It should be noted that m/z+ for inositol from this tested solution is 383, not 181. This number probably represents the sum: 383 = 180 + 180 + 22 + 1, where 22 is the atomic mass of sodium.

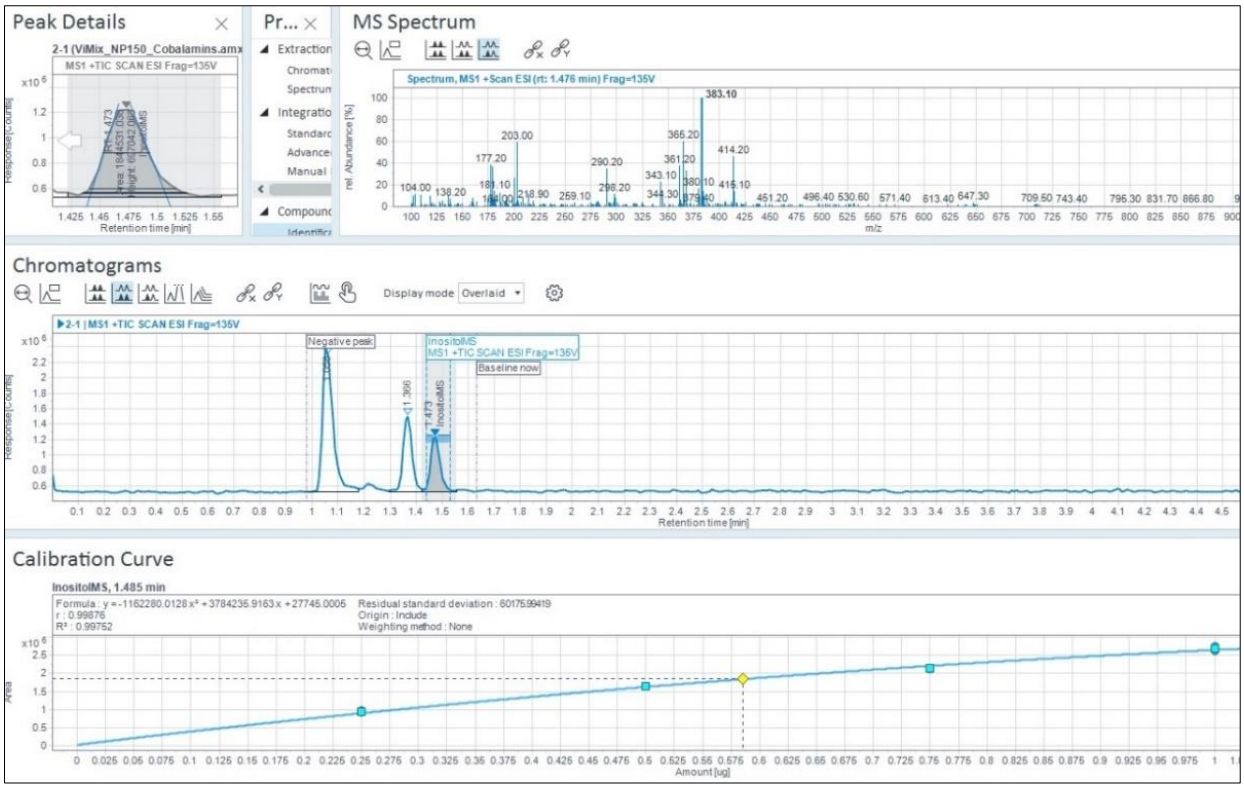

Figure 7: Analysis of inositol in a multicomponent injection solution. Data processing screenshot

The LOD calculation (Table 4) was carried out using formula (2), based on the calibration curve.

Table 4: LOD of inositol calculation using MSD data

MSD	y = ax² + bx + c		
Inositol (µg)	Mean Y (n=	Y calc.	ΔY
0		2947.186	
0.25	271171.8	271171.8	0
0.5	503376.1	495552.9	7823.149
0.75	622773.2	676090.5	-53317.3
1	833519.4	812784.5	20734.95
1.5	947645.7	954641.7	-6995.98
y = ax² + bx + c			
a	-350749		
b	1160586		
c	2947.186		
r	0.999		
Mean DY (n=5)	-6351.04		
S.D. DY (n=5)	28200.81		
LOD (µg)	**0.080**		
"X"- the content of the analyte in the sample; "Y" - the peak area; "Y calc."- the calculated peak area; "DY" - the residues; "a" - the slope of the regression line;"a","b", "c" - the parameters of regression curve; "r" - the correlation coefficient; "S.D. DY" - the residual standard deviation of the regression curve.			

The detection limit of inositol under our conditions is 0.08 micrograms, such accuracy is quite sufficient for the analysis of pharmaceutical products.

CONCLUSION

Electrospray mass spectrometry can be used for both qualitative analysis and, in many cases, quantitative analysis. Mass spectrometry is particularly effective when the analyte is not detectable by a diode detector, as in the case of inositol and glutathione. The maximum correlation coefficient can be obtained if the calibration curve is approximated by quadratic parabola. The analysis mode and mobile phase acidity are selected experimentally. Positive mode and an acidic mobile phase provide the best results in many cases. However, in some cases, negative mode and an alkaline mobile phase work well.

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