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Eco-Friendly Direct GC–MS Method for Estimation of Niacin and Related Impurities Involving Pyridine in Food Supplements

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Abstract: Niacin is a water-soluble vitamin whose deficiency causes many disorders and diseases, including pellagra and high blood cholesterol. Herein, niacin and four common impurities, isonicotinic acid (ISO), 5-ethyl-2-methylpyridine (MP), pyridine-2, 5-dicarboxylic acid (PDC) and pyridine PYR, are simultaneously analyzed, where PYR is known as potentially nephrotoxic and hepatotoxic. The separation of a mixture using gas chromatography–mass spectroscopy (GC–MS) without any derivatization steps was the main target. Many studies have been published to study Niacin and its impurities using colorimetry and HPLC. GC–MS was selected to study the analyzed mixture owing to its known sensitivity and selectivity. In this study, a single quadrupole mass spectrometer operated in selected ion monitoring (SIM) mode at an electron ionization energy of 70 eV was applied for the quantitative analysis of Niacin. Helium was used as the carrier gas and adjusted to run through an HP-5ms (5%-phenyl)-methylpolysiloxane column. Statistical analysis proved that this method is equally effective as the previously reported method. Importantly, this study was eco-friendly as compared to the reported high performance thin layer chromatographic method (HPTLC). Finally, this study provides a new and valid eco-friendly method analysis to determine the concentration of niacin and its common impurities at very low concentration. Conclusion: A simple, rapid accurate and green GC–MS analytical method was developed and validated to determine niacin and its related official impurities.

Keywords: gas chromatography–mass spectroscopy; niacin; pyridine; selected ion monitoring

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1. Introduction

Pyridine-3-carboxylic acid is the IUPAC name of water-soluble vitamin B3 [1], also known as nicotinic acid or niacin (NIA) [1]. Pellagra is a disease that is most prevalent in the world's poorest communities where malnutrition prevails [2]. A severe cellular deficiency of NIA is the main cause of pellagra, which is also known as the 4 "D's": diarrhea, dermatitis, dementia, and death [2,3]. Niacin is the dietary precursor for NAD^+ (nicotinamide adenine dinucleotide), which is a central cofactor for cellular metabolism [4] and essential for DNA synthesis [4,5]. The supplemental form of NIA is the major medication used to treat pellagra. Niacin is also an efficient agent for reducing low-density lipoprotein cholesterol levels and elevating high-density lipoprotein [6,7]. Hence, it is used alongside other lipid-lowering medications to treat high blood cholesterol [6,7].

As an essential nutrient, NIA has attracted significant attention from researchers for a long time. In the 1930s, NIA was successfully determined in foodstuffs using colorimetric methods [8,9]. Several analytical studies followed, using TLC [10–14], spectrophotometric [15–18], and HPLC methods [19–21]. Capillary electrophoresis and HPLC methods were

developed to determine NIA in foods [22] via autoclaving at 121 °C in the presence of 0.8 M sulfuric acid for 2 h. Potentiometric and voltametric studies have also been reported in the literature [23,24]. Although the thermal stability of NIA was a controversial topic [25], many gas chromatographic methods were developed for the determination of NIA in food samples and in enzymes, relying on short analysis time, low molecular weight of NIA and high volatility [25,26]. Pyridine (PYR) is the main impurity in commercial NIA [1]. With respect to the literature, PYR has been reaffirmed to be pneumotoxic, hepatotoxic, and nephrotoxic [27,28]. Additionally, PYR is also a precursor of illegal drugs; for example, it is used in amphetamine and amphetamine-like drugs [29]. In addition to PYR, isonicotinic acid (ISO), 5-ethyl-2-methylpyridine (MP), and pyridine-2, 5-dicarboxylic acid (PDC) have been reported as common NIA impurities; see Figure 1 [1].

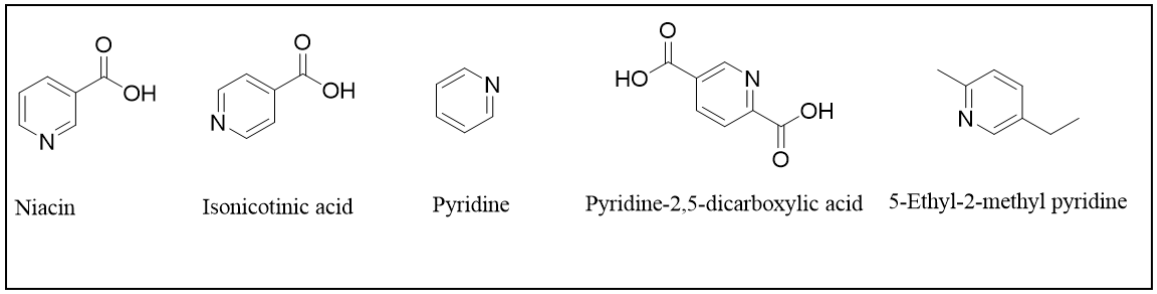

Figure 1. The chemical structures of nicotinic acid and its official impurities.

Few analytical studies concerned with the determination of NIA and its impurities have been reported. Recently, the quintuple mixture containing NIA and its official impurities was analyzed by high performance thin layer chromatographic method (HPTLC) [30] and chemometric models [31]. The presented study is designed to suggest and validate a gas chromatography–mass spectroscopy (GC–MS) method to separate and assess NIA and its related impurities with high accuracy and sensitivity, applying a GC method coupled with a mass spectrometer offering high sensitivity and additional confirmatory results. Unlike previously reported, direct GC analysis offers specificity and sensitivity, reproducibility, linearity and saves costs and time. GC–MS is known for its sensitivity to quantify very small concentrations of compounds (up to pbb or less) and to identify unknown compounds from a complicated matrix [32]. Owing to the high resolution, sensitivity, precision and accuracy of the GC–MS method, it has separative and quantitative features to assess small concentrations of analytes [33,34]. In addition, this study aims to implement the established GC–MS methods to quantify NIA in the marketed pharmaceutical formulation as part of quality control analysis.

Moreover, the presented work aims to offer validation of the developed method by applying USP guidelines [35] and a statistical comparison with a previously reported method [20] to verify its ability to conduct routine analysis of the studied drug.

Achieving a balance between the environmental impact and separation capacity of the analytical method represents great challenges for any analyst. Accordingly, an eco-scale assessment for the newly developed GC–MS and previously reported HPTLC method [30] is conducted to prove the relative greenness of the introduced method.

2. Experimental Analysis

2.1. Instruments

The analyses of NIA, PYR, MP, PDC and ISO samples were performed using a gas chromatograph (TRACETM 1310 GC) equipped with a single quadrupole mass spectrometer (ISQ LT) and AI/AS1310 auto-sampling unit (Thermo Scientific, Waltham, MA, USA). To assist compound identification, the NIST Standard Reference Database 1A was used. Analysis was achieved using an Agilent (19091S-433: 2330.46415) HP-5ms (5%-phenyl)-methylpolysiloxane column (30 m × 250 µm I.D. × 0.25 µm film thickness). The maximum

temperature of the oven of the GC–MS was 325 °C, within which temperature programs were used to optimize separation efficiency.

The Xcalibur program (Thermo Scientific, USA) version 3.1 was used for data analysis.

2.2. Materials and Reagents

2.2.1. Standards

Niacin was kindly supplied by the Amoun Pharmaceutical Company, Obour city, Cairo, Egypt, with a purity of 99.70% according to the company certificate. PYR, PDC, and ISO standards were purchased from Acros Organics Company, Geel, Antwerp, Belgium, with purity of 98% for PYR, and PDC, and 99% for ISO according to the company's certificate. Alfa Aesar Company, Kandel, Germany, was the supplier of MP with a purity of 97% according to the certificate.

2.2.2. Pharmaceutical Formulation

NIACIN® tablets (batch no. 152405-05) were manufactured in the USA by Solgar, Inc., Leonia, NJ, and each tablet was claimed to deliver 100 μg of NIA.

2.2.3. Chemicals and Solvents

HPLC-grade ethanol was purchased from Sigma Aldrich, Darmstadt, Germany.

2.3. Standard Solutions

2.3.1. Stock and Working Standard Solutions

Stock standard solutions of NIA, PYR, MP, PDC, and ISO were prepared separately by accurate weighting of 0.1 g of the respective standards. In sequence, the weighed portions were added to five separate 100 mL flasks. For ultimate dissolution and environmental considerations, ethanol was used as the solvent to prepare 1000 μg mL^{-1} stock solutions. First, 10 mL of each stock solution was accurately transferred to five separate 100 mL flasks to prepare the five respective 100 μg mL^{-1} working standard solutions.

2.3.2. Solutions of Pharmaceutical Formulation

Hard grinding of 20 NIACIN® tablets was performed after accurate weighting using a four-digit balance. An accurately weighed equivalent portion (100 mg) of NIA was placed into a 100 mL volumetric flask, and 75 mL of ethanol was subsequently added to the flask. Ultrasonication of the prepared solution was conducted for 30 min to achieve maximum dissolution. The solution was left to cool at 25 °C for a sufficient time, and ethanol was added to the flask to reach the final volume. Finally, filtration was performed to obtain a 1000 μg mL^{-1} stock solution.

A working solution of 100 μg mL^{-1} was prepared via proper dilution of the stock solution. To apply the standard addition technique, homogeneous blending of the pure standard of NIA with the ground tablets was necessary before repeating the previously described procedure.

2.4. Chromatographic Conditions

Analysis of NIA, PYR, MP, PDC and ISO was achieved using an Agilent (19091S-433: 2330.46415) HP-5ms (5%-phenyl)-methylpolysiloxane (30 m × 250 μm I.D. × 0.25 μm film thickness) column. Helium was pumped as the carrier gas, and the injection volume was 1 μL. Several temperature programs were studied to achieve maximum sensitivity and selectivity. The initial temperature of the oven was firstly set at 120 °C, but the peaks were very broad and tailed, so more decreased temperatures were used to give sharp peaks until reaching a temperature of 90 °C. The best sharp, symmetric peaks were obtained by applying 90 °C as an initial temperature, which was increased gradually to 200 °C according to the specific temperature program mentioned before in the experimental section. Additionally, various trials concerning different helium carrier gas flow rates, such as

0.8, 1 and 1.2 mL min^{-1}, were conducted. Full separation of the five analyzed components with minimal peak tailing was obtained upon application at a flow rate of 1.2 mL min^{-1}.

A total of 9 min was required by the method to analyze the mixture. The injector and transfer line had controlled temperatures of 250 and 280 °C, respectively. Selected ion monitoring (SIM) mode was applied to perform the analysis via electron ionization with 70 eV energy. The injector was used in split-less mode during the entire analysis.

2.5. Calibration Curve Construction

Numerous concentrations of NIA, PYR (its toxic impurity), MP, PDC and ISO, in the ranges 0.5–20 μg mL^{-1}, 0.005–5 μg mL^{-1}, 0.1–5 μg mL^{-1}, 0.5–20 μg mL^{-1} and 0.5–20 μg mL^{-1}, respectively, were prepared via proper dilutions of their corresponding standard working solutions using ethanol as a solvent. Triple injections into the GC–MS for every concentration were performed following the detailed separation procedure (Section 3.1). A calibration graph of each analyte was constructed using the recorded peak areas, and the linear regression equations were calculated.

2.6. Application to Pharmaceutical Formulation

Solutions of the marketed dosage form were freshly prepared before applying the clarified steps under construction of the calibration curve.

3. Validation of the Newly Developed Method

It should be noted that validation of the suggested method was accomplished in view of the USP guidelines [35].

3.1. Linearity

Using the proposed chromatographic conditions, the linearity of the developed method was confirmed by determining the integrated peak area of different concentrations of NIA, PYR, MP, PDC and ISO. Sequentially, calibration graphs were constructed by plotting the peak area as a function of concentration, and the regression equations were then determined.

3.2. Accuracy

The accuracy of the GC–MS method was examined by implementing the introduced method to assess numerous concentrations of pure standards of NIA, PYR, MP, PDC and ISO. By applying the corresponding regression equation of each analyte (Table 1**,** the concentrations of the analyzed samples were obtained; see Table 2. To further confirm the accuracy of the presented method, a standard addition technique was implemented, which resulted in good recoveries, confirming no interventions with excipients; see Table 3.

Table 1. The regression equations and retention times of the separated ingredients.

Components of the Analyzed Mixture	Regression Equation *	Retention Time
NIA	$A_1 = 0.\ 9636\ C_1 + 0.5158$	5.68
PYR	$A_2 = 36.61\ C_2 + 8.2884$	1.67
MP	$A_3 = 10.15\ C_3 - 0.5745$	3.57
PDC	$A_4 = 0.4934\ C4 - 0.0726$	7.00
ISO	$A_5 = 1.1665\ C5 + 0.0874$	7.76

* A_1, A_2, A_3, A_4 and A_5 are the respective integrated peak areas × 10^{-6}; C_1, C_2, C_3, C_4 and C_5 are the concentrations in μg mL^{-1}; r_1, r_2, r_3, r_4 and r_5 are the regression coefficients of NIA, PYR, MP, PDC and ISO, respectively.

Table 2. Analytical validation parameters of the introduced gas chromatography–mass spectroscopy (GC–MS) method for analysis of the quintuple mixture of PYR, MP, NAI, PDC and ISO.

GC–MS Method					Parameter
ISO	**PDC**	**MP**	**PYR**	**NIA**	
0.5–20	0.5–20	0.1–5	0.005–5	0.5–20	Range (μg mL^{-1})
1.1665	0.4934	10.1500	36.6100	0.9636	Slope
0.0874	−0.0726	−0.5745	8.2884	0.5158	Intercept
0.9999	0.9998	0.9999	0.9999	0.9997	Regression coefficient
101.03 ± 1.729	100.95 ± 2.100	100.17 ± 1.064	99.69 ± 1.607	100.49 ± 0.754	Accuracy (Mean ± SD)
2.074	1.305	1.719	1.613	1.513	Precision - Repeatability (RSD%) [a]
2.464	2.434	2.200	2.515	2.122	- Intermediate precision (RSD%) [b]
0.139	0.136	0.030	0.001	0.121	LOD (μg mL^{-1})
0.460	0.450	0.098	0.003	0.400	LOQ (μg mL^{-1})

[a] The intraday precision, mean of 3 different concentrations reanalyzed 3 times during the same day. [b] The intermediate precision, mean of the same three different concentrations reassessed 3 times during 5 consecutive days.

Table 3. Quantitative analysis of NIA in the pharmaceutical tablets via the developed method and applying of standard addition technique.

Recovery % [b]	**Pure Found (μg mL^{-1})**	**Pure Added (μg mL^{-1})**	**Found% [a] Mean ± SD)**	**Claimed Taken (μg mL^{-1})**	**Niacin® Tablets, Batch No. 152405-05**
98.67	2.96	3.0	100.11 ± 1.755	5.0	NIA
99.60	4.98	5.0			
99.33	9.93	10.0			
99.20 ± 0.391	Mean ± SD				

[a] Average of 6 determinations. [b] Average of 3 determinations.

3.3. Precision

The repeatability and intermediate precision for each analyte were tested to confirm the precision of the newly suggested method [35].

Repeatability

Three concentrations of NIA, PYR, MP, PDC and ISO were analyzed on the same day in triplicate using the proposed method. The chosen concentrations for NIA, ISO and PDC were 5, 10 and 20 μg mL^{-1} respectively. For PYR and MP, the chosen concentrations were 0.5, 1 and 2 μg mL^{-1}, respectively. Good RSD% values confirmed the repeatability of the proposed method; see Table 2.

3.4. Intermediate Precision

Over five successive days, the aforementioned procedure was reapplied to analyze the selected concentrations of the studied analytes. Good RSD % values were gained and are listed in Table 2.

3.5. Specificity

Full separation of the five injected components using the proposed GC–MS method showed the specificity of the newly developed method; see Figure 2. In addition, the ability of the proposed method to test the component of interest in a specific and accurate manner

in existence with other interfering ingredients (Table 3) was tested. Acceptable selectivity and resolution values were obtained; see Table 4.

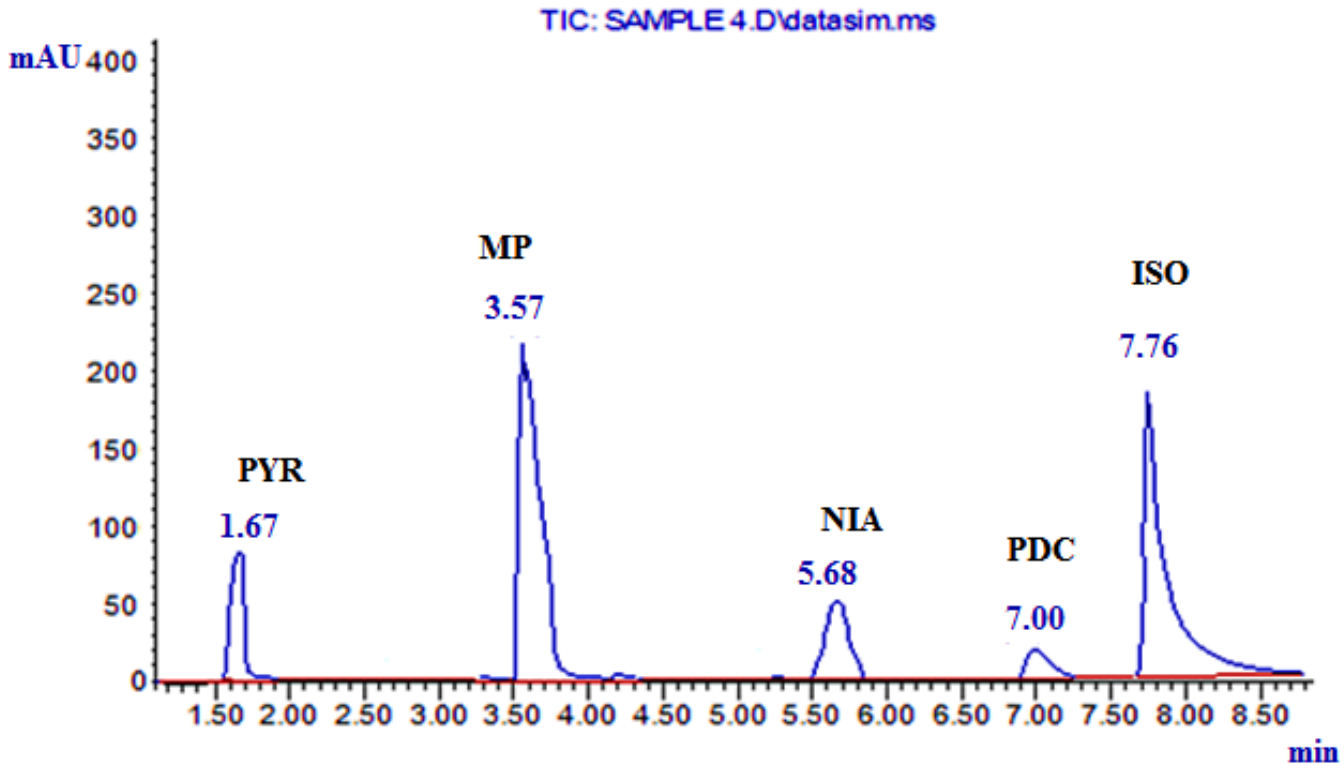

Figure 2. GC chromatogram of the quintuple mixture of NIA (5 µg/mL), PYR (0.05 µg/mL), MP (0.1 µg/mL), PDC (0.5 µg/mL) and ISO (10 µg/mL).

Table 4. System suitability testing parameters of the suggested method for quantitative estimation of quintuple mixture of NIA and its related impurities.

Reference Values [35]	GC/MS								Parameter
	ISO	PDC	NIA	PDC	NIA	MP	PYR	MP	
~1	1.5	1.3	1	1.3	1	1.4	1.3	1.4	Tailing factor (T)
1–10 Acceptable	6.76	6	4.68	6	4.68	2.57	0.67	2.57	Capacity factor (K′)
$R_s > 1.5$	3.185		6.26		2.64		1.67		Resolution (R_S)
>1	3.84		1.82		1.28		1.13		Selectivity(α)
Increase with efficiency of the separation	214,285.71	35,714.29	11,718.75	35,714.29	11,718.75	7092.2	3348.2	7092.2	Column efficiency (N)
The smaller the value the higher the column efficiency	0.014	0.084	0.256	0.084	0.256	0.423	0.896	0.423	HETP = height equivalent to theoretical plate, (cm/plate)

Limits of Detection and Quantitation (LOD and LOQ)

The low obtained values of LOD and LOQ were indicators of the high sensitivity of the proposed method (Table 2), but the presented method is only capable of the impurity testing of pyridine. Both LOD and LOQ were assessed mathematically [35]; LOD = 3.3 × (SD of the response/slope) and LOQ = 10 × (SD of the response/slope).

3.6. System Suitability

System suitability testing was performed based on the concept that analytical procedures, samples, equipment and electronics work as a comprehensive system that can be assessed in its entirety [35]. It is supposed to assess the system performance before and within the time of analysis. Resolution (Rs), peak asymmetry and selectivity factor (α) were examined.

4. Results and Discussion

The quintuple mixture under study was assessed by chemometric models, which were unable to determine all analytes in the mixture, as they determined the active pharmaceutical component (NIA) only [21]. Regarding chromatographic methods, the recently developed HPTLC method was able to separate and assess the mixture of interest [30]. Nonetheless, the developed chemometric and chromatographic methods were less sensitive compared to the presented GC–MS method.

The novelty of this study is driven by the ability of the developed GC–MS method to separate and quantify the studied substances with respect to the USP guidelines [36]. It offers high sensitivity and selectivity for the presented mixture compared to previously published chromatographic methods. The introduced GC–MS method was rapid, direct, sensitive and more eco-friendly as compared to the reported HPTLC method.

4.1. Method Development and Optimization

Several temperature programs were studied to achieve maximum sensitivity and selectivity. The initial temperature of the oven was firstly set at 120 °C, but the peaks very broad and tailed, so more decreased temperatures were used to give sharp peaks until reaching temperature of 90 °C. The best sharp, symmetric peaks were obtained applying 90 °C as an initial temperature, which increased gradually to 200 °C according to the specific temperature program mentioned before in the experimental section. Additionally, various trials concerning different helium carrier gas flow rates, such as 0.8, 1 and 1.2 mL min^{-1}, were conducted. The full separation of the five analyzed components with minimal peak tailing was applying at a flow rate of 1.2 mL min^{-1}. The implemented temperature programs and all the analytical conditions are detailed in the experimental section. Pyridine first appeared at 1.67 min, followed by MP, NIA, PDC and ISO at 3.57, 5.68, 7.00 and 7.76 min, respectively; see Figure 2. The resulting chromatogram showed sharp and symmetric peaks for all analytes, except the ISO peak, which had slight tailing that remained within the permissible limits according to the USP guidelines [36].

For more confirmatory data, a mass spectrometer with electron impact ionization/selective ion monitoring (ES/SIM) mode was used. The mass spectra of the five analytes are shown in Figure S1 (Supplementary Materials). Fragments (*m/z*) were used to quantitatively assess both the active ingredient and impurities. The major mass fragments for NIA and ISO were 123, 105 and 78 *m/z*, while those of PYR were 79, 52 and 51 *m/z*. For MP, the major mass fragments were 106, 121 and 79 *m/z* and those for PDC were 167, 123, 51 and 44 *m/z*.

The calibration curve of each analyte was constructed by plotting the integrated peak area $\times 10^{-6}$ against the respective concentration. The regression equation of each separated ingredient was subsequently calculated; see Table 1. The regression equation parameters are listed in Table 2.

4.2. Results of Figures of Merit

The developed GC method was validated according to the USP guidelines [35], and the results for linearity, accuracy and specificity are presented in Table 2, affirming the validity of this method.

For further evaluation of accuracy, the standard addition method was applied, and the results are presented in Table 3, where perfect recoveries of the pure added fractions were obtained, confirming the accuracy of the method.

Precision was validated at two levels, the repeatability and intermediate precision. The results presented in Table 2 show low RSD% for both affirming low deviations for intraday determinations and day to day variations as well.

Very low limits of detection and quantitation were obtained for the main drug (NIA) and its impurities, as indicated in Table 2, especially the toxic one (pyridine), which could be detected at 0.001 μg mL^{-1} and quantified at 0.003 μg mL^{-1}, indicating the very high sensitivity of the method.

Resolution (Rs), peak asymmetry, capacity factor, tailing factor, HETP and selectivity factor (α) were examined to ensure the system performance and suitability of the suggested method, and results are acceptable and fully presented in Table 4. The system suitability results for the main drug (NIA) were optimum regarding peak symmetry (tailing factor) and resolution from all other peaks.

4.3. Results of Assay of Dosage Form

The results of the assay of the drug product provided optimum recovery for NIA with low standard deviation. Finally, a statistical comparison between the results obtained from the suggested method, previously reported HPLC method and recently developed HPTLC method was performed; see Table 5 [20,30]. The hypothetical t and F values were larger than those that were experimentally obtained, confirming no significant differences between the two methods in terms of both accuracy and precision.

Table 5. Statistical analysis of the values resulted by the developed among the suggested GC–MS method, reported HPLC and reported high performance thin layer chromatographic method (HPTLC) method for assessment of NIA in its commercial dosage form.

Parameter	Reported HPTLC Method [31] **	GC/MS	Reported HPLC Method [21] ***	GC/MS
Mean	99.82	100.11	99.74	100.11
SD	1.673	1.922	0.965	1.922
Variance	2.797	3.697	0.931	3.697
n	6	6	6	6
Student's *t*-test * (2.228)	0.283		0.430	
F-test * (5.050)	1.322		3.970	

* Figures in parenthesis are the corresponding tabulated values at $p = 0.05$. ** The separation was achieved on HPTLC sheets using mixture of ethyl acetate: ethanol: ammonia solution (6: 4: 0.05, by volume) then the dried plates were scanned at 254 nm. *** $C_{18\text{-A}}$ (150 × 4.6 mm, 3 μm particle size) in a single run using combined isocratic and linear gradient elution with a mobile phase consisting of 0.010% trifluoroacetic acid of pH 3.9 (solvent A) and methanol (solvent B) at the flow rate 0.7 mL min^{-1} and UV detection at 280 nm.

4.4. Eco-Scale Assessment of Proposed Method

An eco-scale semi-quantitative tool [36] was recently developed to assess the greenness capacity of analytical procedures [37,38]. An eco-scale comparative study of the newly developed GC/MS method, the reported HPLC [20] and recently published HPTLC [30] method was performed, demonstrating the higher greenness value of the newly presented method; see Table 6. The total score was obtained for each method after subtracting the penalty points of all involved parameters. The total scores obtained were 75, 89 and 92 for the reported HPLC method, the recently published HPTLC method and the introduced GC/MS method, respectively. The proposed method proved to be the greenest analytical method.

Table 6. Ecological comparison among the suggested GC–MS method, reported HPLC and reported HPTLC method applying eco-scale assessment method.

Reagent/Instruments	Penalty Points		
	Reported HPLC Method [20]	**Reported HPTLC Method [30]**	**Proposed GC–MS Method**
Helium	-	-	1
Ethylacetate		4	-
Ethanol		2	-
Methanol	12	-	-
Triflouroacetic acid	8	-	-
Ammonia solution	-	2	-
Technique (Energy used)	0	0	2
Occupational hazard	0	0	0
Waste	5	3	5
Total penalty points	Σ25	Σ11	Σ8
Analytical Eco-scale Total score	75	89	92

5. Conclusions

The mixture of the active ingredient (NIA) and common impurities involving toxic PYR were analyzed by the newly developed simple and selective GC–MS method. The uniqueness of the developed method is its ability to separate and quantitatively analyze the five studied components without intricate derivatization steps and within 9 min. Using the presented method, the components of interest could be assessed at very low concentrations. In addition, the validation of the proposed method was conducted according to the USP guidelines. In addition, environmental aspects were considered by using ethanol, a well-known green solvent, and decreasing waste by ensuring a short analysis time. The total eco-score of the proposed method was higher than that of the previously HPTLC method, confirming its higher greenness capacity. The capacity of the developed method for application in the routine analysis of pharmaceutical formulations containing NIA was confirmed via the successful determination of NIA in NIACIN® tablets, because no interference from the additives was observed.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/separations8040046/s1, Figure S1: Mass spectra of NIA, PYR, MP, PDC and ISO.

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Abbreviations

GC–MS	Gas chromatography–mass spectrometry
ISO	isonicotinic acid
MP	5-ethyl-2-methylpyridine
NIA	niacin
PCD	pyridine-2, 5-dicarboxylic acid
SIM	selected ion monitoring
USP	United States pharmacopeia

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