

Research Article**Simultaneous determination of nine nitrosamines in fermented vegetables by GC-MS/MS**

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Abstract

In this study, nine nitrosamines were identified in fermented vegetables by gas chromatography tandem mass spectrometry (GC–MS/MS), using NDMA-d6 as the internal standard and a DB–17MS capillary column (30 m × 0.25 mm × 0.25 μm). The QuEChERS sample preparation procedure was optimized to achieve improved extraction efficiency for the analytes. The extracted sample was injected in splitless mode with a volume of 2 μL, with helium as a carrier gas at 1 mL/min, an EI ionization source at 280°C, and a collision energy of 50 eV. The method was validated according to EC 2021/808, showing good selectivity; a calibration curve was generated over the range of 0.5–20 ng/mL with correlation coefficient $R^2 \geq 0.995$; recoveries ranged from 83.0 to 119.6%; relative standard deviation (RSD) and relative standard deviation for repeatability (RSDr) ranged from 4.62–11.06% and 7.65–11.9%, respectively, meeting the acceptance criteria. The limits of detection and quantification were 0.15 μg/kg and 0.5 μg/kg, respectively. The method was applied to analyze 108 fermented vegetable samples from the market, of which 14 samples were found to contain nitrosamines. The nitrosamines detected were mainly NDMA, NDEA, and NDBA, consistent with published results related to nitrosamine contamination in fermented foods.

Keywords: Nitrosamine, GC–MS/MS, QuEChERS, fermented vegetables.

1. INTRODUCTION

Cancer is currently one of the leading global public health concerns. According to GLOBOCAN 2022, Vietnam recorded about 180,000 new cases and more than 120,000 cancer-related deaths [1]. In addition to genetic predisposition, most cancer cases are associated with environmental factors, with diet being a significant source of carcinogen exposure. During the processing and storage of commonly consumed foods, various harmful compounds may be generated, potentially increasing cancer risk upon long-term exposure. Nitrosamines are a group of compounds of particular concern due to their potential carcinogenicity, which could be formed in foods when nitrates react with amines under high-temperature or acidic conditions. Nitrite groups may be added directly as preservatives or produced naturally during fermentation. Consequently, nitrosamines are commonly detected in heat-processed and fermented foods, including bacon, cured meats, processed fish, and pickled vegetables. Several previous studies have employed GC–MS/MS techniques to quantify nitrosamines at trace levels in various food matrices.

In Vietnam, previous research has primarily focused on thermally processed foods, whereas data on nitrosamines in fermented vegetables remain limited. According to the International Agency for Research on Cancer (IARC), NDEA and NDMA are classified as Group 2A carcinogens; while NMEA, NPYR, NDBA,

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NPIP, and NMOR belong to Group 2B, and NDPhA is in Group 3 [2]. The concentration limits for nitrosamines are established based on the acceptable daily intake (ADI) of each compound and the recommended daily intake limits for N-nitrosamines, as outlined in FDA guidance. Accordingly, the determined limits are 1.92 ng/mL for NDEA, 0.53 ng/mL for NDMA, and 0.53 ng/mL for NDBA, whereas specific limits for the remaining nitrosamines have not yet been defined [2].

The incidents of nitrosamine contamination in valsartan, ranitidine, and metformin from 2018 to the present have continued to underscore the importance of controlling these compounds in both pharmaceuticals and food products. Major sources of nitrosamine exposure include foods such as meat, heat-processed fish, fermented beverages, and certain vegetables [3, 4]. In foods, this process occurs when nitrite, under acidic conditions, forms nitric anhydride (N_2O_3), which subsequently reacts with amines to produce nitrosamines [5]. In fermented vegetables, nitrite may be formed naturally through bacterial denitrification, thereby increasing the risk of nitrosamine formation during the fermentation process.

Although various analytical techniques for nitrosamines have been reported - including GC-TEA, LC-MS/MS, and GC-MS/MS, GC-MS/MS remains the most suitable approach for the determination of volatile nitrosamines due to its high sensitivity and excellent selectivity. In the context of limited domestic data and the growing need for nitrosamine monitoring in Vietnam, this study was undertaken with two objectives: (i) to develop and validate a GC-MS/MS method for the simultaneous quantification of nine nitrosamines (NDMA, NDEA, NMEA, NDBA, NDPA, NPIP, NPYR, NMOR, and NDPhA) in fermented vegetables; and (ii) to apply the validated method to evaluate nitrosamine exposure levels in selected fermented vegetable products available on the market.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

Fermented vegetable samples were randomly collected from supermarkets and food stores within the Hanoi area and used as analytical matrices. The presence of nitrosamines in the samples was assessed based on nine target analytes, including NDMA, NDEA, NMEA, NDBA, NDPA, NPIP, NPYR, NMOR, and NDPhA - compounds that have been reported to exhibit potential carcinogenicity.

EPA 8270 standard mixture containing nine nitrosamines (NDMA, NDEA, NMEA, NDBA, NDPA, NPIP, NPYR, NMOR, and NDPhA) at a concentration of 2000 $\mu\text{g/mL}$ in methanol (Supelco, USA) was used as the calibration standard. NDMA- d_6 (1000 $\mu\text{g/mL}$ in methanol, LGC, UK) was employed as the internal standard. Dichloromethane (DCM) and acetonitrile (MeCN) of analytical grade (Merck, Germany) were used as extraction solvents. Additional reagents included anhydrous MgSO_4 , NaCl, NH_4Cl , CH_3COONa , Na_2SO_4 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$, and $\text{C}_6\text{H}_6\text{Na}_2\text{O}_7 \cdot 1.5\text{H}_2\text{O}$. Adsorbents used for sample clean-up consisted of PSA, C18, and GCB (Agilent, USA), as well as Florisil (Sigma Aldrich, USA). Ultrapure water used throughout the study was obtained from a Hamilton deionized water system.

2.2. Equipment

The TRACE 1310 gas chromatography system coupled to a TSQ 9000 triple quadrupole mass spectrometer (Thermo Fisher, USA) was used for the analysis. Chromatographic separation was achieved on a DB-17MS capillary column (30 m \times 0.25 mm, 0.25 μm film thickness) (Agilent, USA). Additional laboratory equipment included an analytical balance (Mettler Toledo, Switzerland), a vortex mixer (IKA, Germany), a centrifuge (Hermle, Germany), and other standard laboratory consumables.

2.3. Method

2.3.1. Sample preparation

Based on previous studies [6, 7], the QuEChERS sample preparation procedure was selected for this study. The proposed workflow consisted of the following steps: (i) homogenize the sample and weigh 5.00 ± 0.01 g into a 50 mL centrifuge tube; (ii) add 50 μL of the internal standard solution (NDMA- d_6 , 200 ng/mL); (iii) add 5 mL of extraction solvent and 5 mL of distilled water, followed by vortex mixing for 1 min; (iv) shake horizontally for 30 min, then add the extraction salt; (v) shake vigorously for 1 min and centrifuge at 6000 rpm for 5 min at -10°C ; (vi) transfer 1 mL of the supernatant into a dispersive-SPE tube containing C18, PSA and MgSO_4

(50/50/150 mg); (vii) vortex for 1 min, centrifuge at 13,000 rpm for 5 min, and transfer 500 μ L of the cleaned extract into a GC vial; and (viii) analyze the final extract using the GC-MS/MS system.

The extraction efficiency was optimized among the extraction solvents including MeCN, MeCN–DCM mixture (1:3), MeCN–DCM mixture (1:1), and MeCN–DCM mixture (3:1) and among the extraction salts including Salt-1 (4 g MgSO_4 , 1 g NaCl, 1 g $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ and 0.5 g $\text{C}_6\text{H}_6\text{Na}_2\text{O}_7 \cdot 1.5\text{H}_2\text{O}$); Salt-2 (4 g CH_3COONa and 1 g NaCl); Salt-3 (4 g MgSO_4 and 1 g NaCl); Salt-4 (4 g Na_2SO_4 and 1 g NaCl) and Salt-5 (7.5 g NH_4Cl). The expected recovery efficiency range during the sample preparation, used as a basis for selecting optimal experimental conditions, is not expected to be less than 90%.

2.3.2. GC-MS/MS conditions

A mixed standard solution containing the nine target nitrosamines and the internal standard at a concentration of 20 ng/mL was directly injected into the GC-MS/MS system for instrument calibration and optimization. Helium was used as the carrier gas at the flow rate of 1 mL/min. The following temperature program was used for the chromatographic separation: The column was initially held at 50°C for 2 min. The temperature was then ramped to 160°C at the rate of 10°C/min. This was followed by a ramp to 280°C at 40°C/min and held for 1.5 min; Finally, the temperature was further ramped to 300°C at 40°C/min and maintained for 3 min. The injection volume was 2 μ L via the splitless injection. Transfer line temperature was set at 280°C [7].

The mass spectrometry conditions used were as follows: Electron impact ionization (EI) source with the emission current 20 μ A and at the temperature of 250°C. The analytes were optimized in the MRM mode, selecting a precursor ion and two product ions for each compound with suitable collision energies. The MS/MS conditions were presented in **Table 1**.

Table 1. Optimized mass spectrometry conditions

Analyte	Precursor ion (m/z)	Product ion (m/z)	CE(eV)
NDMA	74	42	15
	74	44*	5
NDMA-d6 (IS)	80	46	15
	80	50*	5
NMEA	88	43	10
	88	71*	5
NDEA	102	56	10
	102	85*	5
NDPA	101	70	5
	130	113*	5
NPYR	100	43	10
	100	55*	5
NMOR	116	86*	5
	116	56	10
NPIP	114	97*	5
	114	84	10
NDBA	158	99	5
	116	99*	5
NDPhA	169	167	15
	169	168*	10

(*) Quantitative ion

2.3.3. Method validation

The post-optimization procedure would be validated in accordance with AOAC guidelines and Commission Implementing Regulation (EU) 2021/808, covering key performance characteristics including specificity, linearity, limits of detection and quantification, repeatability, reproducibility, and recovery.

3. RESULTS AND DISCUSSION

3.1. Investigation of sample preparation

Based on the QuEChERS sample preparation method described in section 2.3.1, the performance of different extraction solvents and extraction salts were investigated using both blank and matrix-spiked samples. The pre-treatment and post-treatment spiked samples at 2.0 µg/kg were analyzed in triplicate for each condition, and the mean values were used for subsequent evaluation.

3.1.1. Selection of extraction solvent

The results of the study showed that the recovery efficiency of the analytes significantly depends on the composition of the extraction solvent (**Figure 1**). When using MeCN as a single solvent, the recovery reached 94.6–104.5% and showed a low level of variation between substances. Meanwhile, the MeCN–DCM mixtures showed a decreasing trend in recovery efficiency as the DCM ratio increased. Specifically, the MeCN–DCM mixture (3:1) reached 86.9–101.0%, the mixture (1:1) reached 79.5–90.4%, and the mixture (1:3) only 77.5–88.2%.

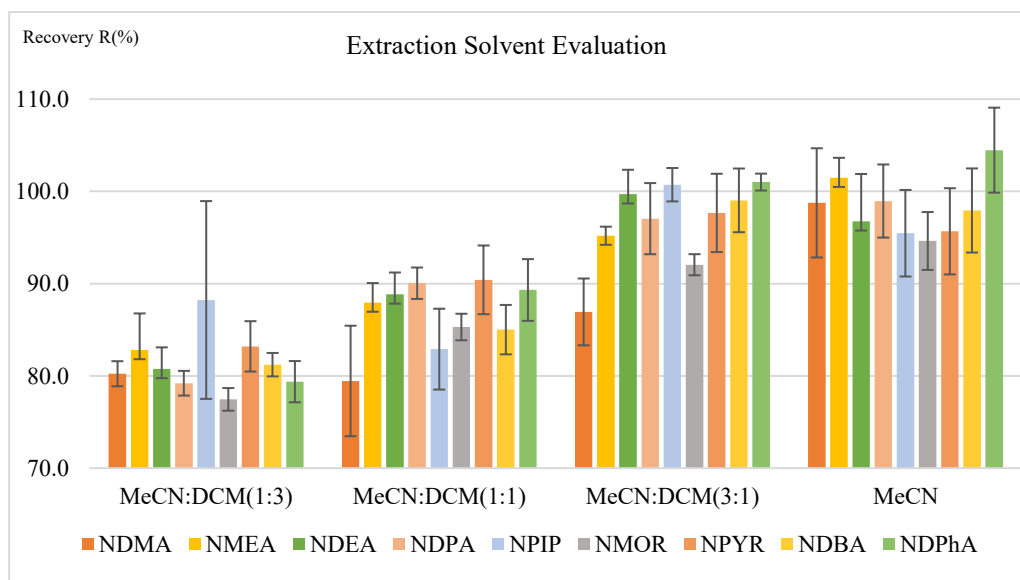


Figure 1. Results of the extraction solvent survey

The differences in recovery among the solvent systems can be attributed to the polarity and miscibility characteristics of MeCN and DCM. Pure MeCN provides strong solvation capacity for polar and semi-polar nitrosamines, promoting efficient partitioning from the sample matrix and resulting in both high and consistent recoveries. In contrast, adding DCM decreases the overall polarity of the solvent system, thereby reducing its affinity for highly polar compounds such as NDMA and NMEA. This explains the progressive decline in extraction efficiency as the DCM proportion increases, particularly in the 1:3 MeCN–DCM mixture (77.5–88.2%). The notable drop when DCM exceeds 50% indicates that the solvent system becomes too non-polar to effectively dissolve low-molecular-weight nitrosamines, leading to poor transfer into the organic phase. Given these solvent–analyte interactions and the superior reproducibility observed, MeCN alone represents the most suitable extraction solvent for this study.

3.1.2. Selection of extraction salts

The use of extraction salts significantly affected the extraction efficiency (**Figure 2**).

The extraction salt composition markedly influenced the salting-out efficiency and recovery of nitrosamines. Salt-1 produced the lowest yields (77.4–87.1%), indicating insufficient dehydration and weak ionic strength, which limited analyte transfer into the organic phase. In contrast, Salt-2 and especially Salt-3 significantly improved recoveries, with NDMA reaching up to 94.6%, due to the strong water-binding capacity of MgSO₄ combined with the phase-separation enhancement provided by NaCl.

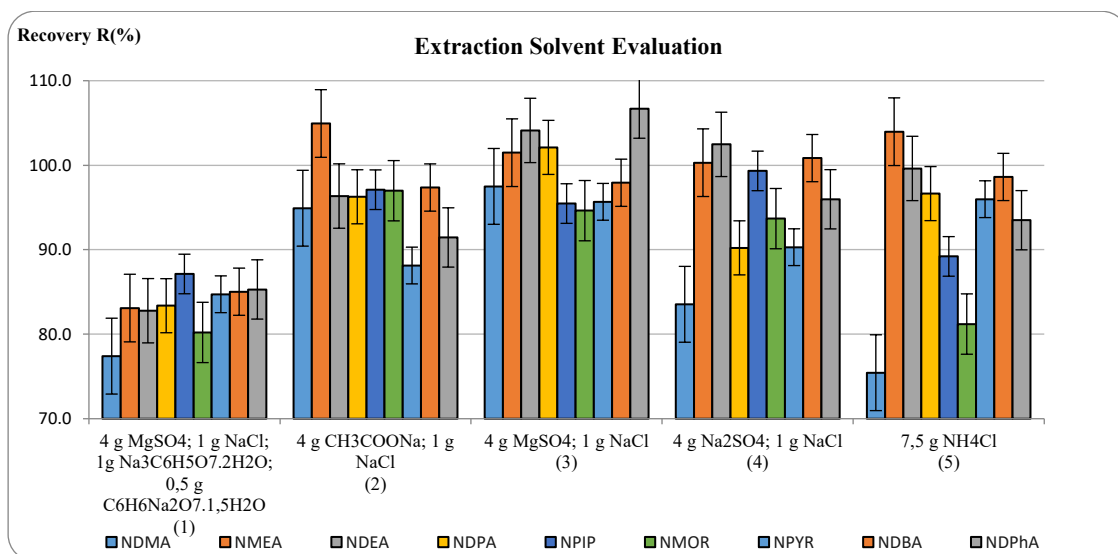


Figure 2. Results of salt extraction survey

Salt-3 also demonstrated the most stable performance (94.6–106.7%), suggesting reduced matrix interference and more efficient partitioning of both highly polar and semi-volatile nitrosamines. Meanwhile, Salt-4 and Salt-5 offered moderate but more variable recoveries, likely due to less optimal ionic balance. Overall, the MgSO_4 – NaCl mixture provided the best compromise between extraction efficiency and reproducibility, supporting its selection for subsequent analyses.

Thus, the optimal sample preparation procedure involves selecting MeCN as the extraction solvent and a salt extraction mixture of 4 g MgSO_4 and 1 g NaCl .

3.2. Method validation

3.2.1. Specificity

The specificity of the method was first evaluated based on the IP point: Analytes have a precursor ion and two product ions, resulting in an IP score of 5. The ion ratios of the analytes were also evaluated and met the requirements for mass spectrometry analysis (EC 2021/808) [9].

Moreover, six consecutive injections of the mixed standard solution containing nine nitrosamines and the internal standard at a concentration of 2 ng/mL were performed. The blank sample, standard solution, and spiked blank sample were analyzed to evaluate the specificity. The chromatographic results showed that no signal corresponding to any analyte was detected in the blank sample at their respective retention times, while the spiked blank sample exhibited clear signals for all target analytes. The retention time deviation between the spiked blank sample and the standard solution did not exceed $\pm 0.5\%$. These results demonstrate that the method meets the specificity requirement and is suitable for the analysis of nine nitrosamines using GC-MS/MS. The typical chromatograms are presented in **Figure 3**.

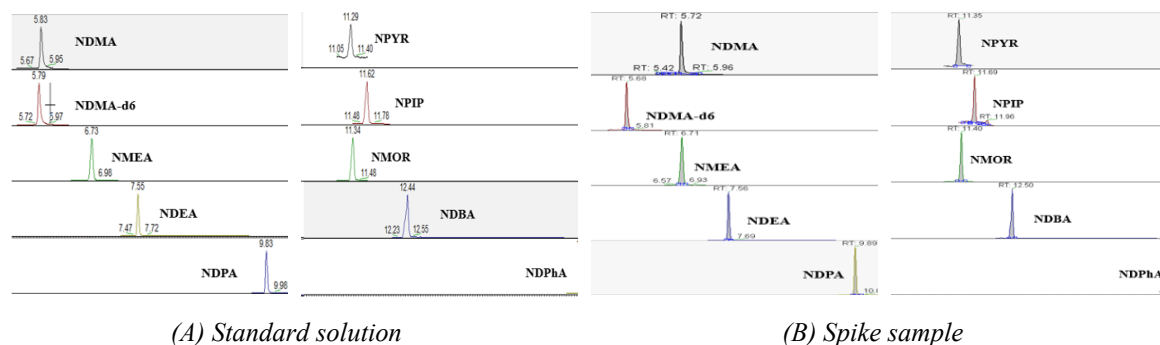


Figure 3. Chromatograms of each nitrosamine in the (A) standard solution and (B) spiked sample

3.2.2. Limit of detection (LOD) and limit of quantification (LOQ)

The blank sample was spiked with nitrosamines to achieve the final contents of 0.5 µg/kg (corresponding to 0.5 ng/mL in the solution, the procedure with a dilution factor of 1) and then analyzed with six replicates to obtain the signal-to-noise ratio (S/N). The S/N ratios obtained was of 10 or higher indicating that the LOQ can be at 0.5 µg/kg. The LOD was extrapolated from the LOQ using the formula ($LOD = LOQ \times 3/10$). The chromatogram and S/N at the 0.5 µg/kg spiked sample are shown in **Figure 4**.

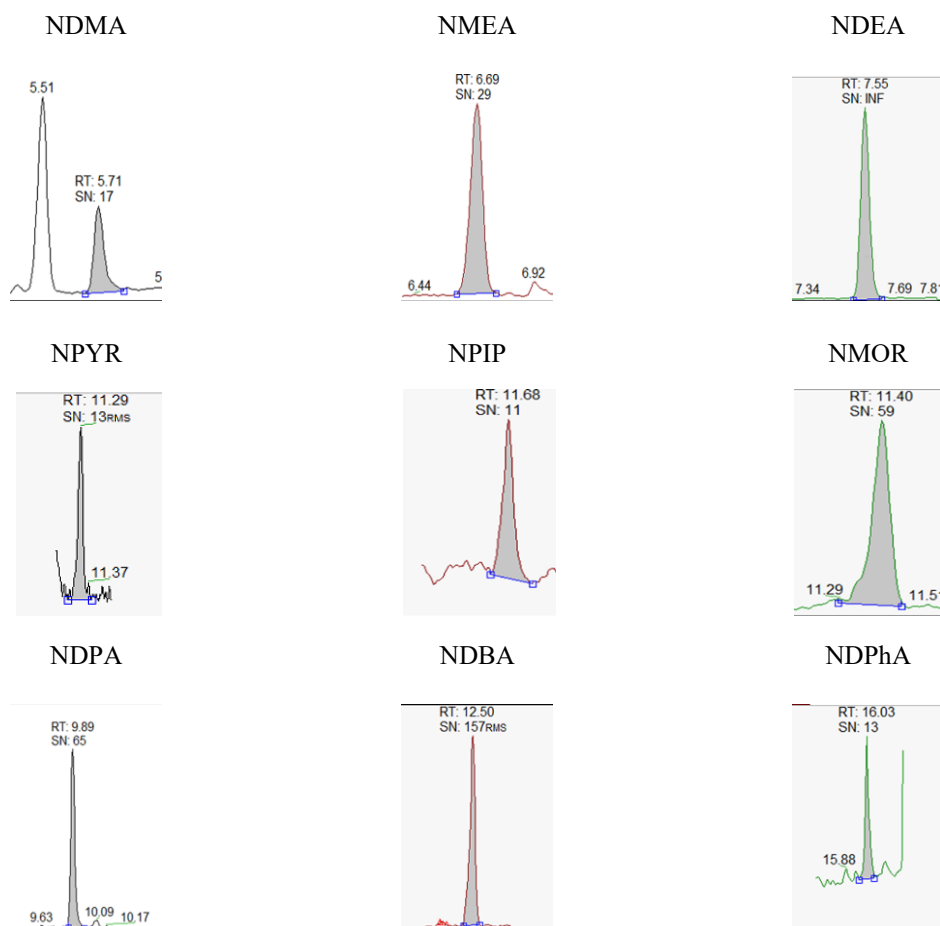


Figure 4. Chromatogram of substances at 0.5 µg/kg (LOQ)

At the spiked concentration level, all target analytes exhibited a signal-to-noise ratio (S/N) is closest to 10. Therefore, the concentration of 0.5 µg/kg was established as the limit of quantification (LOQ) for the method, corresponding to a calculated limit of detection (LOD) of 0.15 µg/kg.

3.2.3. Linearity

Based on the optimized conditions, the calibration curves for nine analytes were constructed at six concentration levels from 0.5; 1.0; 2.0; 5.0; 10.0; 20.0 (ng/mL). The calibration curve was developed by plotting the peak area against the corresponding concentration in **Table 2**.

The results showed that the calibration curves obtained had correlation coefficients (R^2) ≥ 0.995 , and the deviation at all concentration levels did not exceed 15%. This demonstrates that, within the concentration range of 0.5–20 ng/mL (corresponding to 0.5–20 µg/kg sample), a linear relationship exists between the peak area ratio of the analyte to the internal standard and the analyte concentration. Therefore, the method satisfies the linearity requirements according to AOAC.

Table 2. Linearity curve and correlation coefficient for the analytes

Analyte	Concentration range	Linear curve	R ²
NDMA	0.5 – 20.0 ng/mL	$Y = 0.294 \cdot X - 0.0184$	0.9967
NMEA	0.5 – 20.0 ng/mL	$Y = 0.654 \cdot X - 0.0591$	0.9985
NDEA	0.5 – 20.0 ng/mL	$Y = 0.547 \cdot X - 0.0124$	0.9982
NDPA	0.5 – 20.0 ng/mL	$Y = 0.402 \cdot X - 0.0318$	0.9954
NPIP	0.5 – 20.0 ng/mL	$Y = 0.444 \cdot X - 0.0535$	0.9970
NPYR	0.5 – 20.0 ng/mL	$Y = 0.685 \cdot X - 0.0509$	0.9972
NMOR	0.5 – 20.0 ng/mL	$Y = 0.561 \cdot X - 0.0394$	0.9959
NDBA	0.5 – 20.0 ng/mL	$Y = 0.814 \cdot X - 0.0643$	0.9975
NDPhA	0.5 – 20.0 ng/mL	$Y = 6.47 \cdot X + 0.223$	0.9975

3.2.4. Recovery, repeatability and reproducibility

Repeatability was evaluated by analyzing spiked samples at three concentration levels (0.5; 2.0; and 5.0 µg/kg), with six replicates performed at each level. Reproducibility was assessed on different days by spiking the blank matrix at a concentration of 0.5 µg/kg and analyzing six independent replicates. The results were presented in **Table 3**.

Table 3. Results of recovery, repeatability and reproducibility

Analyte	R (%)	RSD (%)	RSDR (%)
NDMA	87.6 – 112.0	7.26 – 8.91	8.64
NMEA	83.7 – 108.6	4.62 – 8.66	10.2
NDEA	88.0 – 108.5	5.88 – 7.86	7.65
NDPA	83.0 – 109.2	5.96 – 7.50	9.10
NPIP	88.8 – 108.3	5.03 – 7.21	8.56
NMOR	90.5 – 112.8	5.40 – 8.79	9.35
NPYR	89.9 – 114.0	6.17 – 8.09	10.4
NDBA	87.8 – 105.7	4.86 – 8.47	9.25
NDPhA	83.9 – 119.6	9.19 – 11.06	11.9

The results show that the recovery values for all 9 analytes are within the range of 83.0- 119.6%, the relative standard deviations range from 4.62 – 11.06% and the reproducibility 7.65- 11.9%. These results indicate that the recovery, repeatability, and reproducibility of the method comply with the performance requirements specified in EC 2021/808 [8].

3.3. Sample analysis

This method was applied to determine the nitrosamine content in 108 fermented vegetable samples randomly collected from traditional markets and supermarkets across six provinces in Vietnam. The analyzed samples included various products commonly consumed in the region: pickled cabbage, grilled mustard greens, pickled radish, fermented bamboo shoots, pickled stem vegetables, stir-fried pork fat with pickles, salad mixes, fermented shrimp, pickled fig, Thai-style fermented fish sauce, and fermented bean curd. The results are presented in **Table 4**.

Table 4. Results of real sample analysis

Nitrosamine	Number of detected samples	Ratio (%)
NDMA	11	10.19
NDEA	12	11.11
NPYR	4	3.70
NPIP	1	0.93
NDBA	10	9.26
NMOR	5	4.63
Total	14	12.96

Among the 108 samples analyzed, nitrosamines were detected in 14 samples (12.96%), with N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) emerging as the most frequently occurring compounds, in which NDMA was detected in 11 samples (10.19%); NDEA was detected in 12 samples (11.11%), representing the highest occurrence; NDBA was found in 10 samples (9.26%); NMOR was detected in 5 samples (4.63%); NPYR appeared in 4 samples (3.70%); NPIP was detected in only 1 sample (0.93%). Although the overall detection frequency was relatively low, the presence of multiple carcinogenic nitrosamines in several samples underscores the need for continuous monitoring and improved control over fermentation conditions and raw material quality. When compared with recent international investigations, the nitrosamine profile of Vietnamese fermented vegetables shows both similarities and important contrasts. A large-scale Korean study assessing nitrosamines in 1,320 food products reported a substantially higher overall detection frequency of 72%, with NDMA, NDEA, NPYR, and NDBA identified as key contributors to dietary exposure. While the Korean data encompass a broader range of food categories – including fish products, seasonings, and processed meats the predominance of NDMA and NDEA in both studies suggests that these compounds remain the most chemically favored and widely occurring nitrosamines across different Asian dietary patterns[10]. In contrast, the Danish study focused specifically on leafy green vegetables with naturally high nitrate levels. After processing (pureeing) and storage under ambient, refrigerated, and frozen conditions, several nitrosamines including NDMA, NPYR, NPIP, and NMOR were detected, particularly in vegetables stored at higher temperatures. This indicates that nitrosamine formation is not limited to fermented products but may also occur in minimally processed vegetables due to endogenous nitrate–nitrite conversion and storage-related biochemical reactions [11].

4. CONCLUSION

The study successfully developed and validated a method for simultaneous analysis of nine nitrosamines in fermented vegetables by GC–MS/MS. The method fully met the validation requirements according to EC 2021/808 and AOAC 2016. The validation results showed that the method has high sensitivity and reliability, suitable for implementation in nitrosamine testing at analytical facilities. Analysis of 108 fermented vegetable samples collected from the market showed that 14 samples were positive for nitrosamines, with the most frequently detected compounds being NDMA, NDEA, and NDBA, followed by NMOR, NPYR, and NPIP. Therefore, establishing standardized reference materials and harmonized analytical protocols remains essential to ensure accuracy and improve comparability across future studies.

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