Analytical Methods

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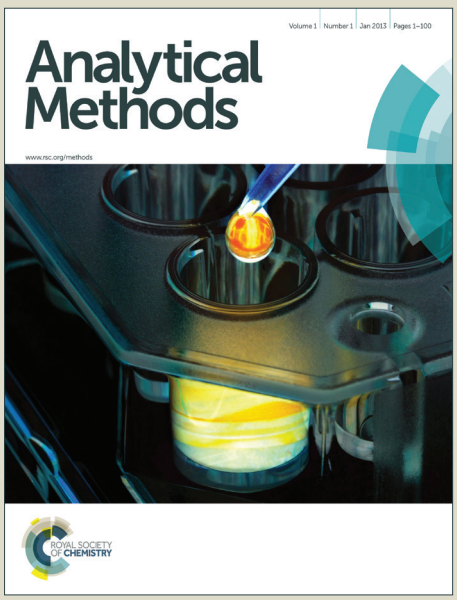

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ARTICLE

Simultaneous Determination of β-methy-β-nitrostyrolene and Its Related Substances by PS-DVB Chromatographic Column and Structural Analysis of Its Photo-isomerization Product

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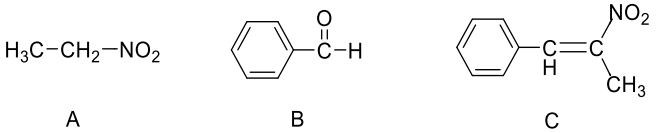
A method of high performance liquid chromatography (HPLC) was established to determine β-methy-β- nitrostyrolene (MNS) and its related substances and to investigate the stability of MNS in acetonitrile solution. In this chromatographic method, MNS and its related substances were separated using polystyrene-divinyl-benzene column (PS-DVB) (25 cm • 4.6 mm i.d. 6 μm). Acetonitrile/water was served as mobile phase with gradient elution of (0-8 min, ACN 74→92%), maintaining the flow rate at 1.0 mL/min with UV detection wavelength at 214 and 250 nm. The selected chromatographic conditions were found to effectively separate MNS, benzaldehyde and nitroethane with retention time of 3.66, 5.51 and 8.94 min, and perfect resolution of 6.76 and 8.69. The linearity range of MNS, benzaldehyde and nitroethane were found in the range of 25-125, 0.16-15.71 and 0.16-15.67 μg/ml, with the Limits of detection 0.031, 0.006 and 0.011 μg/ml, respectively. Recovery studies showed good results for all examined compounds (from 96.5% to 99.9%) with RSD ranging from 0.31% to 0.94%). MNS was unstable in acetonitrile when exposed to nature light, and new product appeared. The new product was purified and identified as α-methyl - styrolene nitrite by GC-MS and NMR, which was the photo-isomerization product of MNS. The proposed method was found to be accurate, precise, reproducible and specific and it could also be used for routine quality-control analysis of MNS.

1. Introduction

β-methyl-β-nitrostyrene (MNS, Figure 1), is usually synthesized via Henry reaction of aromatic aldehyde with nitroalkanes followed by dehydration of the resultant nitroalcohols [1]. MNS is vital starting material for the synthesis of a variety of useful building blocks and valuable intermediates of numerous products. Since the nitroethane is a strong electron-withdrawing group that can lead to polarization of conjugated double bonds and become readily transformed into a series of different types of compounds, such as amine, hydroxylamine, nitrile, alcohol, aldehyde, ketone, etc. [2, 3]. MNS also has effective antitumor and antibacterial functions [4, 5, 6]. Normally, it is more inhibitive to the gram-positive than to the gram-negative bacteria [7].

The main related substances in the synthesis of MNS were benzaldehyde and nitroethane. The determination of benzaldehyde typically employs high-performance liquid chromatography (HPLC) [8], UV absorption method with single wavelength or gas chromatographic method[9]. The general determination method of nitroethane is gas chromatography [10]. There has no other determination method about MNS except high performance liquid chromatography (HPLC) method which was reported only in the literature [7]. The chromatography conditions were as follows: C_{18} chromatographic column was used, the UV detection wavelength was 214 nm, acetonitrile /water served as the mobile phase with the flowrate of 1.0 mL/min, and a gradient of 10→100% (v/v) ACN (0-35 min) was chosen. The literature has detected was the existence of MNS but not the analysis of the mixture of the three compounds. Furthermore, the detection process had no details. A notable issue was that the simultaneous determination of MNS and its related substances together using a single analytical tool had not been reported.

High performance liquid chromatography (HPLC) acts as the most common and convenient technology in the chromatographic world and are well known for providing high selectivity and sensitivity when it is used for determination recently. So the aim of this article was to develop a simple, sensitive, selective and reproducible RP-HPLC-UV method for simultaneous determination of MNS and its related substances for routine quality-control analysis using polystyrene-divinyl-benzene (PS-DVB) column and to determine the stability of MNS in ACN solution with the attendance of nature light. In this study, double wavelength detection model and the optimization of mobile phase gradient were investigated to obtain satisfactory consequence. The GC-MS and NMR were used for further study of the unstable product.



A B C

Figure 1 Chemical structures of MNS and related substances
A: nitroethane; B: benzaldehyde; C: MNS

2. Experimental section

2.1 Chemicals and reagents

MNS standard sample was purchased from Sigma–Aldrich (Germany); MNS sample was synthetized by ourselves; benzaldehyde and nitroethane were purchased from Sinopharm Chemical Reagent Co., Ltd. (China); ACN, of HPLC grade, was purchased from Merck (Germany). Throughout the whole study, ultrapure deionized (DI) water was used, which was made by an Easypure RODI water system (Barnstead Int.).

2.2 RP-HPLC analysis

LC analyses were performed on a Dionex (USA) HPLC system consisting of a pump (P680 HPLC), an injection valve with 20 μL sample loop (7725i) and UV detector (UVD-170U). Chromatographic separation was achieved on a polystyrene-divinyl-benzene column (PS-DVB) (25 cm • 4.6 mm i.d. 6 μm).

The mobile phase containing acetonitrile and water was prepared daily and filtered (Millipore, 0.45 μm) under vacuum. Flow-rate was 1 mL/min and the detection wavelength was set at 214 and 250 nm. All assays were performed at ambient temperature.

2.3 GC-MS analysis

GC–MS analyses were carried out using a Thermo Trace DSQ/ISQ equipped with 30 m • 0.25 mm • 0.25 μm i.d. MS capillary column (TR-5MS). The GC oven temperature ramp was: 80 ^{o}C, hold 1 min, increase to 300 ^{o}C at a rate of 20 ^{o}C/min and hold for 20 min at the final temperature. The interface temperature was 280 ^{o}C. Electron ionization (EI) spectra were obtained at 70 eV at 280 ^{o}C. Helium carried gas at a constant flow-rate of 1.0 mL/min. The injection volume was 1 μL, with a splitless mode. The MS was operating in FASST mode (SCAN and SIM modes simultaneously) with the scan range of 50-600. All of these response data was analyzed by two software standard mass spectral databases (Nist and Main Lab).

2.4 NMR analysis

NMR spectra were recorded at 37 ^{o}C on a 300 MHz Bruker spectrometer using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal reference.

2.5 Preparation of standard solutions

MNS standard stock solution was prepared by dissolving 125 mg standard MNS in 10 mL acetonitrile. The stock solution was further diluted with acetonitrile to get standard MNS solutions ranging between 25 and 125 μg/mL.

Benzaldehyde and nitroethane standard stock solutions were prepared in acetonitrile to give 10.4 and 10.5 μg/mL, respectively. A mixed stock solution of benzaldehyde and nitroethane was prepared in acetonitrile in which the concentration of benzaldehyde and nitroethane were 104 and 105 μg/mL, respectively. The mixed stock solution was further diluted with acetonitrile and MNS solution was added to get standard mixture solutions. In those solutions, the concentration of MNS maintains at 125 μg/mL.

2.6 Preparation of MNS sample solutions

MNS sample stock solution was prepared by dissolving 125 mg MNS sample in 10 mL acetonitrile. The stock solution was further diluted with acetonitrile to get MNS sample solutions.

3. Results and discussion

3.1 Optimization of UV wavelength

The maximum absorption wavelength of benzaldehyde and nitroethane were 214 and 250 nm, respectively. [11, 12, 13] Considering of the large wavelength gap between the two substances, the maximum detection wavelength of benzaldehyde and nitroethane (214 and 250 nm) were both selected as the detection wavelengths to improve the detection sensitivity. MNS could achieve significant absorption at this wavelength range because of the large yield. So there was no need to choose its maximum absorption wavelength, as long as the good separating degree can be achieved. Double-wavelength detection model achieved the simultaneous estimation of the three substances without worrying about the different amount of every component and the low detection sensitivity.

3.2. Optimization of separation conditions

3.2.1 The preliminary selection of gradient elution

Based on the detection method of MNS reported in literature [7], the gradient of 10→100% (v/v) ACN (0-35 min) was chosen to separate the standard mixture solution, in which the concentrations of nitroethane, benzaldehyde and MNS were 10.50, 10.40, and 125.00 μg/mL, separately. The wavelength of 214 nm was chosen. Although MNS could be detected (peak-4), but the related substances (nitroethane and benzaldehyde, peak-1 and peak-2) were overlapped partly as shown in Figure 2. In addition, the separation analysis cost for a long time over 30 min. Therefore, the mobile phase gradient was optimized as following in order to improve the separation efficiency and enhance the column efficiency (number of theoretical plates, N).

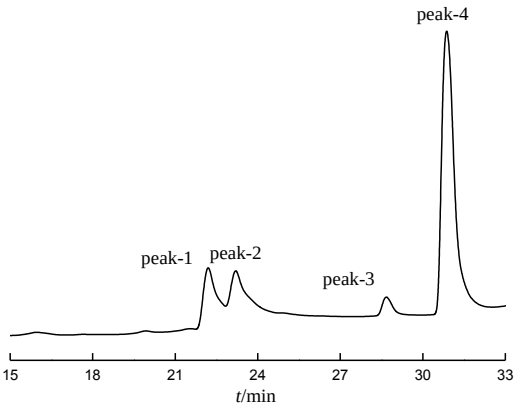


Figure 2 The HPLC chromatogram of ACN 10%→100% (0-35 min)

3.2.2 Optimization of the gradient elution

Based on the findings of 3.2.1, the gradient elution system was optimized to improve resolution and to reduce the time needed for analysis. In an attempt to achieve perfect peak symmetry, separation and column efficiency, different mobile phase gradients were tested. HPLC Chromatograms of different gradients were shown in Figure 3. The perfect separation of nitroethane and benzaldehyde could not be achieved using the

gradient of 10→100% (v/v) ACN (0-35 min). When preliminarily increased the starting concentration of ACN and decreased the gradient time, every substance was separated well, and the column efficiency was improved. Then further increased the starting concentration of ACN, such as the gradient of 74→92% (v/v) ACN (0-8 min), a better column efficiency was achieved. But when the gradient elution of 80→92% (v/v) ACN (0-8 min) was used, the number of theoretical plates reduced. From the discussions above, it could be concluded that the best experimental condition was 74→92% (v/v) ACN (0-8 min), with which the three substances could be separated successfully and the run time was just10 min. Thus the best condition was chosen to take simultaneous determination of MNS and its related substances.

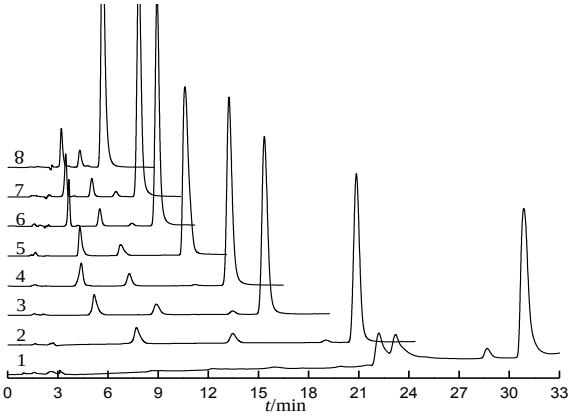


Figure 3 The HPLC chromatograms of different gradients
1: ACN 10%→100% (v/v) (0-35 min); 2: ACN 30%→92% (v/v) (0-20 min); 3: ACN 50%→92% (v/v) (0-15 min); 4: ACN 60%→92% (v/v) (0-15 min); 5: ACN 60%→92% (v/v) (0-8 min); 6: ACN 74%→92% (v/v) (0-8 min); 7: ACN 80%→92% (v/v) (0-8 min); 8: ACN 92% (v/v) isocratic elution

3.3 Specificity

To evaluate the specificity of the method, chromatograms of the pure standard solutions were compared with the MNS sample at the same determining condition by injecting the following solutions: (1) MNS sample solution after light exposure for four days, (2) standard solution of MNS, (3) standard solution of nitroethane, (4) standard solution of benzaldehyde and (5) the solvent (ACN)[14]. No peak was observed in the solvent chromatogram, indicating an absence of interference from the solvent. As could be seen from Figure 4, the retention times of all substance were almost the same in both standard solutions and MNS sample. The developed HPLC method had good specificity to MNS and its related substances and guaranteed obtaining well-shaped peaks both for the related substances and coexisting photo-isomerization product.

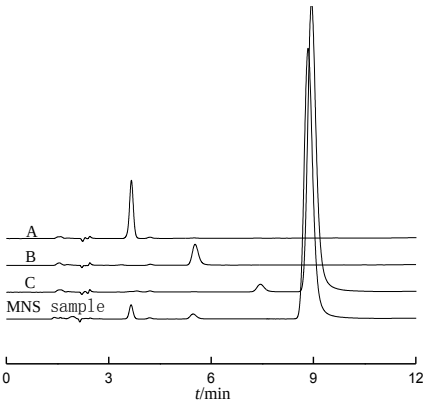


Figure 4 The HPLC chromatogram of MNS sample compared with standard samples
A~C were shown in Figure 1

3.4 Linearity and limit of detection (LOD) of MNS and its related substances

As mentioned above, it could not determinate the three substances sensitively unless choose 214 and 250 nm as the detection wavelength simultaneously, because of the difference of maximum absorption wavelength between benzaldehyde and nitroethane. Considering of the detection sensitivity of both substances, double wavelength detection model was used to reduce the detection limitation.

To determine linearity, a series of standard mixture solutions containing all the analytes and MNS standard solution were injected in triplicate under optimum chromatographic condition. A linear relationship existed between the peak area(Y) and the corresponding concentration (X) of MNS. In addition, a linear relationship existed between the peak area(Y) and the corresponding concentration(X) of the related substances to obtain the calibration graphs. The limits of detection (LOD) were calculated from signal-to-noise ratios (S/N) of all the standard solutions using the definition S/N=3[15]. The LOD of benzaldehyde was 0.006 μg/ml and much lower than literature [16]. Concentration ranges and calibration parameters were reported in Table 1. Good relationship was found as indicated by the coefficient of determination ≥ 0.999 1.

3.5 Accuracy and precision

The accuracy of the method was assessed by detecting the recovery of known amounts of spiking analytes (A, B and C) in MNS sample. The concentration of MNS in the MNS sample was 0.12 mg/mL. Spiked samples were prepared in quintuplicate at three levels and analyzed according to optimum chromatographic condition. As it can be seen in Table 2, quantitative recovery was obtained in each instance (recovery = 96.5-100.4%), and had low RSD values (RSD ≤ 0.94%) [17].

Table 1 The linearity, limit of detection of MNS and its related substances (S/N=3)

Compound	Regression equation	Correlation coefficients	Linear range (μg/mL)	Detection limit (μg/mL)
A	Y=0.814 X+0.034	0.999 7	0.16-15.71	0.031
B	Y=2.265 9X+0.136 4	0.999 9	0.16-15.67	0.006

C	Y=0.738X+0.565 0	0.999 1	25-125	0.011

Y: peak area; *X*: mass concentration, mg /L. A~C were shown in Figure 1.

Table 2 Recovery, RSD of A , B and C

compound	fortified level (μg/mg)	average of testing (μg/mg)	average of recovery (%)	RSD (%, n=5)
A	6.72	6.48	96.5	0.72
	10.50	10.34	98.5	0.94
	20.10	19.79	98.5	0.31
B	6.66	6.54	98.2	0.51
	10.40	10.12	98.0	0.36
	20.08	20.06	99.9	0.87
C	756	757	100.1	0.74
	960	964	100.4	0.71
	1165	1151	98.8	0.22

3.6 Analysis of practical sample

The developed method was applied to a practical MNS sample. The sample contained nitroethane and benzaldehyde at rate of 0.08% and 0.22%. The results showed that the method had thus been demonstrated to be a stability-indicating method and be suitable for assay for commercial products.

3.7 The light stability of MNS and its related substances

Stability is a key problem of every substance during their analysis [18]. The stabilities of standard MNS solution and standard mixture solution were evaluated with the effect of the nature light, in order to find the best storage method.

The standard mixture solution was prepared in triplicate and stored at ambient temperature, exposed to nature light and tested at intervals of 0, 4, 8, 12, 24 and 48 h. The concentration of nitroethane, benzaldehyde and MNS were 5, 2.5 and 125μg/mL, respectively. The results in Figure 5 (A, B) showed that no significant changes of the related substances of the stock standard mixture solution were observed. New product (peak-3) was observed at 7.45 min (Figure 6), which had different retention time compared with MNS. The conversion rate of MNS to the new product was measured as 0, 2.24%, 5.35%, 6.77%, 9.27% and 15.98% via this proposed HPLC method, and it increased with the increasing exposure time.

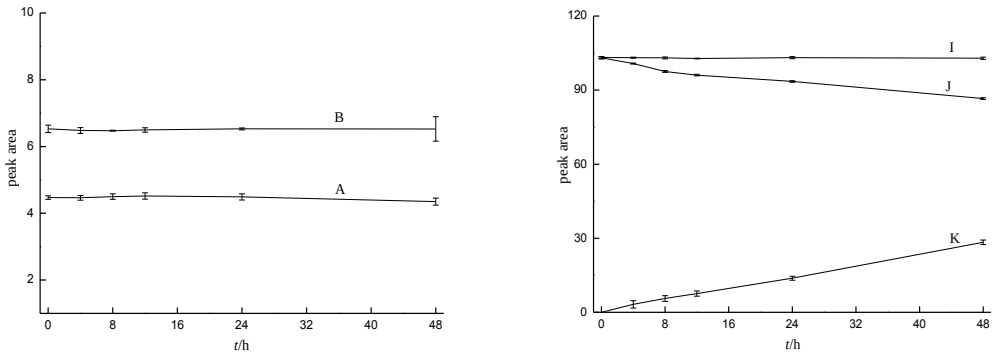


Figure 5 The stability of benzaldehyde, nitroethane and MSN in standard mixture solution
A: nitroethane; B: benzaldehyde; I: peak-4 in dark control; J: peak-4 upon nature light irradiation; K: peak-3 upon nature light irradiation

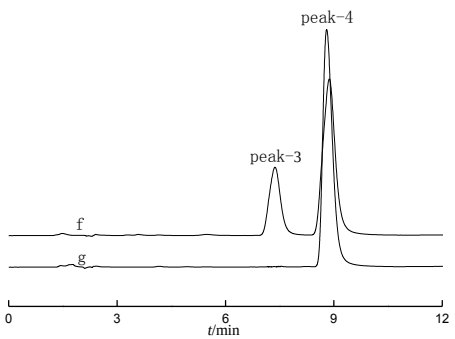


Figure 6 The HPLC chromatograms of fresh and storage (exposed to nature light for 72 h) standard MNS solution

f: standard MNS solution exposed to nature light for 72 h; g: fresh standard solution of MNS

The standard MNS solution was prepared and detected in the same condition of the above standard mixture solution except for the dark control. No product was observed in the dark control (Figure 5I) suggesting that MNS was unstable under the influence of nature light and new product appeared. The RSD values of every detection point in Figure 5 were between 0.11% and 1.47%.

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3.8. The structural analysis of peak-3

3.8.1. Preliminary study by GC-MS

A mixture of peak-3 and peak-4 was obtained by placing the MNS standard solution in nature light for 7 days. The pure peak-3 was achieved after the separation and purification of the mixture with HPLC method. MNS, the mixture and the pure peak-3 were detected under the condition of section 2.3. A total ion current (TIC) chromatogram for these three samples (Figure 7) was obtained and the selected-ion monitoring results were shown in Figure 8.

It is apparently in the Figure 7 that MNS and the peak-4 of the mixture held the same retention time of 6.84 - 6.86 min, and the pure peak-3 and the peak-3 of the mixture held the same retention time of 6.21-6.25. To the conclusion, the purification of peak-3 and peak-4 in the mixture achieved the expected result. Moreover, peak-3 and MNS were not the same substance and they could be efficiently separated under the GC-MS detecting condition. The most important, the retention time of peak-3 was shorter than MNS, thus the boiling point of peak-3 was lower than MNS.

As shown in Figure 8, the mass spectrogram of peak-3 behaved similarly to those of MNS with the same m/z 163, 115, 91, 97 and 146. According to the standard spectral library, the matching results of both peak-3 and peak-4 were MNS. The matching rate of peak-4 was 91%, but the matching rate of peak-3 was only 79%. The result was incredible unless the matching rate was above 90%. Therefore, the molecular structure of peak-3 and MNS were very similar, but not the same. Peak-3 was suspected to the isomerization of MNS.

MNS structure contains double-bond and nitro, so its isomers have two forms containing double-bond isomeride and nitro isomeride. The double-bond isomeride has the same boiling point. But the boiling point of peak-3 was lower than MNS, therefore peak-3 was not the double-bond isomeride of MNS. The literature[18, 19] showed that photo-isomerization of MNS was apt to generate nitrous acid ester, and the boiling point of nitrous acid ester was lower than nitro compounds. For example, the boiling point of ethyl nitrite was 17.2°C which was much lower than nitroethane whose boiling point was 114°C. By inference, the photo-isomeride of MNS was α-methyl-styrolene nitrite whose proposed structure was shown in Figure 9(the photo-isomeride).

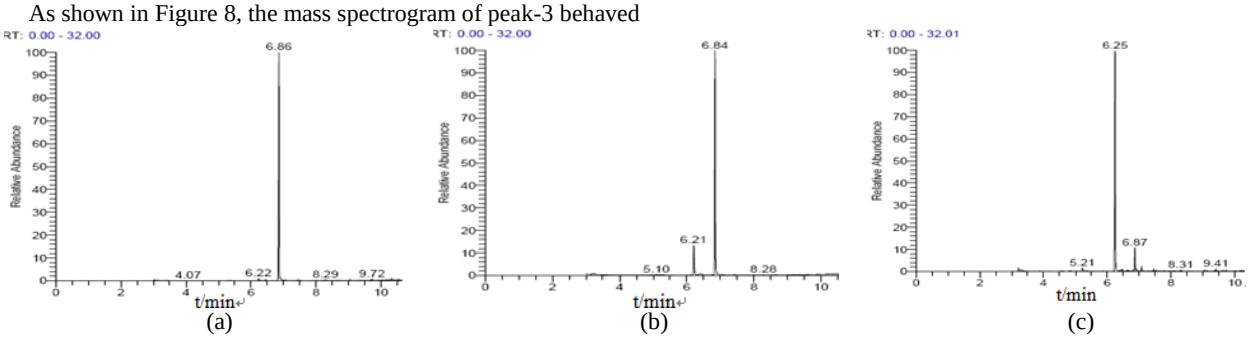

Figure 7 Total ion current chromatograms (TIC) of MNS, the mixture and peak-3

a: MNS; b: the mixture; c:peak-3

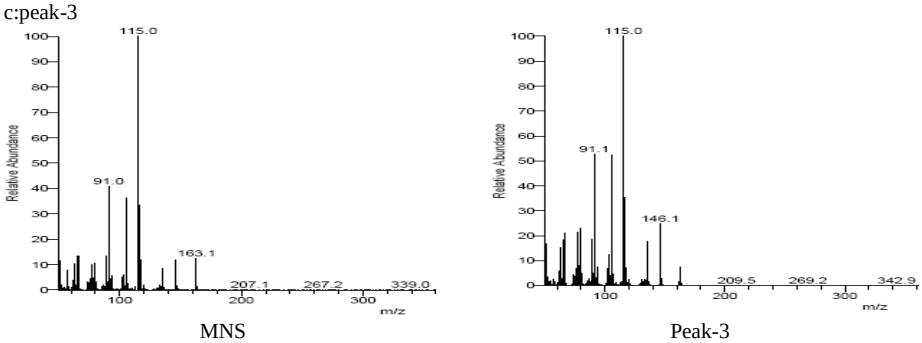

Figure 8 The full scan mass spectra of MNS and peak-3

3.8. 2 Further validation by ^{1}H NMR

According to the structure inferred by GC-MS above, the mixture and MNS were transferred into NMR tube and dried with N_2, dissolved with 0.5 mL $CDCL_3$ and detected by ^{1}H NMR. ^{1}H NMR chemical shifts of the peak-3 and MNS were shown in Figure 9 and a summary of the peaks observed in the spectra could be found in Table 3.

For the purposes of discussion, we started our analysis with MNS whose NMR signals could be assigned from the data reported in literature [19].

Thus, in ^{1}H NMR of the mixture, the singlet at 2.44-2.46 and 8.01-8.1 ppm were attributed to the methyl H and double-bond H on MNS, while the singlet at 2.34-2.35 and 6.46-6.47 ppm were attributed to the methyl H and double-bond H on the photo-isomeride of MNS. The multiplet at 7.3-7.47 ppm was due to the aromatic group on MNS and its photo-isomeride.

Since the electron-withdrawing effect of the nitro group, N of nitro group display electropositive, and the O of nitrite group display electronegative, the nitrite group would promote the

resonances absorbtion of H protons on its adjacent C to the upfield. So the methyl H and double-bond H of photo-isomeride displayed at upfield compared with MNS. The displacement rule of H proton was coincided with that simulated by ChemDraw software. Then it could be concluded that the photo-isomerization of MNS was α-methyl - styrolene nitrite (the photo-isomeride in Figure 9) and the molar ratio of MNS and its photo-isomeride were detected as 1:1.6 with the area ratio of methyl H (or double-bond H) between two compounds.

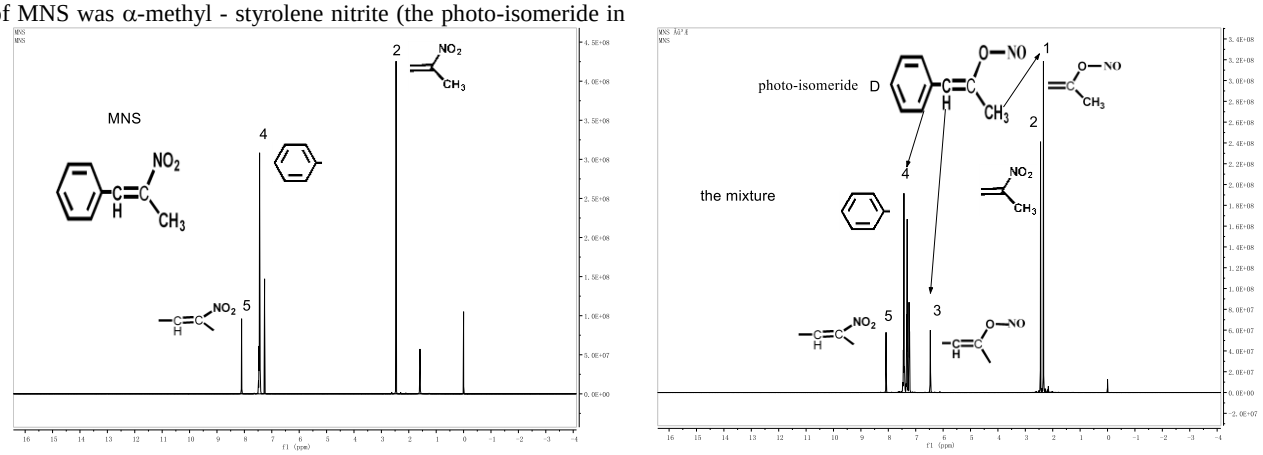

Figure 9 ^{1}H NMR spectrum of MNS and the mixture

Table 3 The assignation of ^{1}H NMR signals in the mixture

No.	5	4	3	2	1
assignation	**H**-C=C-NO_2	Ar-**H**	**H**-C=C-ON	O_2N-C-C**H**$_3$	NO-C-C**H**$_3$
MNS (ppm) / Intensity ratio	8.1 / (1)	7.3-7.45/(5)	-	2.44-2.46/(3)	-
MNS reference[19] (ppm)	8.1/ (1)	7.3-7.5/(5)	-	2.46/(3)	-
D simulated(ppm)	-	7.33-7.6/(5)	6.63/ (1)	-	1.99/ (3)
the mixture (ppm) / Intensity ratio	8.08-8.1/ (1)	7.3-7.47/ (13)	6.46-6.47/ (1.6)	2.44-2.46/(3)	2.34-2.35/ (4.8)

4 conclusion

A fast, reliable, selective and effective RP-HPLC-UV method was developed using polystyrene-divinyl-benzene (PS-DVB) column and validated for the simultaneous determination of MNS and its related substances. Suitable experimental parameters for the separation and detection of the MNS sample were determined, and this method had been demonstrated to have high sensitivity, specificity, linearity and precision. Moreover the method was applied to practical MNS sample and be demonstrated to be a stability-indicating method. The method is simple and rapid makes it a candidate for use in routine analysis in monitoring programs.

The MNS was unstable in acetonitrile solution when exposed to nature light and underwent photo-isomerization. Photo-isomerization of MNS increased with increasing exposure time.

The studies performed by GC-MS and NMR allowed determining the probable chemical structure of photo-isomerization product of MNS, which could be determined using the developed HPLC-UV method. The photo-isomerization product of MNS was identified as *α*-methyl-styrolene nitrite.

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Notes and references

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