

Calculation of Mass Balance Chemical Stressed Active Pharmaceutical Ingredients by Example of Oxidized Nicotinamide Dinucleotide

Stanislav V. Yefimov^{1*}¹Pharmetric Laboratory LLC, 7265 Ulmerton Road Largo, FL USADOI: <https://doi.org/10.36347/sajp.2026.v15i10.003>

| Received: 13.08.2026 | Accepted: 08.10.2026 | Published: 13.10.2026

*Corresponding author: Stanislav V. Yefimov

Pharmetric Laboratory LLC, 7265 Ulmerton Road Largo, FL USA

Abstract

Original Research Article

A mass balance calculation for active pharmaceutical substances subjected to chemical stress was performed using oxidized nicotinamide adenine dinucleotide (NAD⁺) as an example. Calculations were carried out for low, moderate, and extensive degradation of NAD⁺ induced by basic hydrolysis. The applicability of using the mass balance as a criterion for the reliability of an analytical method is discussed.

Keywords: Mass balance, API analysis, NAD⁺, HPLC/MS.

Copyright © 2026 The Author(s): This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (CC BY-NC 4.0) which permits unrestricted use, distribution, and reproduction in any medium for non-commercial use provided the original author and source are credited.

INTRODUCTION

Calculating the mass balance of active pharmaceutical ingredients subjected to chemical stress is becoming a popular feature in publications describing analytical methods (Marden S, *et al.*, 2024), (Hong P, *et al.*, 2016), (Mackaplow M, 2010). Aware of this trend, clients and analytical laboratory managers often require the inclusion of mass balance data in new analytical methods. A range of 95%–105% is now accepted as the standard for mass balance. However, this criterion is frequently not met—a fact which, as this paper will demonstrate, does not justify deeming the method inadequate.

MATERIALS AND METHODS

Chemicals. Water HPLC grade purchased from Agilent. HPLC-grade solvents were used. NAD⁺ 98.2% Medisca INC, and Niacinamide USP reference standards were used.

Mobile phase: ACN/H₂O 3/7 +0.1% Formic acid.

Standard solution preparation: Weight 20 mg standard dissolve in 10 ml mobile phase, filtration 0.45µm pore syringe filter.

Instrument. Agilent HPLC/DAD/MS instrument consists of the following components: Diode Array Detector (DAD), quaternary pump, single quadrupole mass selective detector (MSD) with electrospray ionization and 150 V fragmentor, the gas

temperature is 300°C, the capillary voltage is 4000 V, and the nebulizer is 15psi.

Column - Ascentis Express 90A OH5 150 x 4.6, 2.7mm, Linear range: (0.1, 1.5) µg. Calibration standards: from 0.1 to 1.2 µg

HPLC/MS conditions: column temperature 40°C, flow rate 1ml/min, flow time 5 min, DAD 262, samples injection volume – 0.5 µL; m/z+: 664 for NAD⁺, and 123 for niacinamide.

Quantitative analysis was done using a calibration curve built for each of the components (Fig. 1, and 2).

Calibration curve and coefficient of correlation (r). The range was chosen so that, the calibration curve should be strictly linear (r≥0.999).

The forced degradation study involved incubating samples of NAD⁺ with ammonium hydroxide of different concentrations. The % of recovery which is equivalent to 100 minus % of degradation was determined.

Mass balance (Mb) of API was calculated by formula [1]:

$$Mb = \frac{A+B}{C} \times 100\% [1]$$

Were A – mass of API in sample after chemical stress; B – sum of mass of all products of degradation; C - mass of

API in the sample before chemical stress.

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. The Least-squares regression analysis was used. In most cases, the

calculation was performed automatically by the OpenLAB CDS software of the instrument.

RESULTS AND DISCUSSION

First, we determined calibration curve, range of linearity, API and degradants spectra (Figure 1 and 2).

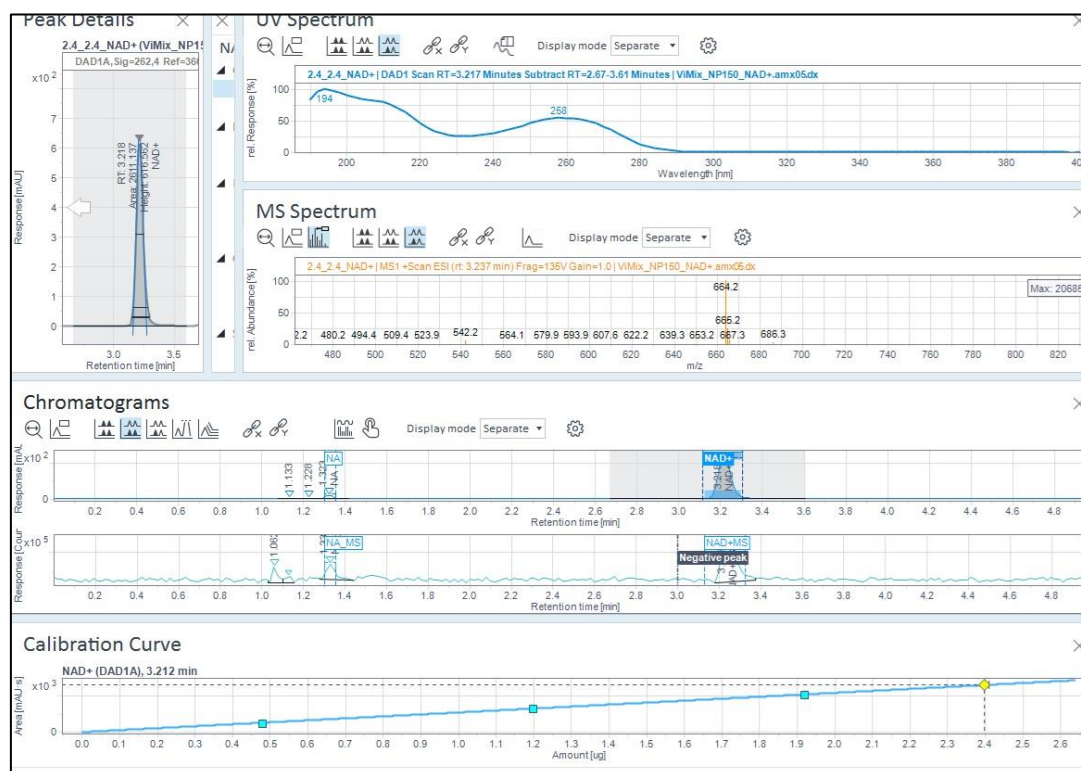


Figure 1: NAD⁺ chromatogram, spectra, calibration curve, range of linearity

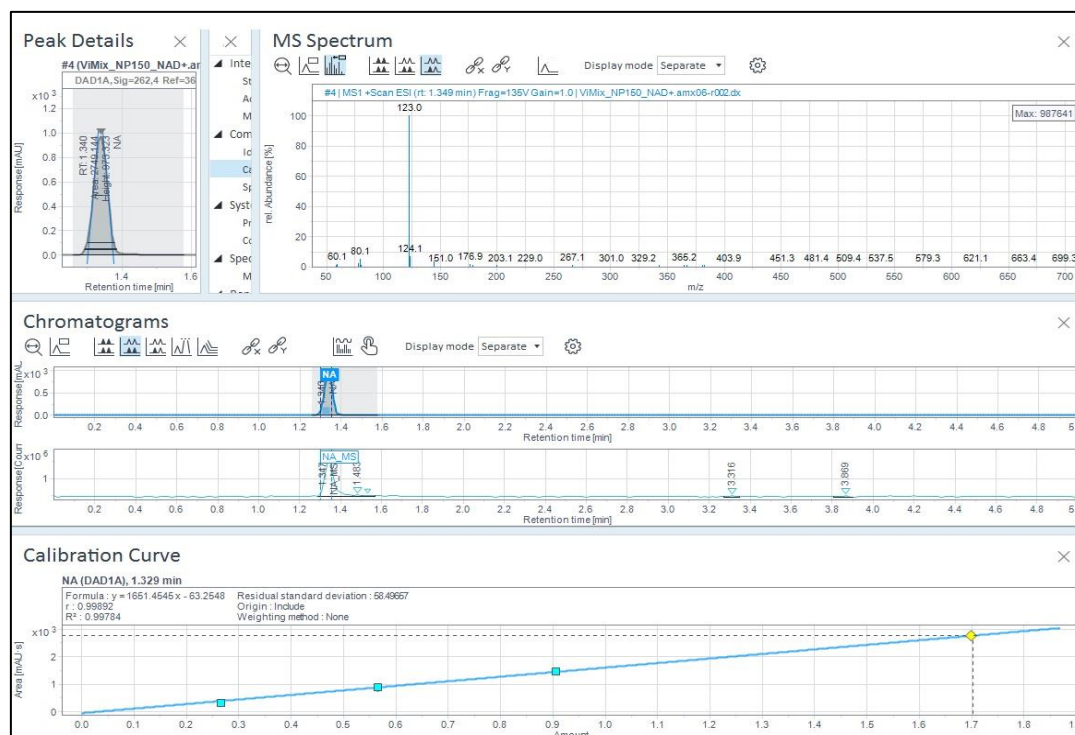


Figure 2: Niacinamide chromatogram, MS spectra, calibration curve, range of linearity

As a control we use the freshly prepared NAD⁺ solution. Analysis of this solution reveals that it contains 98.6% of API and small, not quantitatively detected niacinamide (NA) contamination (Table1., Figure 1 and 2).

Table 1: Control. Fresh prepared solution NAD⁺ 1.47 g/l

RT (min)	Signal Description	Area	Area %	m/z+	Recovery (%)
1.324	DAD	36.7	1.35	-	
1.341	MSD	128272.2	7.05	-	
3.230	DAD	2675.6	98.36	664	98.6
3.255	MSD	1533492.4	84.25	-	

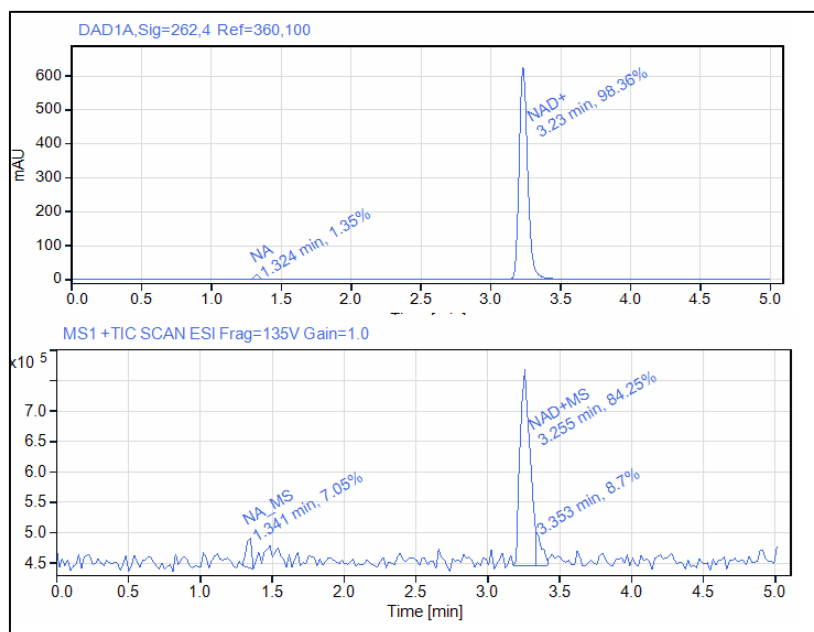


Figure 3: Control. Fresh prepared solution NAD⁺ 1.47 g/l. Chromatograms DAD and MSD

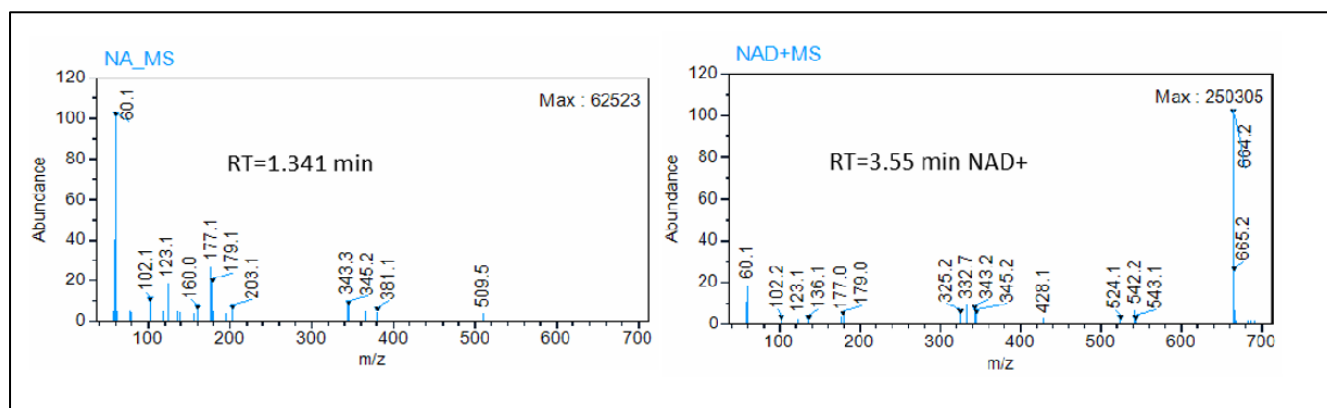


Figure 4: Control. Fresh prepared solution NAD⁺ 1.47 g/l. MS Spectra

NAD⁺ solution which was stored at 4°C for 14 months we used as an example of light chemical stress. The degradation product (NA) is detectable (Figs. 5 and 6),

and its quantity was used to calculate the material balance. Only in this single instance out of three does the material balance fall within the acceptable range (95%–105%) (Table 2, 3, 4).

Table 2: Light degradation. NAD⁺ water solution pH=7.0, 4.5 g/l, 4°C, 14 months

RT (min)	Signal Description	Area	Area %	m/z+	Recovery (%)	mass balance (%)
1.32	DAD	86.50	3.16	123.00		
1.34	MSD	234573.80	16.04	123.00		
3.22	DAD	2645.00	96.63	664.00	98.0	101.1
3.25	MSD	1227977.70	83.96	664.00		

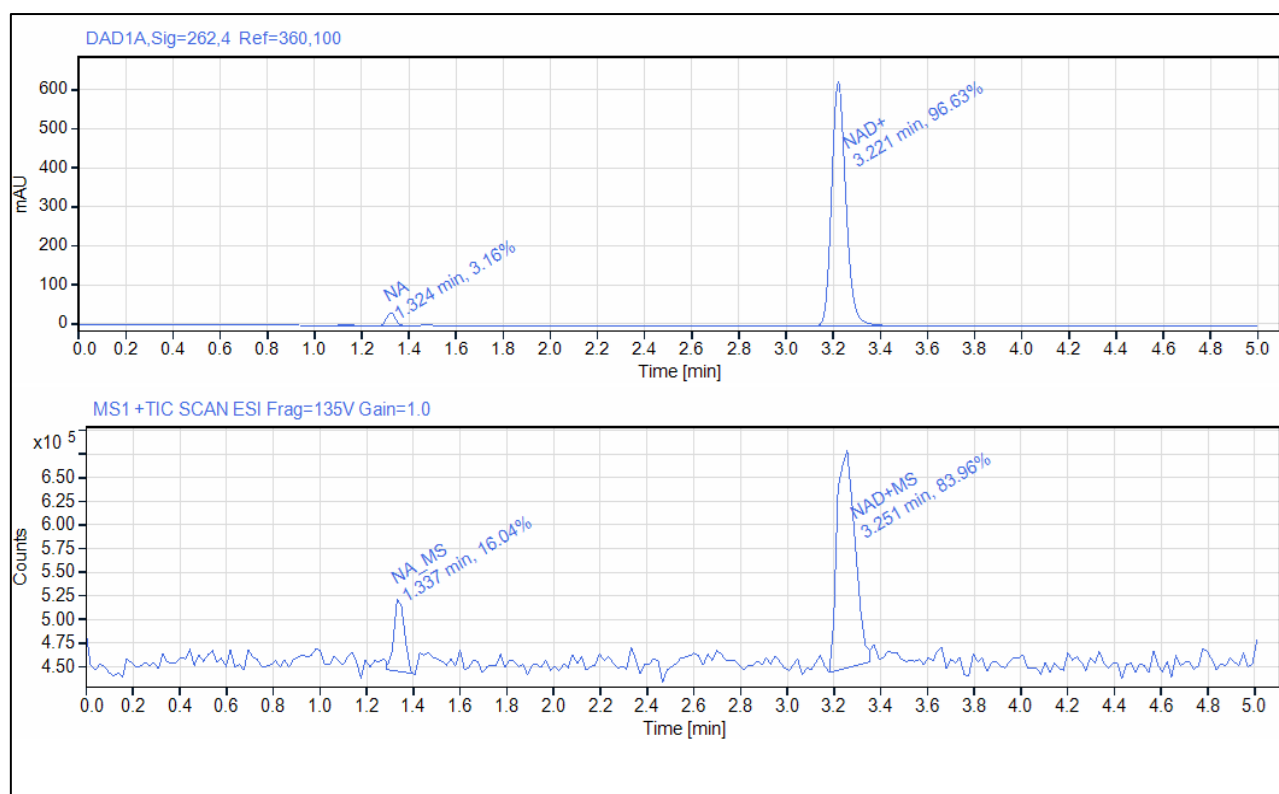


Figure 5: Light degradation. NAD⁺ water solution 4.5 g/l, 4°C, 14 months. Chromatograms DAD and MSD

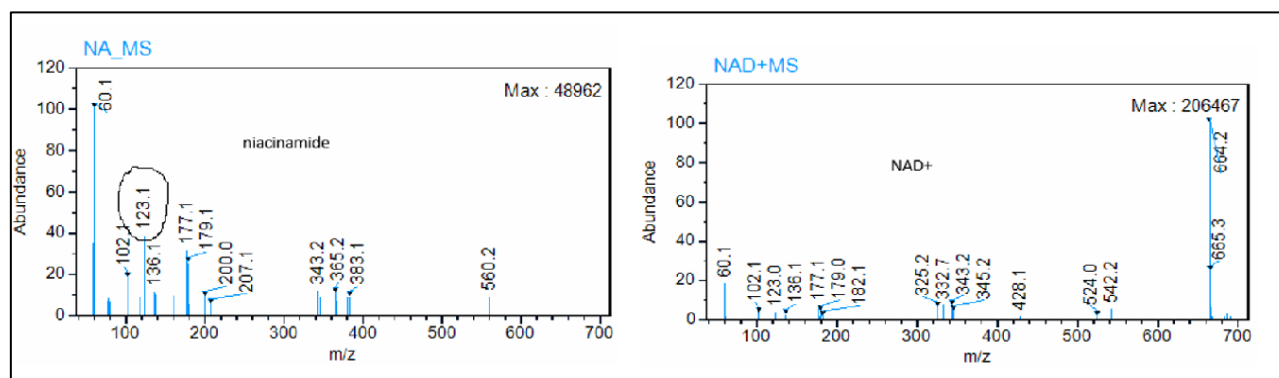


Figure 6: Light degradation. NAD⁺ water solution 4.5 g/l, 4°C, 14 months. MS Spectra

NAD⁺ is quickly degraded in basic solution (3% NH₄OH solution). After 30 minutes the NAD⁺ % recovery was only 14.6. The material balance calculation, accounting for only one known and

detectable degradation product (NA), yields a value of 30.4%. This value indicates the presence of unidentified and undetectable degradation products.

Table 3: Intermediate degradation. NAD⁺ water solution 4.5 g/l, 3% NH₄OH, 25°C, 30min

RT (min)	Signal Description	Area	Area %	m/z+	Recovery (%)	mass balance (%)
1.37	DAD	1102.10	54.57	123		
1.37	MSD	1123318.80	61.06	123		
3.12	DAD	731.20	36.21	664	14.6	30.4
3.15	MSD	401214.00	21.81	664		

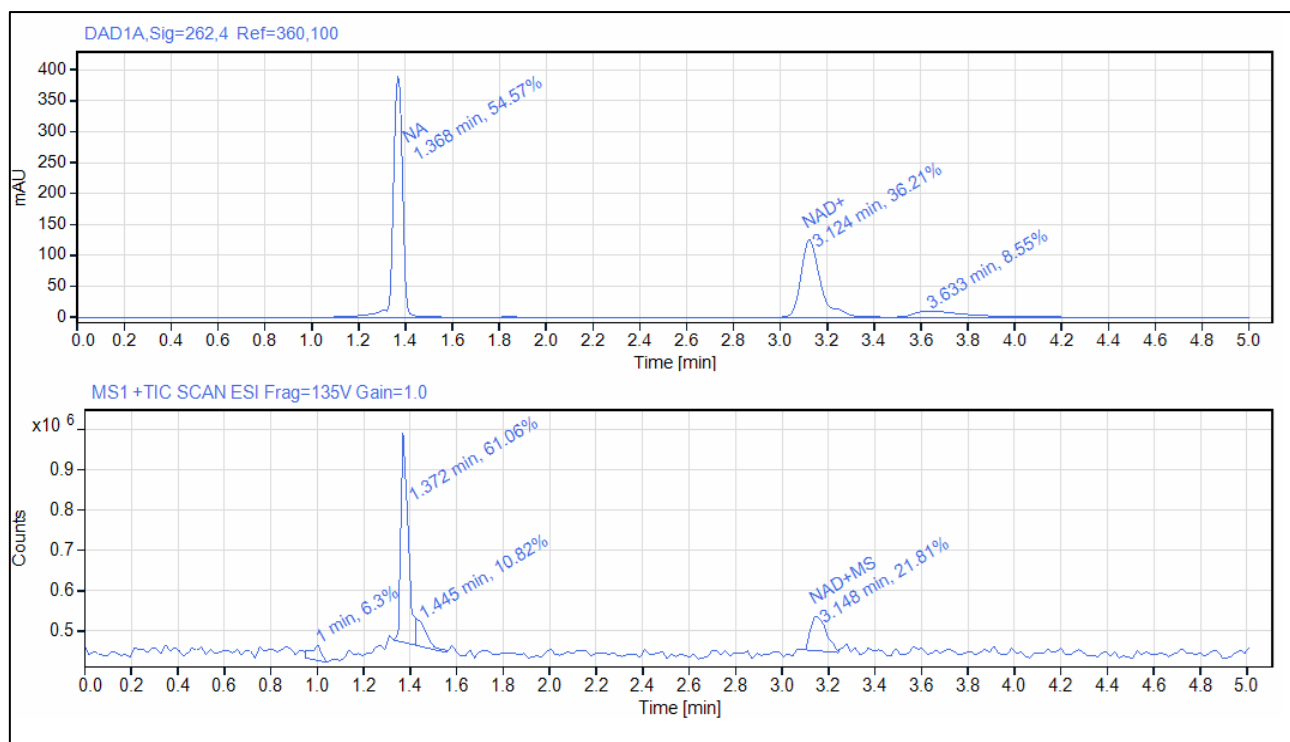


Figure 7: Intermediate degradation. NAD⁺ water solution 4.5 g/l, 3% NH₄OH, 25°C, 30min. Chromatograms DAD and MSD

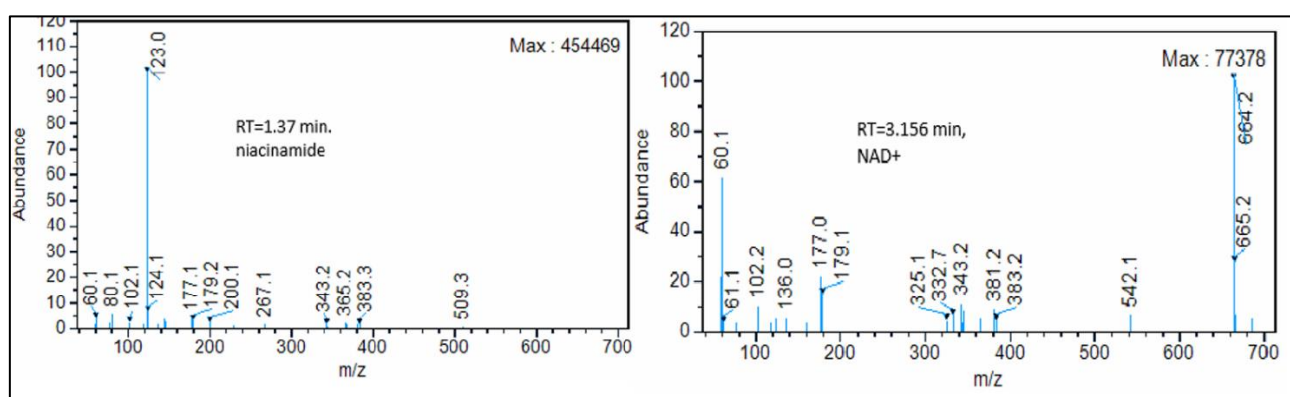


Figure 8: Intermediate degradation. NAD⁺ water solution 4.5 g/l, 3% NH₄OH, 25°C, 30min. MS Spectra.

The deepest degradation of NAD⁺ was observed in an alkaline solution (15% NH₄OH). After 60 minutes of incubation, the recovery of NAD⁺ was only 0.6%. A material balance calculation accounting for only

one known detectable degradation product (NA) yields a value of 7.3%. This figure indicates that most of the degradation products remain unidentified and undetectable (Table 4, Figures 9, 10).

Table 4: Deep degradation. NAD⁺ water solution 2.25 g/l, 15% NH₄OH, 25°C, 60 min

RT (min)	Signal Description	Area	Area %	m/z+	Recovery (%)	mass balance (%)
1.360	DAD	256.8	5.09	123	-	-
1.371	MSD	252323.4	10.88	123	-	-
3.101	DAD	4562.5	90.40	664	0.6	7.3
3.120	MSD	845843.5	36.46	664	-	-

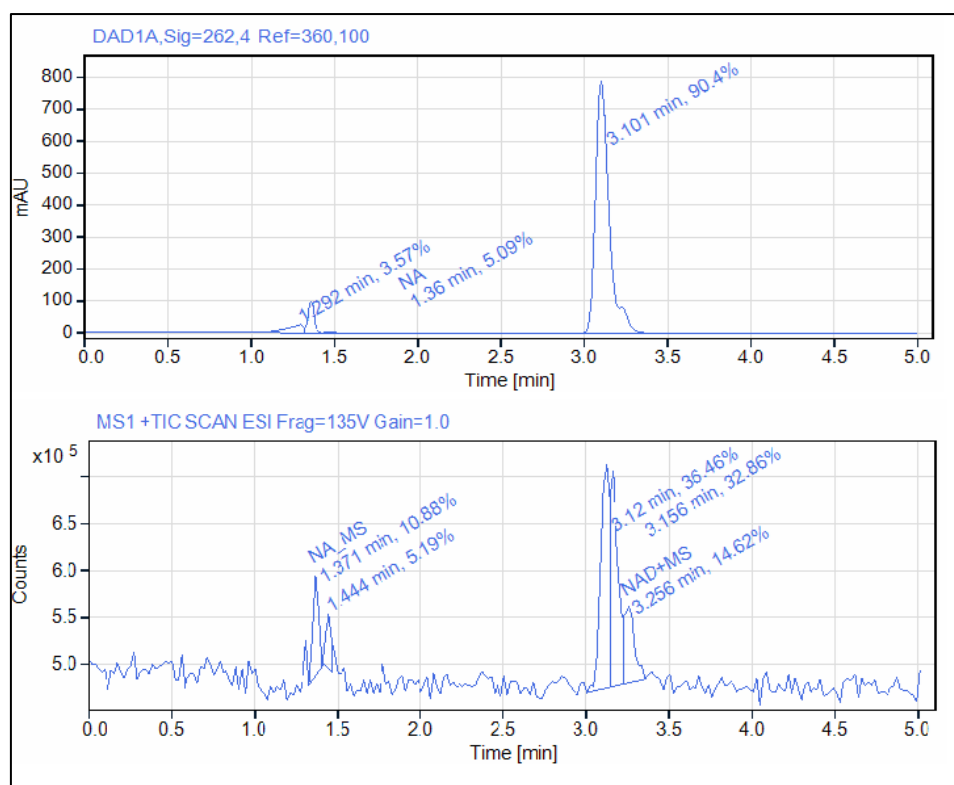


Figure 9: Deep degradation. NAD⁺ water solution 2.25 g/l, 15% NH₄OH, 25°C, 60 min. Chromatograms DAD and MSD

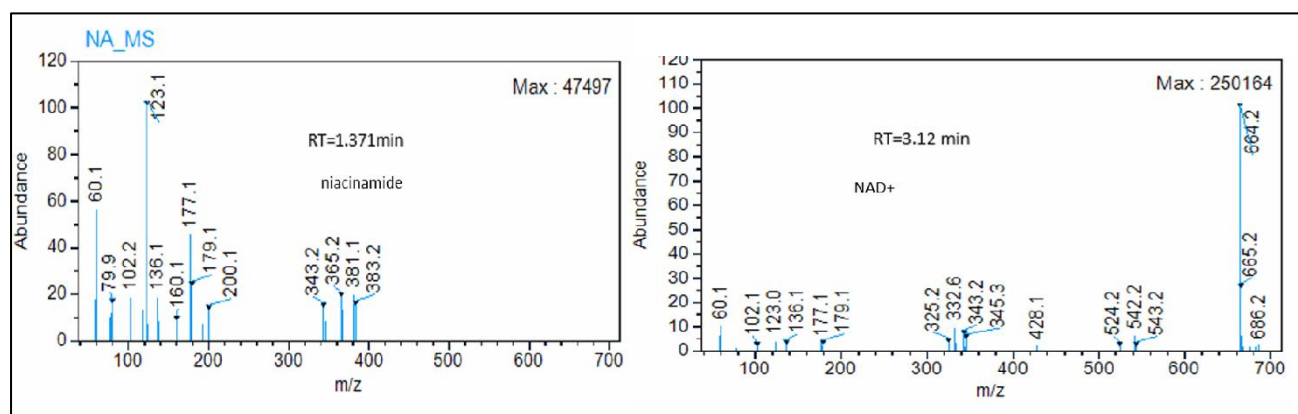


Figure 10: Deep degradation. NAD⁺ water solution 2.25 g/l, 15% NH₄OH, 25°C, 60 min. MS Spectra

The enzymatically generated breakdown products of intracellular NAD⁺ are known (Kulikova V, et al, 2019), (Hachisuka S, et al, 2017): adenosine diphosphate ribose (559.32 g/mol), adenosine monophosphate (347.22 g/mol), and ribose (230.11 g/mol). These products are not detected in the present case. Of all possible degradation products, only nicotinamide is reliably detected, as it is the most resistant to chemical stress (Yefimov S, 2024). Unlike the sequential enzymatic transformations occurring in a living cell, the chemical degradation of a pharmaceutical product is uncontrolled, and the degradation products are unpredictable. Therefore, in the general case, the mass balance calculation is impossible or incorrect. The information necessary and sufficient for pharmacists and chemists is contained in the API recovery rate following stress testing. Based on this information, conclusions are

drawn regarding the storage conditions, and measures are taken to protect the API from the identified degradative effects.

CONCLUSION

When validating analytical methods for pharmaceutical products, the mass balance value may be used as an additional, optional parameter in cases where its calculation is feasible. However, the recovery percentage of the active pharmaceutical ingredient should be considered a primary, mandatory parameter.

Abbreviations:

DAD -Diode Array Detector

MSD - mass selective detector

MP-mobile phase

RSD – relative standard deviation

SQ - single quadrupole

API - Active Pharmaceutical Ingredients

LOD – limit of detection

ACN - Acetonitrile

REFERENCES

1. Marden, S., Campbell, J., Adams, N., Coelho R., Foti, C., Franca, J., Hostyn, S., Huang, Z., Ultramari, M., Zelesky, T., Baertschi, S. (2024) Mass Balance in Pharmaceutical Stress Testing: A Review of Principles and Practical Applications. AAPS J. 26(5), 96. doi: 10.1208/s12248-024-00961-3. <https://pubmed.ncbi.nlm.nih.gov/39174806/>
2. Hong, P., McConville, P. (2016) USE OF A TRIPLE DETECTION (UV-ELSD-MS) SYSTEM FOR MASS BALANCE IN FORCED DEGRADATION OF PHARMACEUTICALS. Waters Corporation. https://labrulez.com/pdf/720008909_d5eff48c0f.pdf
3. Mackaplow, M. (2010) Using a mass balance to determine the potency loss during the production of a pharmaceutical blend. AAPS PharmSciTech. 1(3):1045-53. doi: 10.1208/s12249-010-9466-0. <https://pubmed.ncbi.nlm.nih.gov/20556674/>
4. Hachisuka, S., Sato, T., Atomi, H. (2017). Metabolism dealing with thermal degradation of NAD⁺ in the hyperthermophilic archaeon *Thermococcus kodakarensis*. J Bacteriol. 199. 162-17. <https://doi.org/10.1128/JB.00162-17>.
5. Kulikova, V., Shabalin, K., Nerinovski, K., Yakimov, A, Svetlova, M., Solovjeva, L., Kropotov, K., Khodorkovskiy, M., Migaud, M., Ziegler, M., Nikiforov, A. (2019) Degradation of Extracellular NAD⁺ Intermediates in Cultures of Human HEK293 Cells. Metabolites. 9(12). 293. doi: 10.3390/metabo9120293
6. Yefimov, S. (2024) HPLC/MS Analysis of Riboflavin Sodium Phosphate Consisting of Several Structural Isomers Together with Thiamine HCL, Pyridoxine HCL, Dexpanthenol, and Nicotinamide in A Multi-Vitamin Injection Solution. Sch Acad J Pharm, 13(2): 42-54. DOI: 10.36347/sajp.2024.v13i02.001