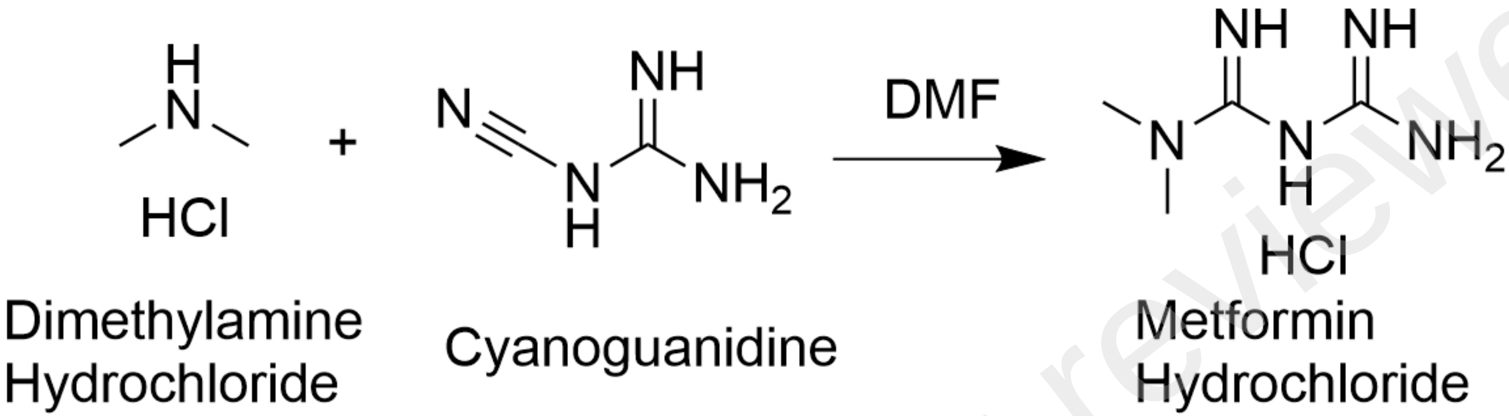
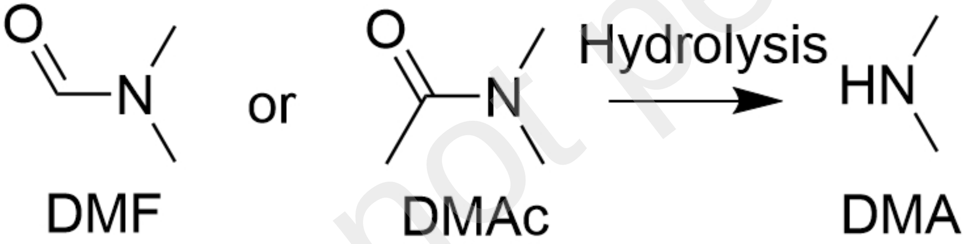
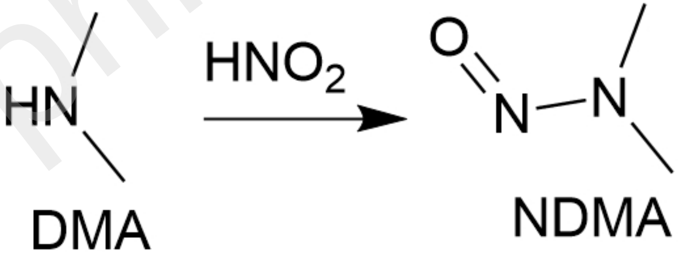
1a.


HCl
Dimethylamine Hydrochloride
+
Cyanoguanidine
DMF
HCl
Metformin Hydrochloride

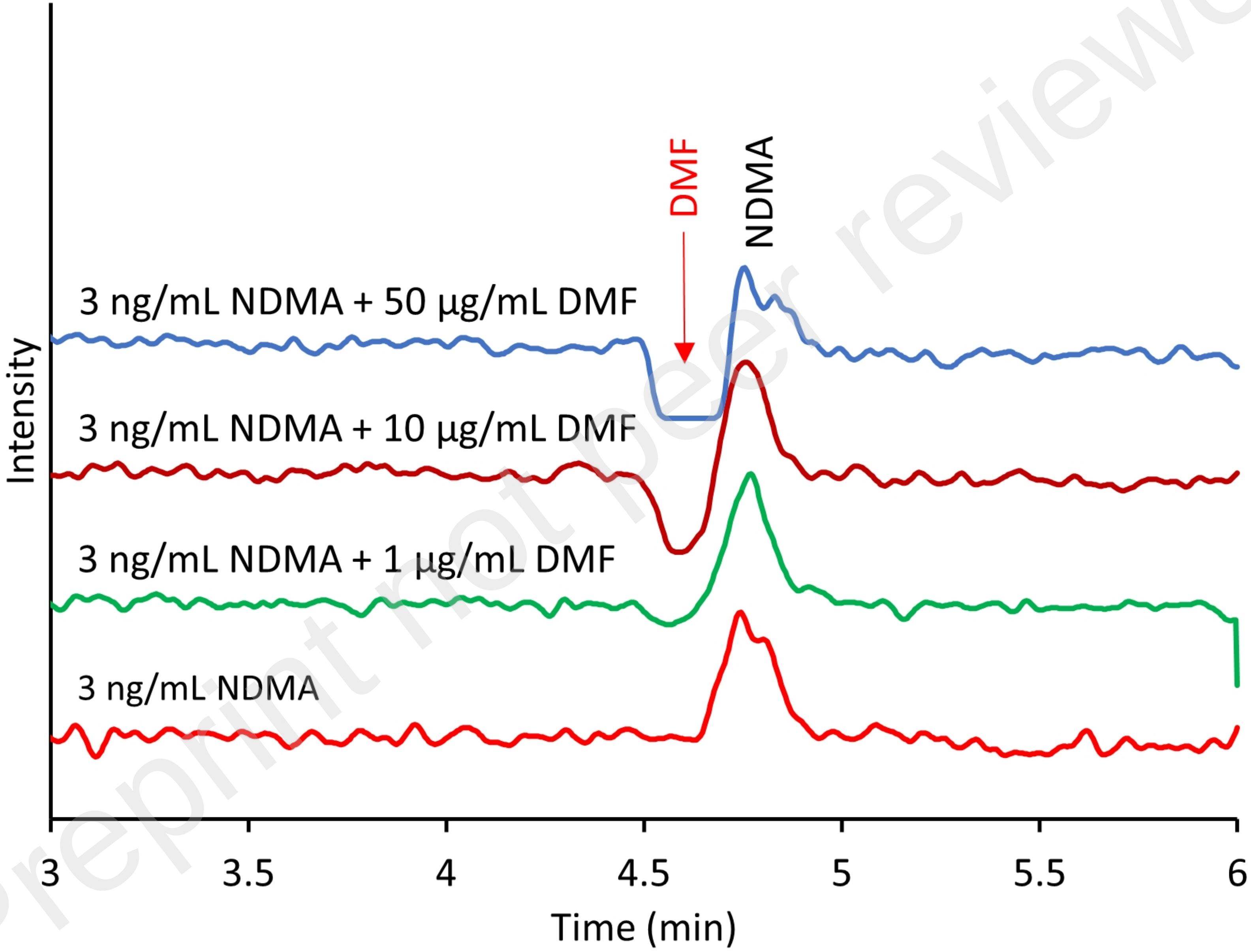
1b.


DMF
or
DMAc
Hydrolysis
DMA

1c.

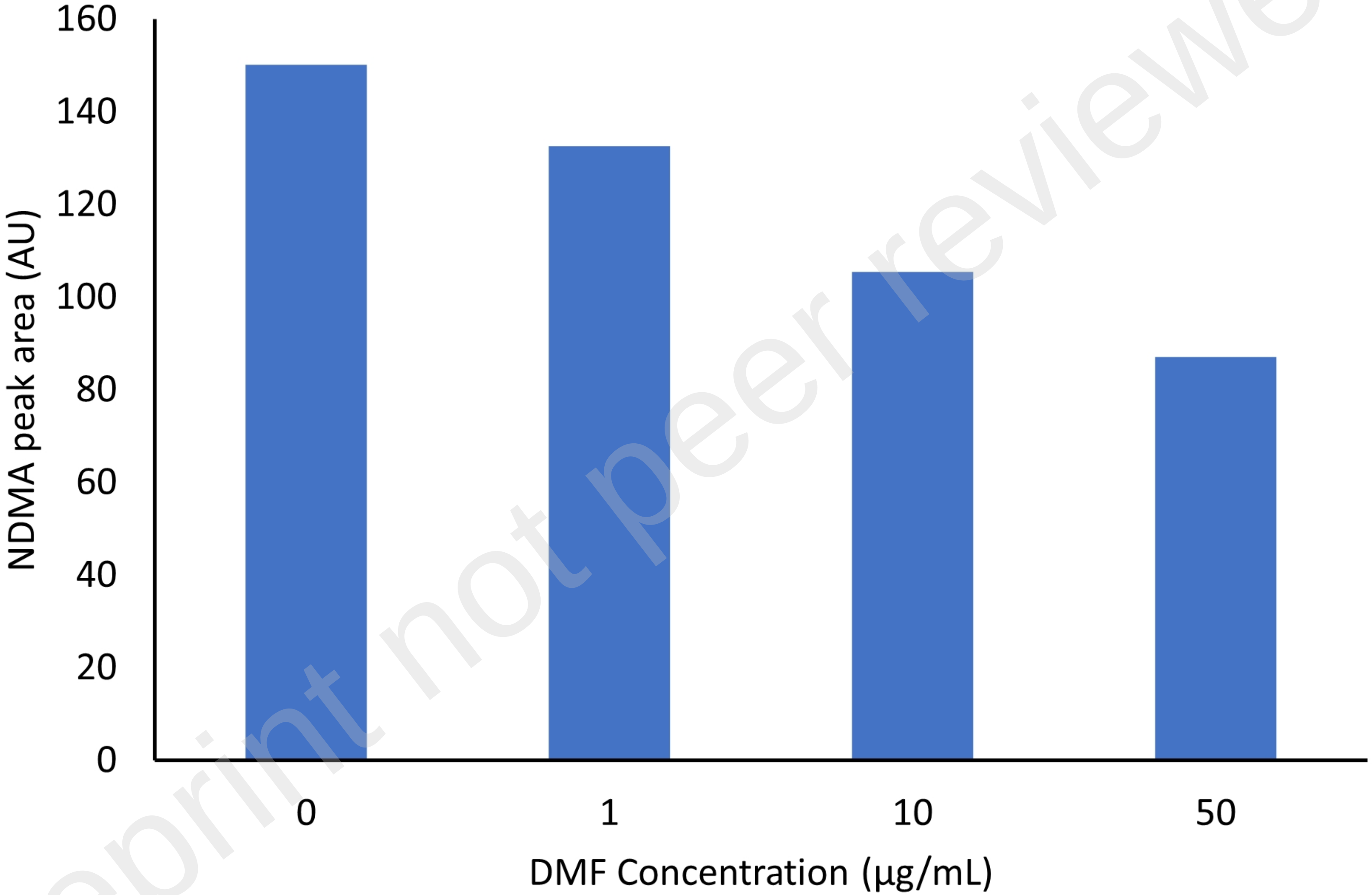

DMA
HNO2
NDMA

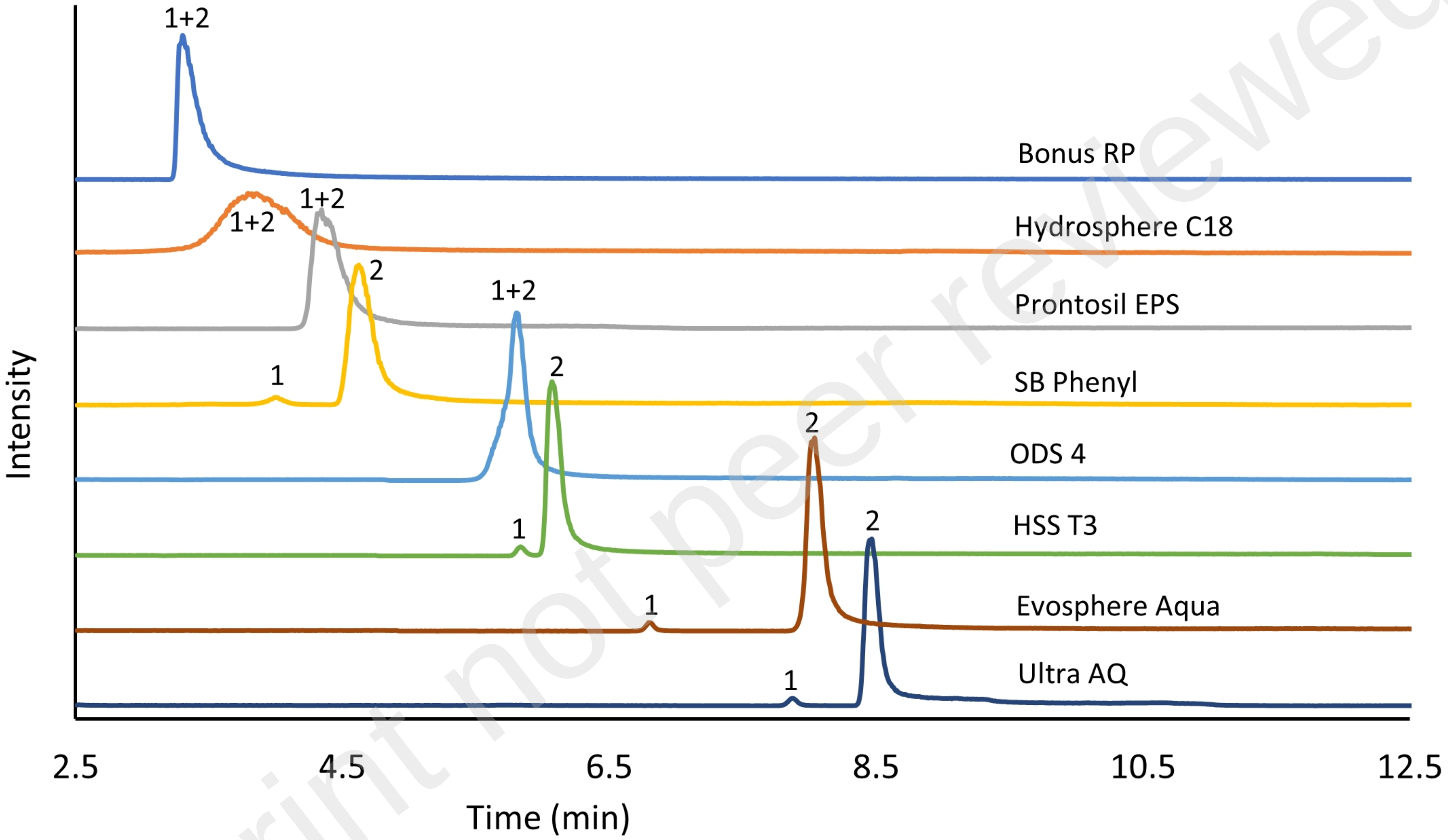
2a.



DMF
NDMA
3 ng/mL NDMA + 50 µg/mL DMF
3 ng/mL NDMA + 10 µg/mL DMF
3 ng/mL NDMA + 1 µg/mL DMF
3 ng/mL NDMA
Intensity
3
3.5
4
4.5
5
5.5
6
Time (min)

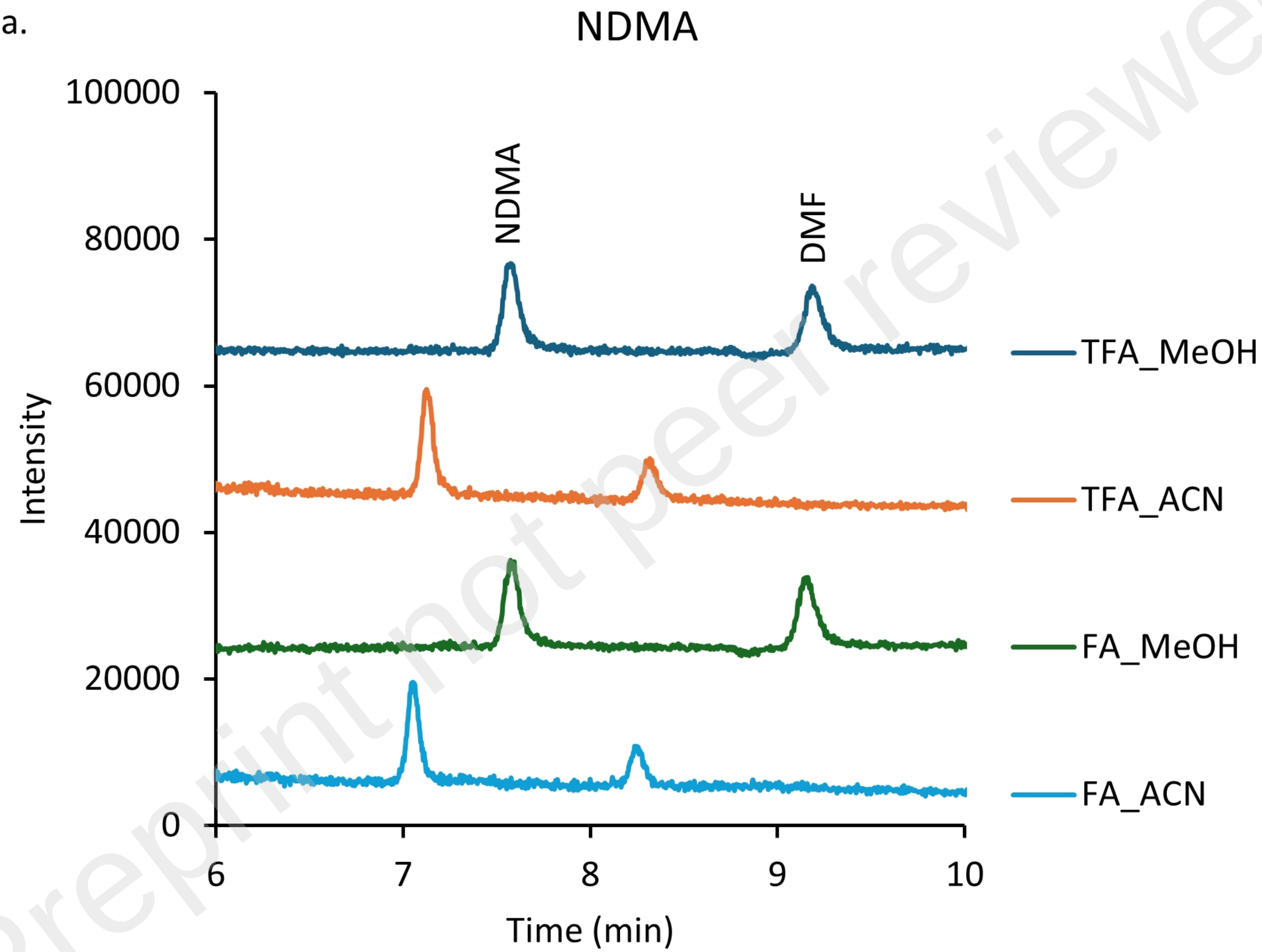
2b.


160
140
120
100
80
60
40
20
0
NDMA peak area (AU)
0
1
10
50
DMF Concentration (µg/mL)

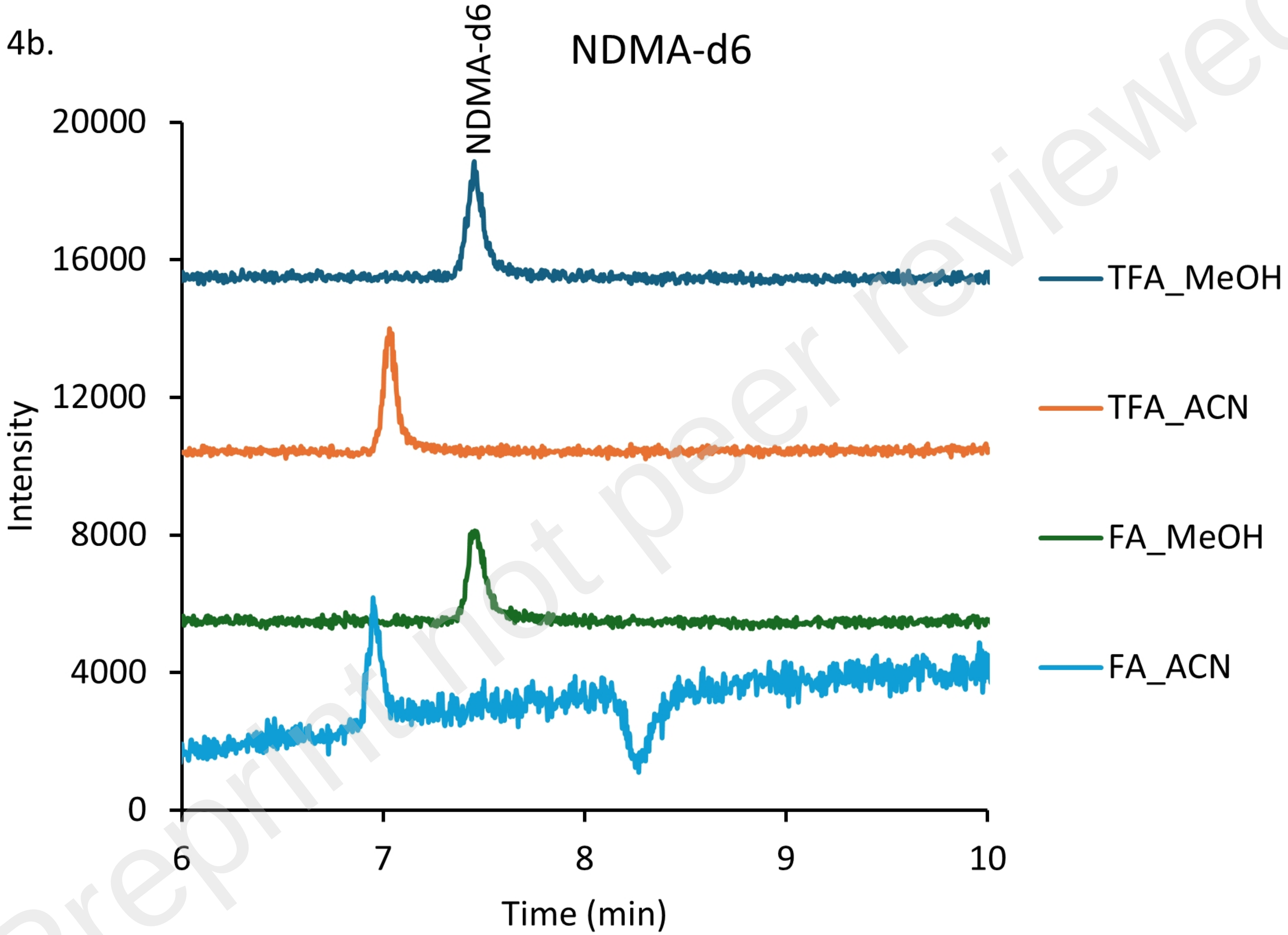


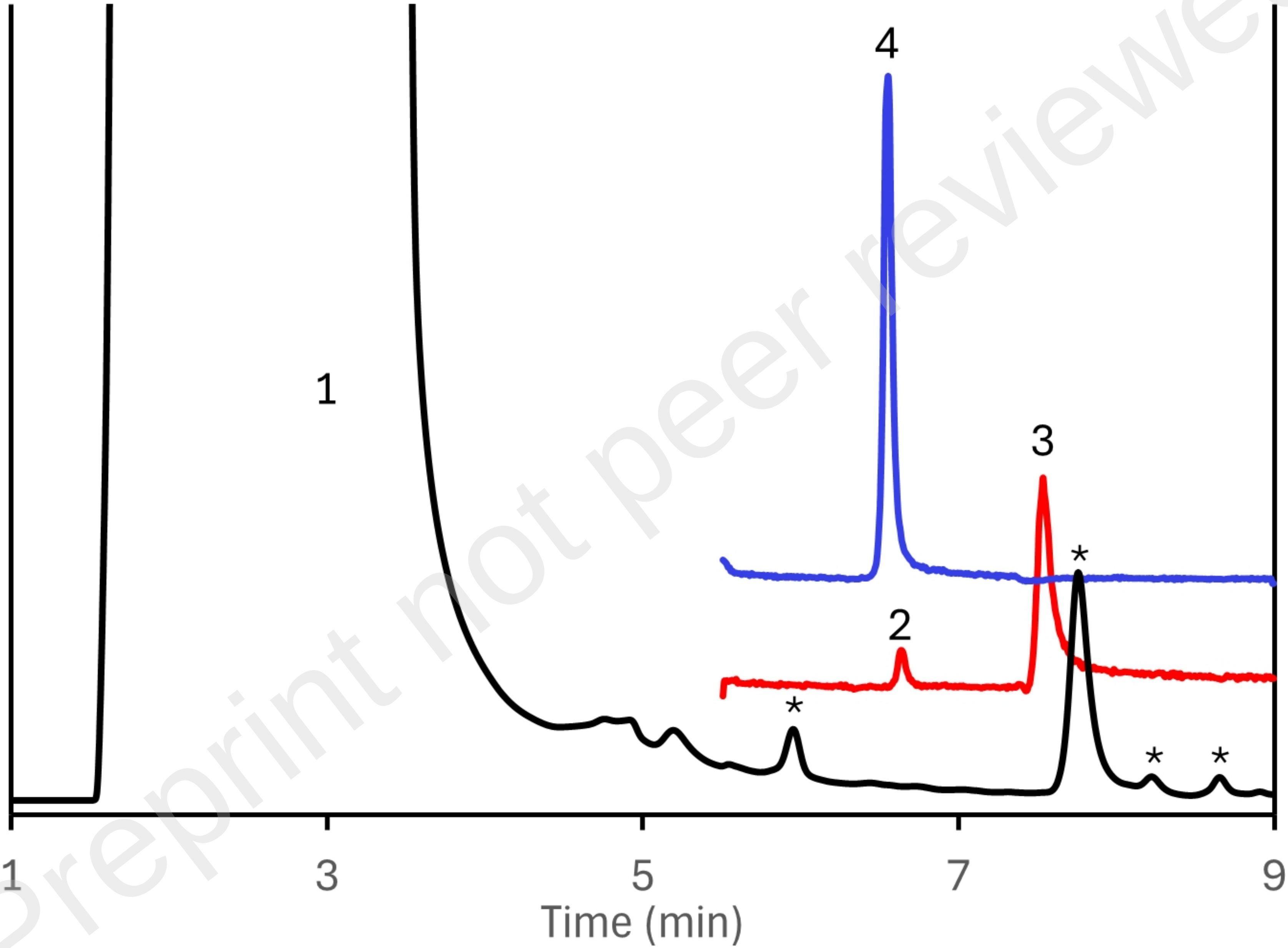
1+2
Bonus RP
1+2
1+2
Hydrosphere C18
2
Prontosil EPS
1+2
1
SB Phenyl
2
ODS 4
2
1
HSS T3
2
1
Evosphere Aqua
1
Ultra AQ
Intensity
2.5
4.5
6.5
8.5
10.5
12.5
Time (min)

4a.

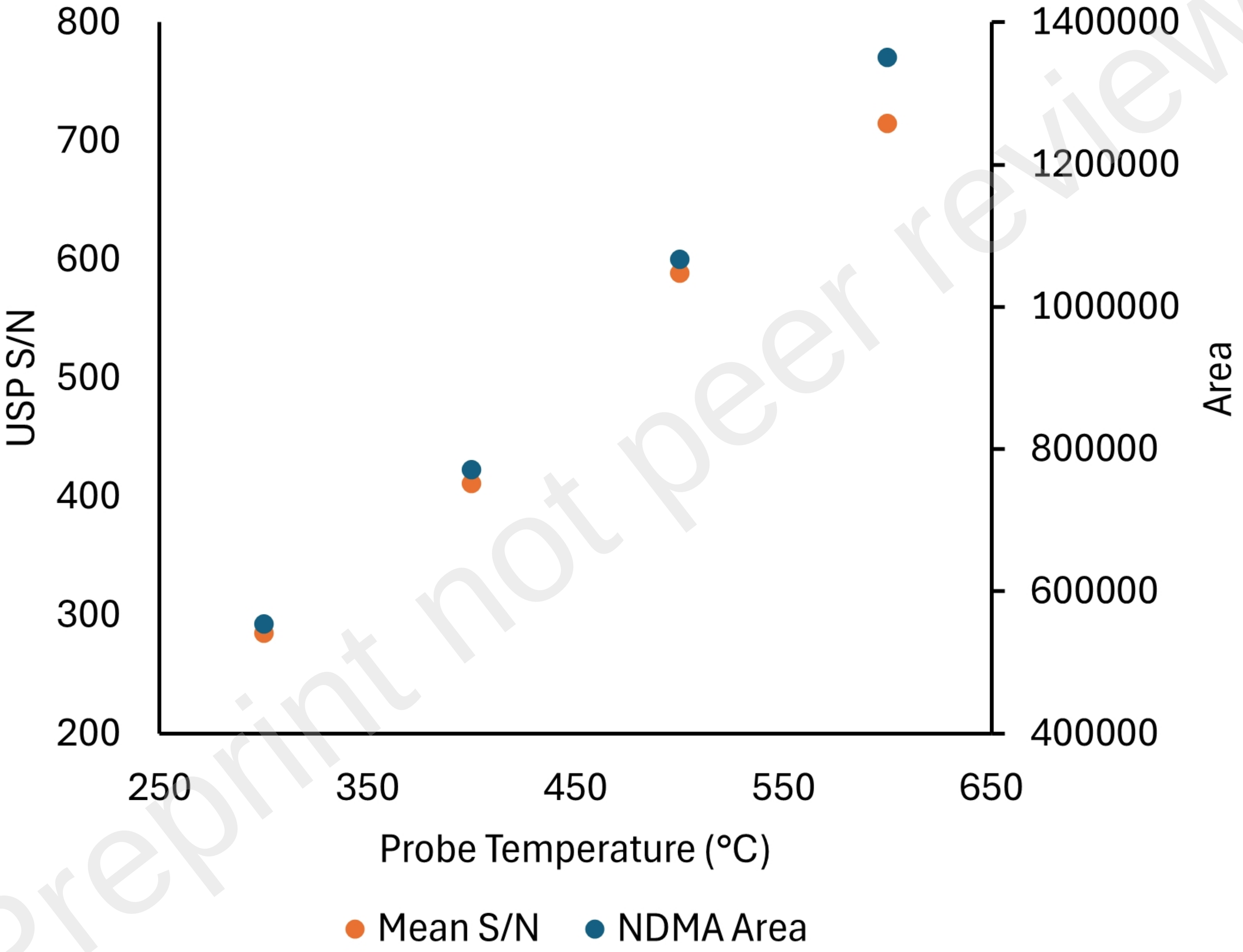

NDMA
Intensity
100000
80000
60000
40000
20000
0
Time (min)
6
7
8
9
10
NDMA
DMF
TFA_MeOH
TFA_ACN
FA_MeOH
FA_ACN

4b.


NDMA-d6
NDMA-d6
Intensity
20000
16000
12000
8000
4000
0
6
7
8
9
10
Time (min)
TFA_MeOH
TFA_ACN
FA_MeOH
FA_ACN

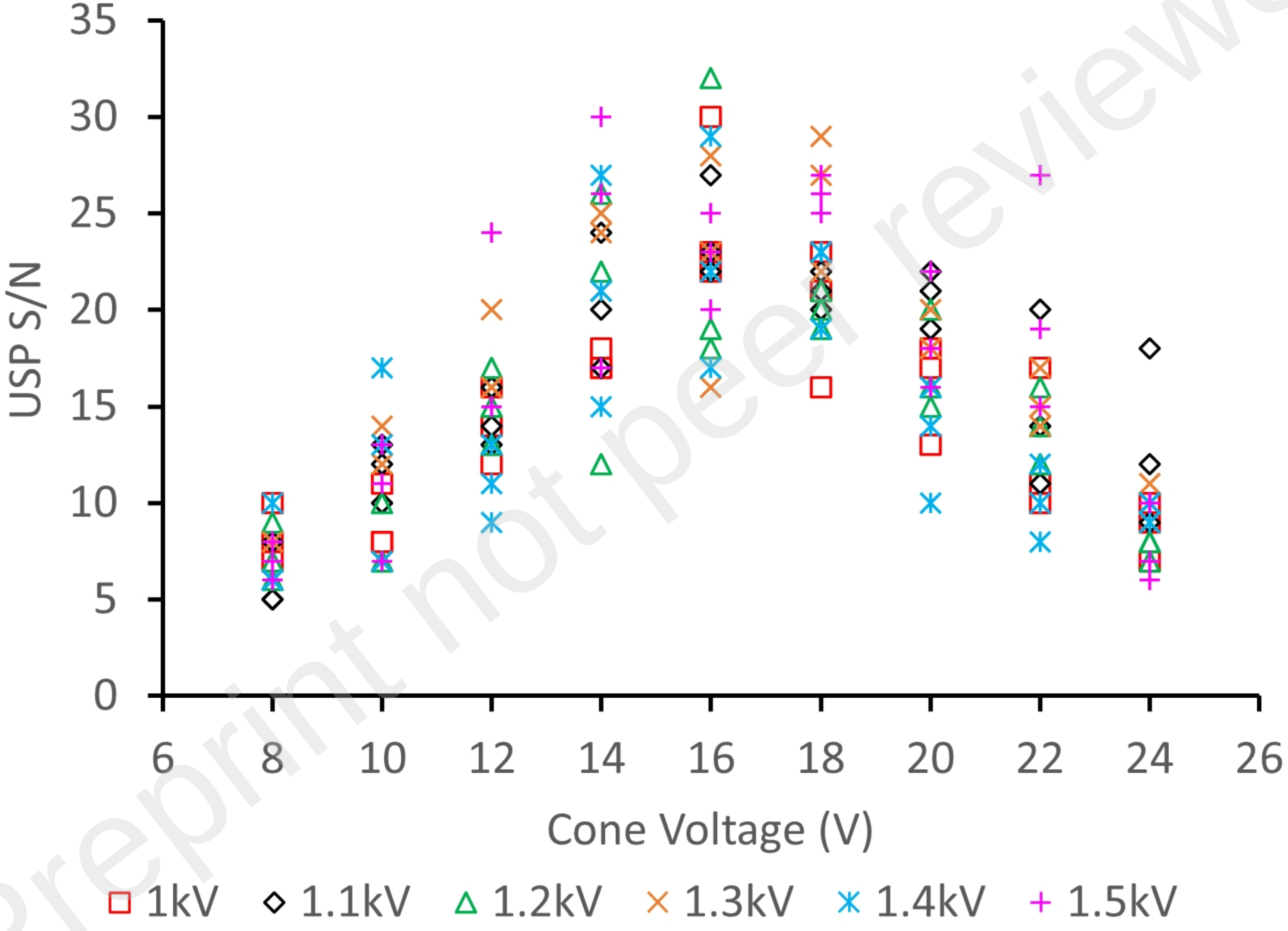

4
1
3
2
*
*
*
*
1
3
5
7
9
Time (min)

6a.



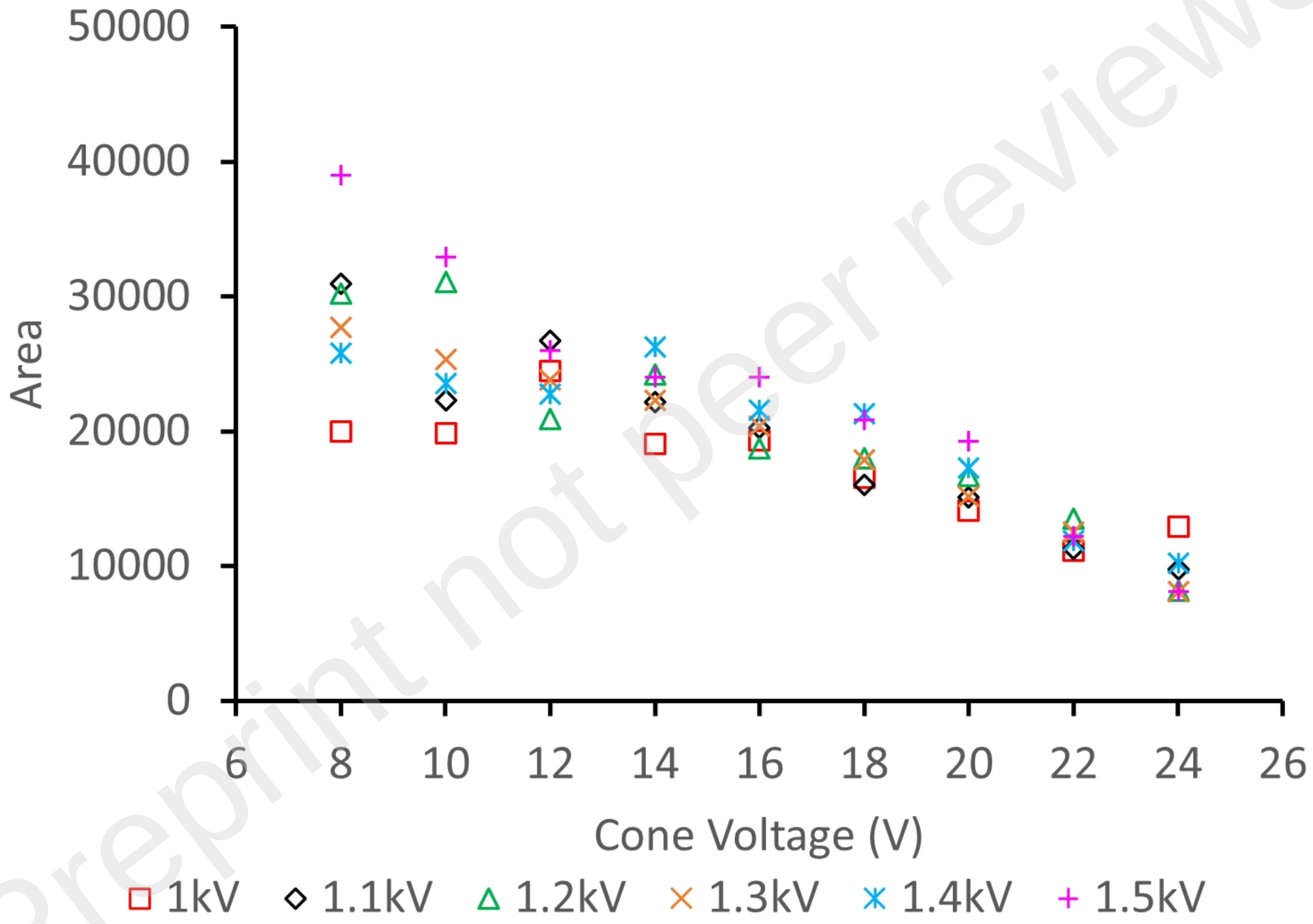
800
700
600
500
400
300
200
USP S/N
1400000
1200000
1000000
800000
600000
400000
Area
250
350
450
550
650
Probe Temperature (°C)
● Mean S/N
● NDMA Area

6c.

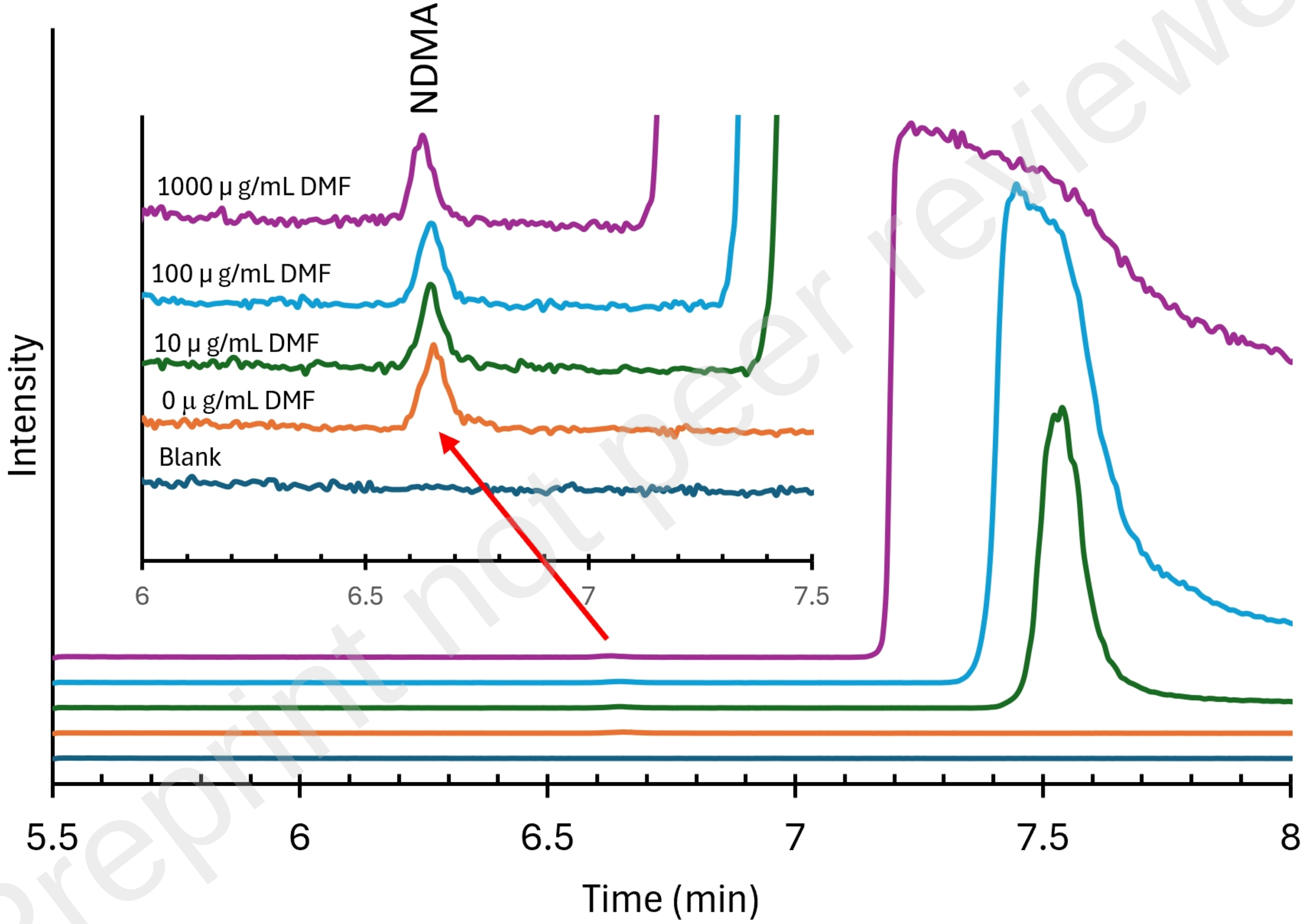


USP S/N
35
30
25
20
15
10
5
0
6
8
10
12
14
16
18
20
22
24
26
Cone Voltage (V)
□ 1kV
◇ 1.1kV
△ 1.2kV
× 1.3kV
✳ 1.4kV
+ 1.5kV

6b.

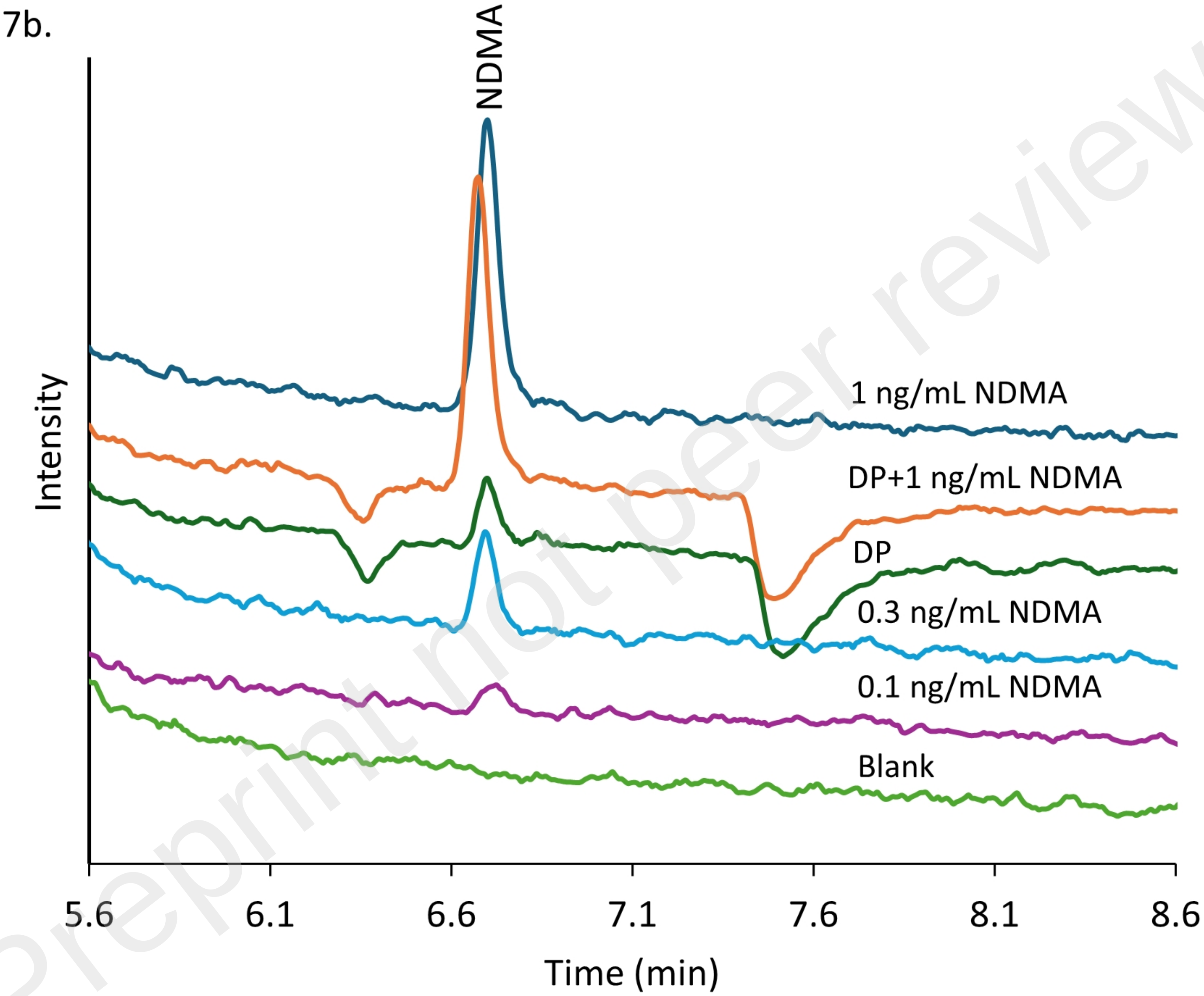

Area
50000
40000
30000
20000
10000
0
6
8
10
12
14
16
18
20
22
24
26
Cone Voltage (V)
□ 1kV
◇ 1.1kV
△ 1.2kV
× 1.3kV
✳ 1.4kV
+ 1.5kV

7a.



NDMA
1000 μ g/mL DMF
100 μ g/mL DMF
10 μ g/mL DMF
0 μ g/mL DMF
Blank
6
6.5
7
7.5
Intensity
5.5
6
6.5
7
7.5
8
Time (min)

7b.


NDMA
Intensity
1 ng/mL NDMA
DP+1 ng/mL NDMA
DP
0.3 ng/mL NDMA
0.1 ng/mL NDMA
Blank
5.6
6.1
6.6
7.1
7.6
8.1
8.6
Time (min)

Title: Analysis of N-Nitrosodimethylamine (NDMA) in Pharmaceutical Products in the Presence of High Concentration N,N-dimethylformamide (DMF)

Mikhaila D. Ritz[a], Ana I. Martinez[a], Timothy Yaroshuk[b], Xihui Liang[a], Xiaoyi Gong[a], Mark D. Mowery[a], and Jinjian Zheng[a]*

[a] Analytical Chemistry in Development and Supply, MMD, Merck & Co., Inc.; 126 E. Lincoln Ave, Rahway, NJ 07065, USA

[b] Department of Chemistry and Environmental Science, New Jersey Institute of Technology, Newark, New Jersey 07102, USA

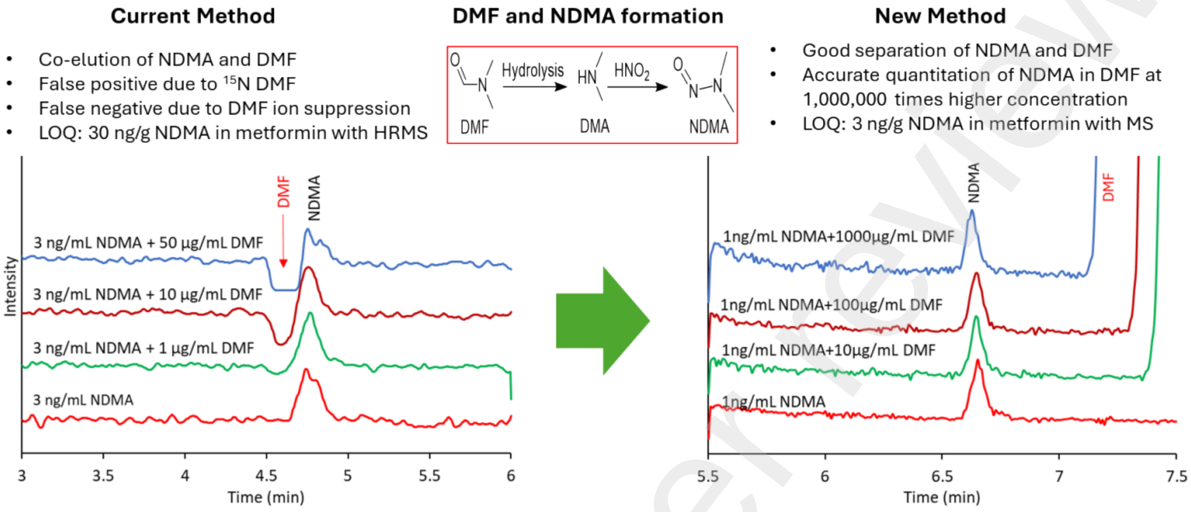
*Corresponding author. Email: jinjian.zheng@merck.com

Abstract: The presence of nitrosamines in pharmaceutical products presents significant challenges for the industry, primarily due to the potential carcinogenic risks they pose to patients. Among these, N-Nitrosodimethylamine (NDMA) has garnered attention as one of the first nitrosamines identified in products like Valsartan, Ranitidine, and Metformin. NDMA is a polar compound with weak retention on most stationary phases, making it susceptible to matrix interferences. A prevalent issue is the co-elution of NDMA and N,N-dimethylformamide (DMF), a common solvent and precursor for NDMA. DMF is typically present in much higher concentrations, which can lead to false positives and an overestimation of NDMA concentration due to interference from ^{15}N DMF and ^{13}C DMF. Conversely, elevated DMF concentrations can induce ion suppression, resulting in false negatives. Consequently, accurately quantifying NDMA remains a challenge, even when utilizing high-resolution or tandem mass spectrometry techniques.

To address these issues, we developed a robust HPLC-MS method employing an Evosphere AQUA column, which enables good separation of NDMA from DMF and other sample matrices. This method permits accurate quantification of NDMA in the presence of DMF at concentrations up to 1,000,000 times greater. We achieved a detection limit of 0.1 ng/mL using a single quadrupole mass spectrometer, such as QDa, which corresponds to 1 ng/g relative to a 100 mg/mL Metformin HCl sample concentration. The method has been successfully validated according to ICH guidelines, demonstrating specificity, sensitivity, accuracy, precision, and robustness. The application of this method was further illustrated

through the analysis of NDMA in Metformin drug products including both immediate release and extended release formulations.

Graphical abstract



Keywords: HPLC-MS; N-Nitrosodimethylamine (NDMA); Nitrosamine; N,N-dimethylformamide (DMF); Pharmaceutical Ananalysis; Metformin

Highlights:

- Analysis of NDMA using HPLC with a single quadrupole mass spectrometer
- Good separation of NDMA from DMF
- Accurate quantitation of NDMA with DMF at 1,000,000 times higher concentration
- LOQ of 3 ng/g NDMA for immediate release and extended release metformin products
- Practical solution with potential for easy implementation in quality control labs

1. Introduction:

N-nitroso dimethylamine (NDMA) is the smallest and most prevalent nitrosamine, recognized as a potent carcinogen in animal studies[1, 2]. The acceptable intake (AI) of NDMA has been established at 96 ng/day based on TD50 data[1]. While NDMA has been detected in the environment and food[3], its presence in pharmaceutical products was first identified in 2018 when it was found in Valsartan, a hypertension medication[4]. Since then, NDMA has been discovered in various pharmaceutical products, including sartans, metformin[5], and ranitidine[6], leading to numerous recalls and disruptions in the supply of these essential medications.

NDMA is commonly found in pharmaceutical products because dimethylamine (DMA), its precursor, is often present in active pharmaceutical ingredients (APIs) at trace levels[7]. DMA is frequently used as a building block in synthetic processes, and residual DMA can carry over into the API. For instance, in the synthesis of metformin hydrochloride, DMA reacts with cyanoguanidine to form metformin (see Figure 1a). Although most DMA can be removed from the metformin API, trace levels remain, with a specification limit of 500 ppm[8].

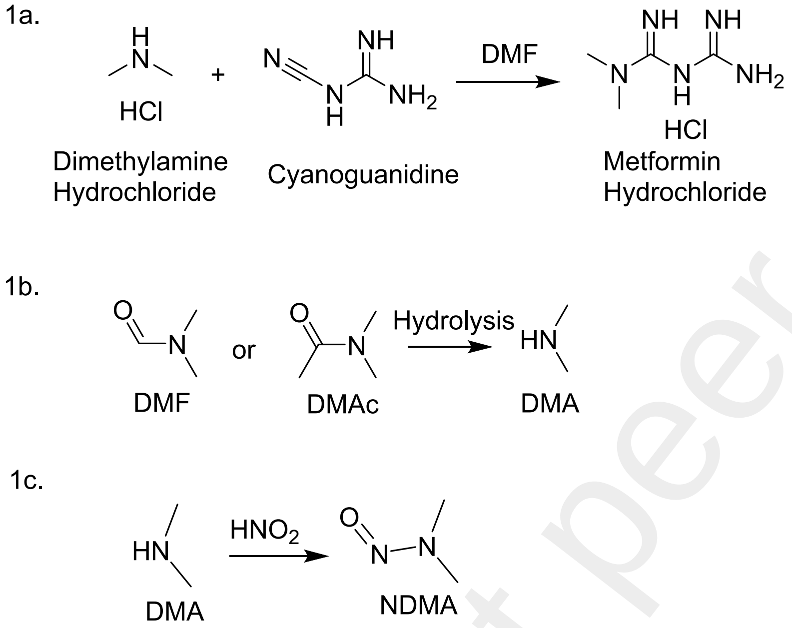


Figure 1. Formation of N-nitrosodimethylamine (NDMA). 1a. DMF as reaction solvent for the synthesis of metformin; 1b. Hydrolysis of DMF or DMAc to form DMA; 1c. Nitrosation of DMA to form NDMA.

Another source of DMA is the hydrolysis of N,N-dimethylformamide (DMF) or N,N-dimethylacetamide (DMAc), two common solvents used in pharmaceutical synthesis (see Figure 1b)[9]. Notably, DMF has been identified as the primary source of NDMA contamination in valsartan drug substances[10, 11]. Consequently, regulatory agencies often require confirmatory testing for NDMA when DMF is involved in the synthesis process.

Trace levels of DMA in drug substances can react with nitrosating agents, such as nitrite[12], which is commonly found in pharmaceutical excipients (see Figure 1c)[13]. Some batches of metformin products have been recalled due to NDMA levels exceeding the AI limit[5].

Numerous analytical methods have been developed to detect NDMA, including LC-MS[11, 14-16], GC-MS[16, 17], and GC-NPD[18, 19]. We have developed a sensitive method for analyzing NDMA in pharmaceutical products using full evaporation headspace gas chromatography with nitrogen-phosphorus detection (FE-HSGC-NPD). However, this method may not be suitable for thermally labile products[18]. On the other hand, HPLC coupled with mass spectrometry does not have this limitation, and is often used for NDMA analysis due to its high sensitivity and selectivity.

One challenge in analyzing NDMA by LC-MS is its polar nature, which results in weak retention on most reversed-phase liquid chromatography (RPLC) columns. As described by the Purnell equation[20], inadequate retention complicates the separation of the analyte from matrix interferences:

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k}{k+1}$$

For LC-MS analysis of NDMA in metformin products, matrix interference can arise from several sources, including metformin API, DMF, and excipients. A high concentration of metformin may suppress ionization or contaminate the mass spectrometer if it is not separated from NDMA. Additionally, DMF may co-elute with NDMA (M+H: 75.0553), and the presence of ^{15}N DMF (M+H: 75.0571) can lead to false positives in NDMA quantitation[11]. Conversely, high DMF concentrations may suppress NDMA ionization, resulting in false negatives.

Given that DMF can cause both false positives and negatives, improving the separation of NDMA and DMF is essential to mitigate interference. Although several attempts have been made to enhance this separation, satisfactory results have yet to be achieved, primarily due to the inadequate retention of both NDMA and DMF[21, 22].

In this study, we present a systematic approach to develop a robust LC-MS method for analyzing NDMA in metformin HCl products using an Evosphere AQUA column. This method effectively retains and separates NDMA from DMF and other sample matrices, offering superior sensitivity even with a simple single quadrupole mass spectrometer. The method has been successfully validated according to ICH Q2(R2) guidelines and applied to the analysis of NDMA in both instant release and extended release metformin HCl drug products.

2. Experiment

Evosphere AQUA column is manufactured by Fortis Technologies (Neston, UK) and purchased from Mac-Mod (Chadds Ford, PA). Acetonitrile (Optima LC-MS grade), methyl tert-butyl ether (MTBE, HPLC grade) and formic acid (Optima LC-MS grade) is purchased from Fisher Chemical (Pittsburgh, PA). NDMA (5 mg/mL in methanol) is purchased from MilliporeSigma (Burlington, MA) and NDMA-d6 (1 mg/mL in methanol) is purchased from Agilent Technologies (Santa Clara, CA).

2.1. **Instrumentation**:

All studies were performed using a Waters H–Class UHPLC system with QDa detector (Milford, MA). All data were acquired using Waters Empower 3 software with Feature Release 5 (Milford, MA).

The metformin HCl tablets were ground using IKA Tube Mill Model C-S001 (NC, USA). For the analysis of NDMA in metformin HCl extended release tablets, a SpeedVac (Model SPD140DDA, Thermo Scientific) was used to remove MTBE as described in sample preparation section.

2.2. **Standard Preparation:**

Diluent: 20 ng/mL NDMA-d6 in Milli-Q water. Prepared through sequential dilution of NDMA-d6 stock solution using Milli-Q water.

NDMA standard solution (3 ng/mL) and limit of quantitation solution (0.3 ng/mL) were prepared through sequential dilution of NDMA stock solution using Diluent.

2.3. **Sample Preparation:**

Sample preparation for immediate release metformin HCl tablets: Grind 3 or more metformin HCl tablets into fine powder. Weigh the powder equivalent to 500 mg metformin HCl into a 50-mL plastic centrifuge tube. Pipet 5.0 mL Diluent into it. Vortex for 2 minutes to extract NDMA into aqueous solution. Centrifuge at 4700 RPM for 10 minutes. Transfer the supernatant into an HPLC vial for analysis.

Sample preparation for extended release metformin HCl tablets: Grind 3 or more metformin HCl tablets into fine powder. Weigh the powder equivalent to 500 mg metformin HCl into a 50-mL plastic centrifuge tube. Pipet 10.0 mL MTBE into it. Cap and shake for 30 minutes. Centrifuge at 4700 RPM for 10 minutes. Pipet 4.0 mL supernatant into a 20-mL scintillation vial. Pipet 2.0 mL Diluent into it. Use SpeedVac to remove MTBE at 40°C and 3mTorr vacuum for 10 minutes. Transfer the remaining aqueous solution into a 1.5-mL centrifuge tube. Centrifuge at 13.3k RPM for 5 minutes. Transfer the supernatant into an HPLC vial for analysis.

2.4. HPLC Conditions for Finalized Method:

NDMA is separated using an Evosphere AQUA column (150x4.6 mm I.D., 3µm particle size). The mobile phase A contains 0.05% trifluoroacetic acid in Milli-Q water and mobile phase B is 100% acetonitrile. The flow rate is 1.0 mL/ min. The method uses gradient elution by holding at 0% acetonitrile for 1 minute, ramping from 0% to 10% acetonitrile in 9 minutes, washing at 90% acetonitrile for 2 minutes, followed by re-equilibration at 0% acetonitrile for 13 minutes for a total run time of 25 minutes. Column temperature is held at 25°C and the injection volume is 50 µL.

2.5. QDa Parameters:

NDMA and NDMA-d6 are detected with a QDa detector (Waters, MA) in positive mode with Single Ion Recording (SIR) at m/z= 75 for NDMA and m/z= 81 for d6 NDMA.

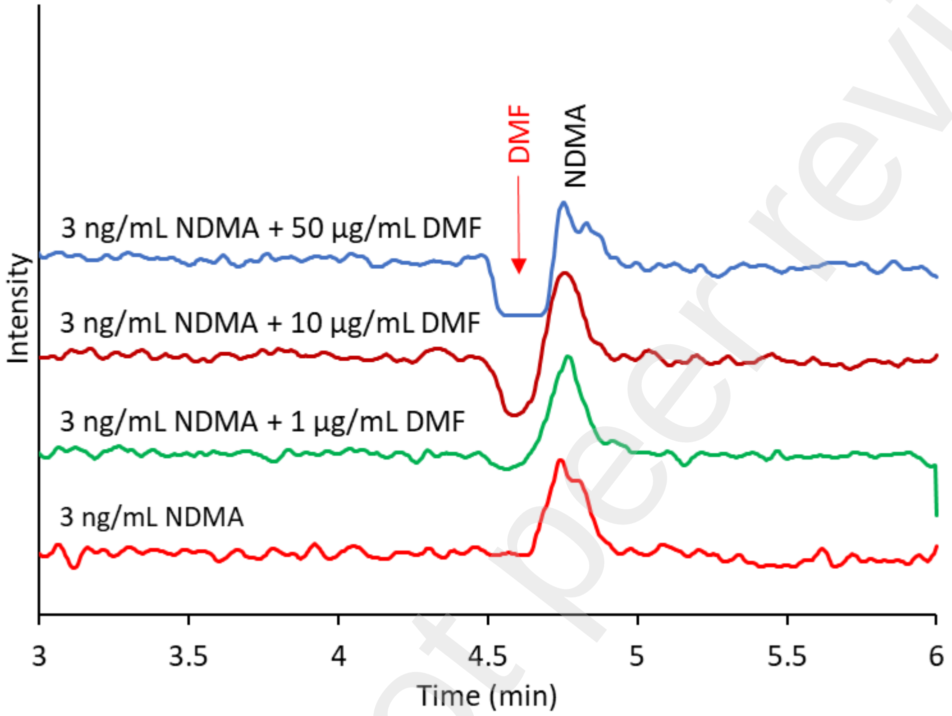
3. Results and Discussion:

3.1. Interference of DMF on the quantitation of NDMA

False positive on NDMA analysis caused by the interference of DMF has been discussed in detail in the publication by FDA in response to a Citizen Petition filed by Valisure[11]. The m/z of protonated ^{15}N DMF is 75.0571, which is within 24 ppm of m/z of protonated NDMA, 75.0553. When DMF co-elutes with NDMA, ^{15}N DMF may be misidentified as NDMA, contributing to false positive if insufficient mass resolution is used. Although the abundance of ^{15}N is only 0.365%, ^{15}N DMF can still contribute to significant false positive for metformin products if DMF co-elutes with NDMA because the specification of DMF is 880 µg/g, which is 27,500 times higher than the AI of 0.032 µg/g for NDMA based on 3 g maximum daily dose for diabetes indication. In addition, the ^{13}C isotope of DMF (M+H: 75.0634) may contribute to false positive of NDMA if a low resolution mass spectrometer is used. Yang et al has shown that this interference can be minimized by using high resolution mass spectrometer such as QExactive™ (Thermoscientific, PA) with a narrow mass isolation range[11]. However, this solution has its limitations when large amount of DMF is present, which may suppress the ionization of NDMA.

Shown in Figure 2a are the chromatograms of 3 ng/mL NDMA spiked with 0, 1, 10 and 50 µg/mL of DMF using the FDA method with a QExactive™ mass spectrometer[14]. The responses of NDMA are summarized in Figure 2b, which shows that NDMA peak area decreases with increasing DMF concentration. It was also noticed that there is a baseline dip at the retention time of DMF and right before NDMA peak in Figure 2a, which is another indication of ion suppression due to DMF. These results clearly demonstrated that high concentration of DMF may suppress the ionization of NDMA, causing false negative. It is worth noting that the ion suppression is instrument dependent[23], and in some cases, NDMA cannot be detected at all. Refer to Supplemental Information 1 – 3 for overlaid chromatograms of NDMA in the presence of DMF concentrations from 0 to 100 μg/mL. To overcome this issue, better separation of NDMA and DMF is desired.

2a.



2b.

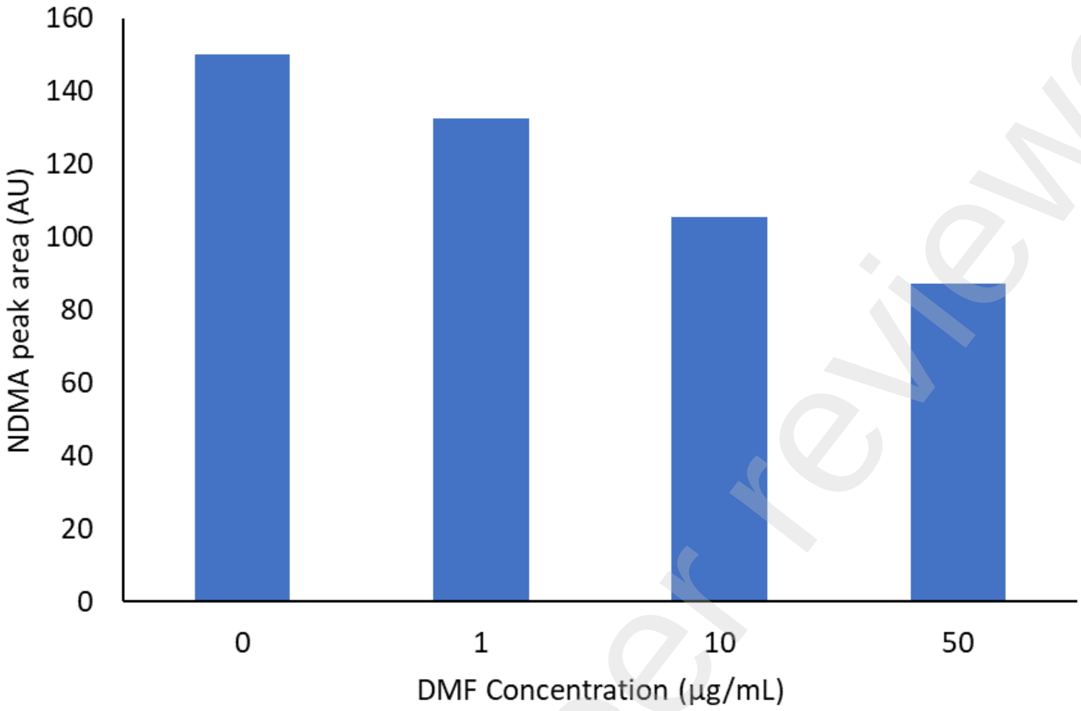


Figure 2. Baseline disturbance and ion suppression caused by co-elution of NDMA with DMF. 2a. Overlaid chromatograms of NDMA in the presence of different concentrations of DMF; 2b. Peak area of NDMA with respect to DMF concentration in the sample.

3.2. Method development:

3.2.1. Column screening

Column screening was performed to identify a column that provides sufficient separation of NDMA from DMF and sample matrix. Considering both NDMA and DMF are polar compounds with negative LogP (LogP = -0.57 for NDMA calculated using ACD/Labs), we only selected columns that are compatible with 100% aqueous mobile phase for screening. Those columns include Evosphere AQUA (Fortis Technologies, UK), Hydrosphere C_{18} (YMC, Japan), Bonus RP (Agilent Technologies, US), SB-Phenyl (Agilent Technologies, CA), Xselect HSS T3 (Waters, MA), Inertsil ODS-4 (GL Sciences, Japan), Prontosil C_{18} ace-EPS (Bischoff, DE) and Ultra Aqueous C_{18} (Restek, PA). The column dimension is 4.6x150 mm for all columns. The particle size is 3 µm for all columns except for Xselect HSS T3, Zorbax SB Phenyl and Zorbax Bonus RP columns, which are 3.5 µm. The column temperature is controlled at 25°C. Mobile phase A is 0.1% formic acid and mobile phase B is acetonitrile, and the flow rate is 1 mL/min. The gradient is as follows: hold at 0%B for 1 min, ramp from 0%B to 15%B in

18 min. The sample contains mixture of 10 ng/mL NDMA + 10 μg/mL DMF, and the injection volume is 5 μL. NDMA and DMF are detected using QDa in positive mode with single ion recording (SIR) at m/z 75 for NDMA, ^{13}C DMF and ^{15}N DMF.

The overlaid chromatograms are shown in Figure 3. The best retention of NDMA and best separation of NDMA and DMF were obtained using the Fortis Evosphere AQUA column. Baseline separation can also be obtained using the Restek Ultra Aqueous C_{18} column and Agilent Zorbax SB Phenyl column. However, the best resolution was obtained using an Evosphere AQUA column. Since the retention time of DMF decreases when its concentration increases due to mass overloading and may cause DMF to co-elute with NDMA, high resolution between NDMA and DMF is preferred. Therefore, Evosphere AQUA column was selected for further optimization.

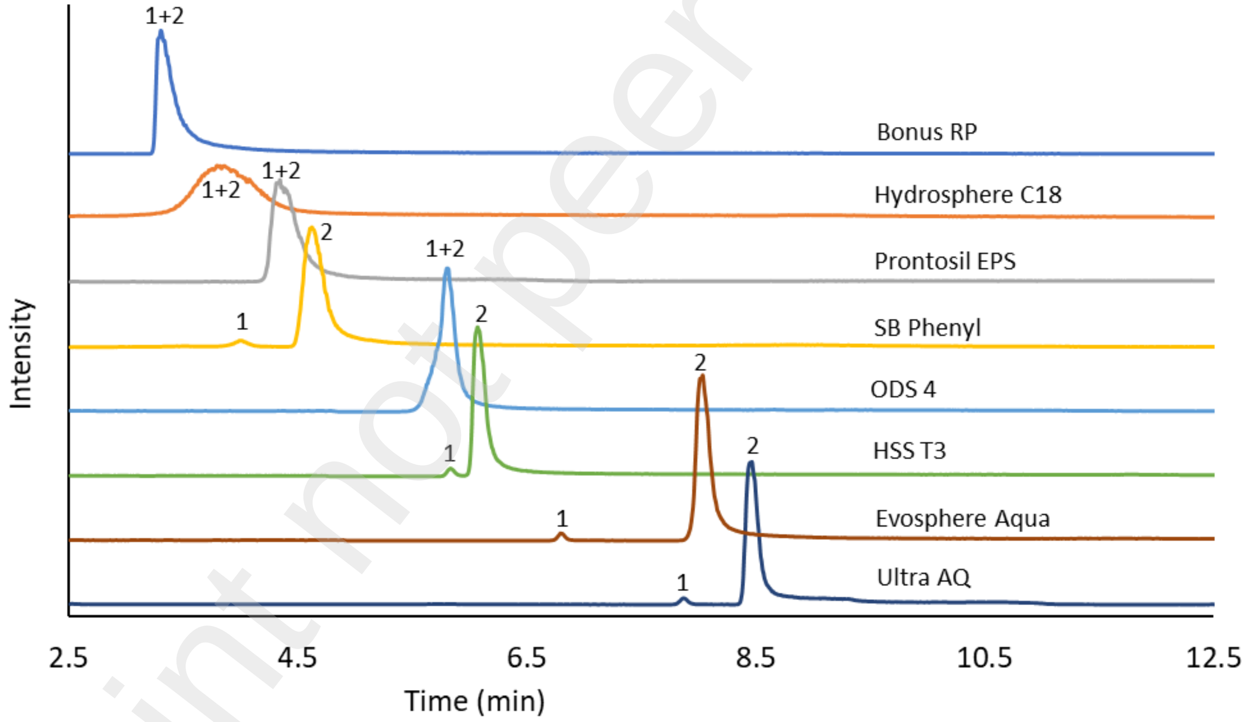


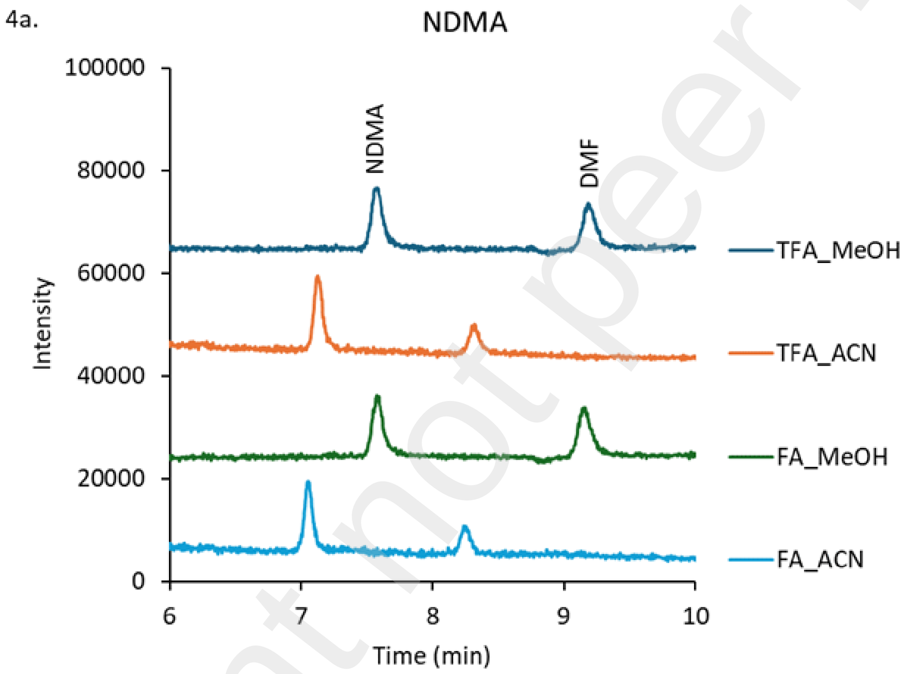
Figure 3. Overlaid chromatograms from column screening. Peak labeling: 1- NDMA; 2 -DMF.

3.2.2. Mobile phase screening

The impact of organic solvent (acetonitrile vs methanol) and mobile phase additive (formic acid vs trifluoroacetic acid, TFA) on method performance was evaluated including method sensitivity and resolution of NDMA and DMF.

Satisfactory separation of NDMA and DMF was obtained using acetonitrile or methanol as mobile phase as shown in Figure 4. However, better peak shape of NDMA was obtained using acetonitrile as mobile phase.

The impact of acidic additives on method performance was evaluated using 0.1% formic acid and 0.05% trifluoroacetic acid (TFA). No significant differences were observed in the separation of NDMA and DMF, nor in the sensitivity of NDMA, as shown in Figure 4a. However, when 0.1% formic acid and acetonitrile were used as mobile phases, slightly higher baseline noise was observed for NDMA-d6, the internal standard employed to compensate for matrix effects during the sample preparation of extended-release metformin HCl tablets, as illustrated in Figure 4b. Consequently, 0.05% TFA and acetonitrile were selected as the mobile phases for further optimization.



4b.

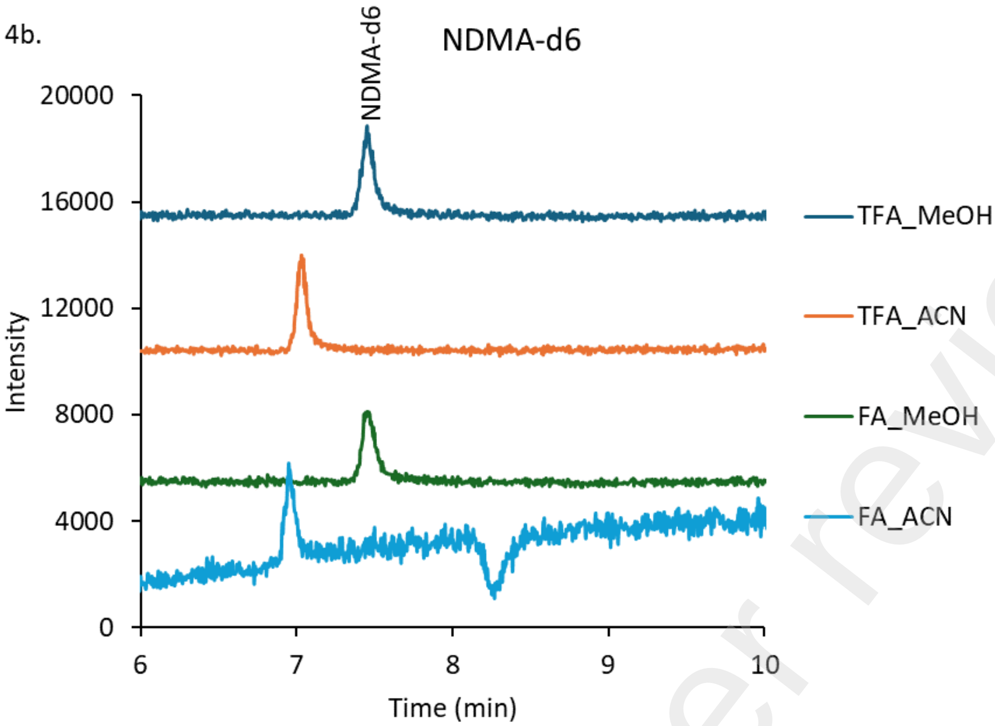


Figure 4. Screening of organic solvents and acidic modifiers. 4a. NDMA (m/z 75); 4b. NDMA-d6 (m/z 81). Sample: metformin tablet spiked with 100 ng/mL NDMA. Injection volume: 2 µL. Mobile phase A1: 0.1% formic acid. Mobile phase A2: 0.05% trifluoroacetic acid. Mobile phase B1: acetonitrile. Mobile phase B2: methanol. Gradient with Mobile phase B1: hold 0%B for 1 min, ramp 0%B to 10%B in 12 min. Re-equilibrate for 7 minutes. Gradient with Mobile phase B2: hold 0%B for 1 min, ramp 0%B to 25%B in 12 min. Re-equilibrate for 7 minutes. QDa parameters are the same as described in experimental section.

3.2.3. Optimization of TFA-acetonitrile method

Using the retention data from two gradients on Evosphere AQUA column including retention time and peak width, we were able to optimize the separation of NDMA, DMF and matrix peaks using ACD/Labs LC Simulator, a chromatography modelling software. The retention data for NDMA, NDMA-d6 and DMF was based on MS detection while the retention data for matrix peaks was based on UV detection. Although matrix peaks cannot be detected by MS, it is desirable to separate them from NDMA to avoid potential ion suppression and contamination to MS instrument.

The optimized method was shown in Figure 5. Good separation of NDMA from DMF and matrix peaks was obtained.

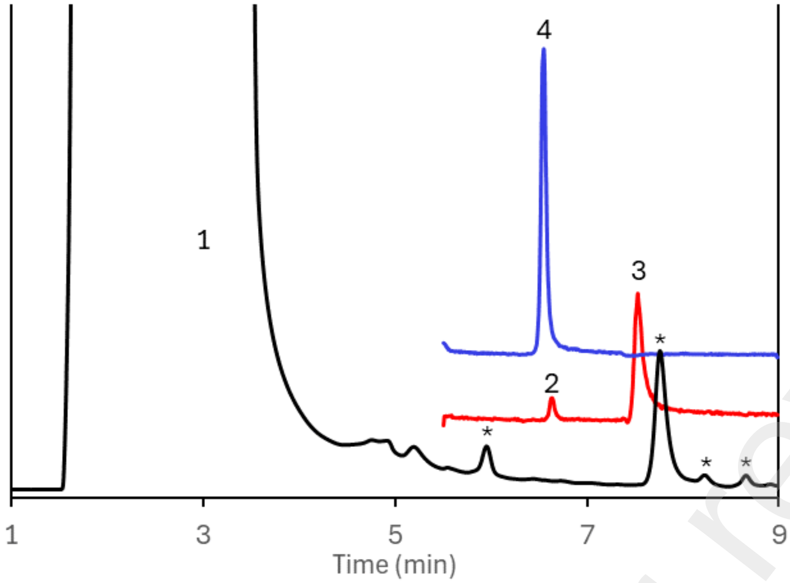


Figure 5. Chromatograms using optimized method. NDMA, NDMA-d6 and DMF were detected by MS while other matrix peaks were detected by UV. Peak identity: 1 – metformin; 2 – NDMA; 3 – DMF; 4 – d6 NDMA; * – matrix peaks

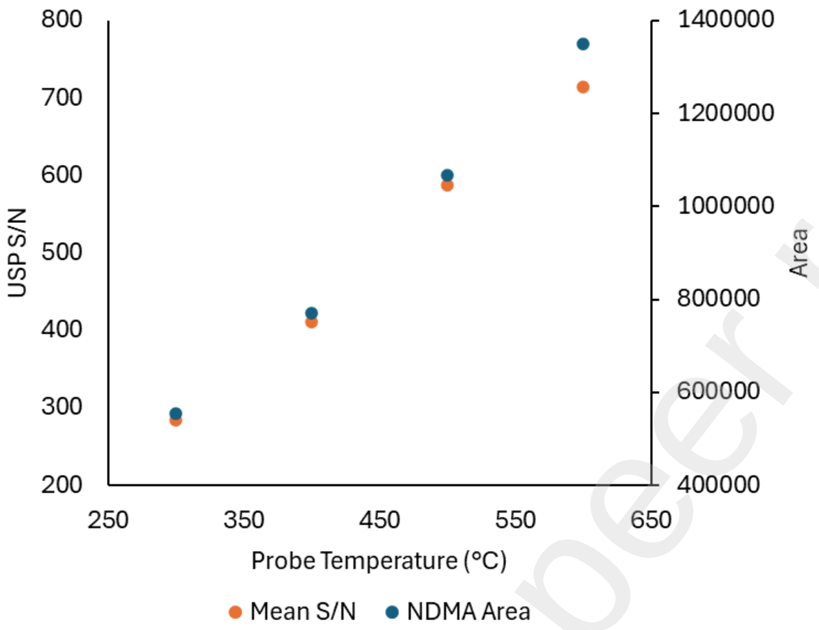
3.2.4. Optimization of QDa parameters:

The QDa parameters were optimized including capillary voltage, probe temperature and cone voltage. Both peak area and signal-to-noise (S/N) ratio ratio of NDMA were evaluated. The S/N ratio of NDMA was calculated using peak-to-peak noise from three different baseline regions to account for random fluctuation in baseline noise. Both NDMA peak area and S/N ratio increase with increasing probe temperature as shown in Figure 6a. Therefore, the probe temperature was set to 600 °C, the highest setting for the QDa.

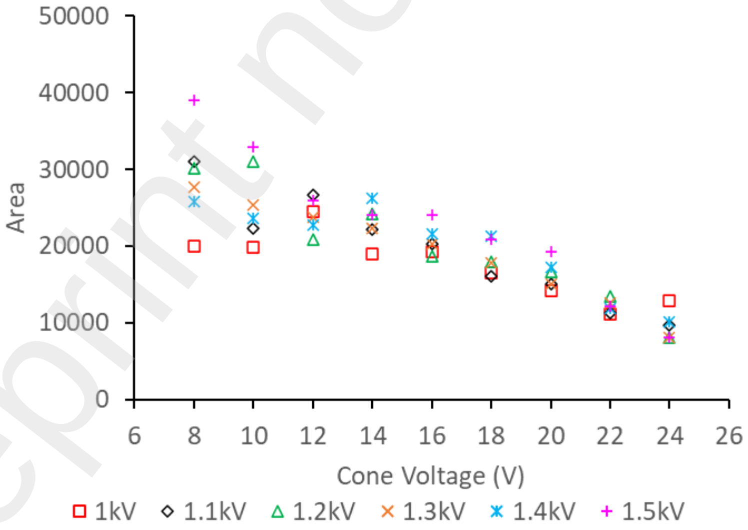
The cone voltage was varied from 8 V to 24 V. The peak area of NDMA decreases with increasing cone voltage, probably due to in source fragmentation[24] of NDMA at higher cone voltage as shown in Figure 6b. However, the S/N ratio does not follow the same trend, and the highest S/N ratios were obtained at cone voltage between 16 V and 18 V as shown in Figure 6c. Therefore, the cone voltage was set at 17 V.

The capillary voltage was varied from 1.0 kV to 1.5 kV. NDMA peak area increased about 30% when the capillary voltage was increased from 1.0 kV to 1.5 kV. However, there is no significant difference in S/N ratio except that S/N ratio is slightly lower when the capillary voltage is set at 1.0 kV. For the final method, the capillary voltage was set at 1.5 kV.

6a.



6b.



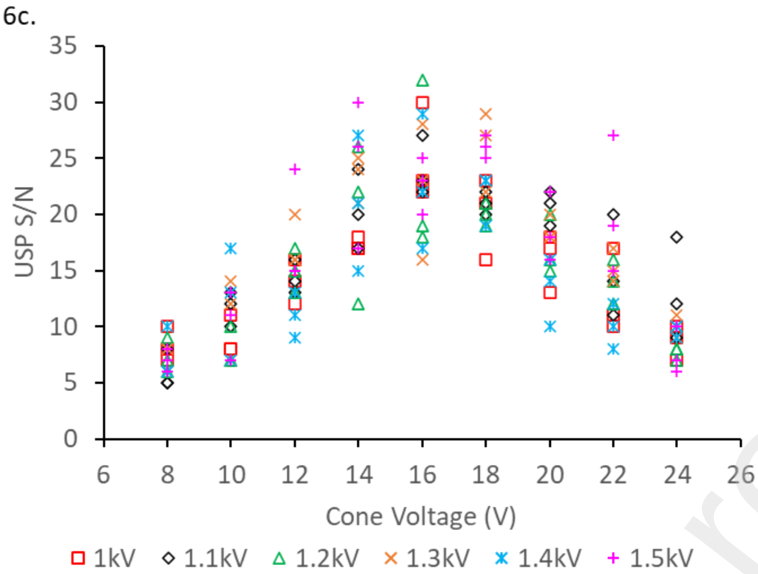


Figure 6. Optimization of QDa parameters. 6a: method sensitivity vs probe temperature; 6b: NDMA peak area vs capillary voltage and cone voltage; 6c: method sensitivity vs capillary voltage and cone voltage

3.3. Method characterization

3.3.1. Interference of DMF

As discussed above, one of the biggest challenges for NDMA analysis is how to eliminate the interference of DMF. Figure 7a shows the overlaid chromatograms of 1 ng/mL NDMA spiked with different DMF concentrations from 10 μg/mL to 1000 μg/mL using the optimized method. It is noticed that DMF peak did not return to the baseline and spanned a wide range from 7.1 min to after 9 min. This may create a problem if NDMA elutes on the tail of DMF peak and no sufficient separation was achieved. It is also noticed that the retention time of DMF decreases with increasing concentration, and may interfere with the quantitation of NDMA in methods where NDMA elutes before DMF but is not adequately separated from DMF [21].

In our new method, NDMA elutes before DMF and is well separated from it. The quantitation of NDMA remains unaffected by DMF, even at concentrations up to 1,000,000 times higher, as demonstrated in Figure 7a. For details on the peak area of NDMA relative to DMF concentrations, please refer to Supplemental Information 4. In the metformin HCl drug substance, DMF is controlled to ≤ 880 ppm [8], which corresponds to a maximum of 88 µg/mL in a 100 mg/mL metformin HCl sample used for NDMA analysis. Therefore, there is no risk of DMF interfering with the quantitation of NDMA.

Shown in Figure 7b are representative chromatograms of blank, LOD (0.1 ng/mL NDMA), LOQ (0.3 ng/mL NDMA), metformin drug product, metformin drug product spiked with 1ng/mL NDMA and 1 ng/mL NDMA standard. No interference from blank or sample matrix was observed, demonstrating satisfactory method specificity. The retention time of NDMA decreased slightly (~0.05 minute) in metformin drug product, which is likely due to the injection of large amount of metformin. However, it is not a concern as it does not affect the identification or integration of NDMA peak.

7a.

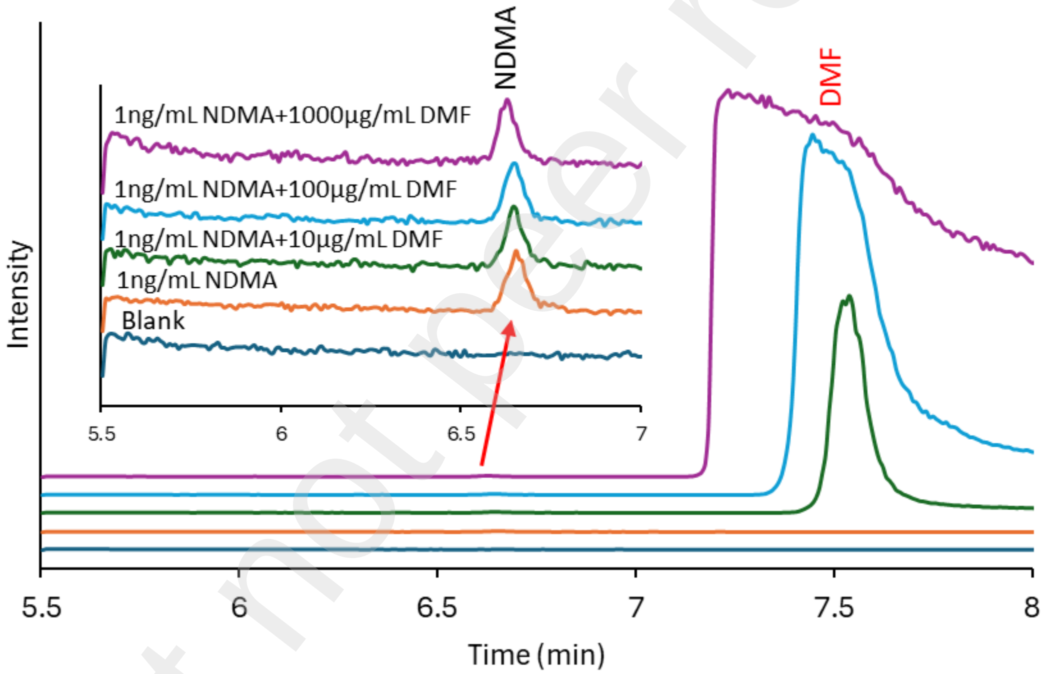


Figure 7a. Overlaid chromatograms of 1 ng/mL NDMA spiked with 10, 100 and 1000 µg/mL DMF. Refer to Supplemental Information 4 for peak area of NDMA in different samples.

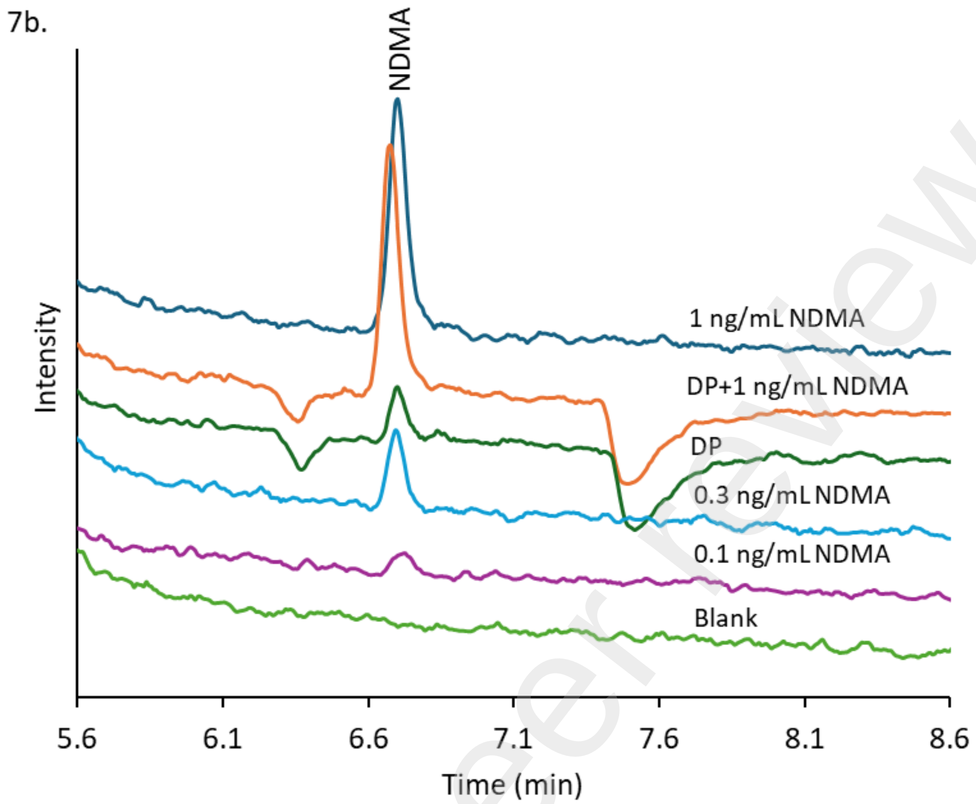


Figure 7b. Representative chromatograms of blank, LOD (0.1 ng/mL NDMA), LOQ (0.3 ng/mL NDMA), DP, DP+1ng/mL NDMA and 1 ng/mL NDMA.

3.3.2. Method validation:

The method has been successfully validated per ICH Q2(R2) guideline including specificy, linearity/range, accuracy, LOQ/LOD and precision[25]. The results are summarized in Table 1.

Good sensitivity was obtained even with a basic single quadrupole mass spectrometer such as QDa. The LOD is 0.1 ng/mL or 1 ng/g with respect to 100 mg/mL metformin HCl concentration. The LOQ is 0.3 ng/mL, corresponding to 3 ng/g with respect to 100 mg/mL metformin HCl concentration, or 10% of the acceptable intake of 96 ng/day or 32 ng/g relative to maximum daily dose of 3 g metformin HCl.

The range was established to be 3 ng/g to 100 ng/g by successfully meeting linearity, accuracy and precision requirements.

The validation results were obtained using Water QDa, a basic single quadrupole mass spectrometer. The method can be adapted on most single quadrupole mass spectrometers with the same or better sensitivity.

The method has been successfully applied for both immediate release and extended release metformin drug products. Refer to the Experimentation section for sample preparation procedures for each product. For extended release tablets, the sample preparation procedure involves a solvent switch from MTBE to water using nitrogen blow down. To minimize variability caused by loss of NDMA or water during MTBE removal, NDMA-d6 was used as internal standard. Refer to Supplemental Information 5 and 6 for overlaid chromatograms for NDMA in metformin HCl immediate release and extended release drug products.

4. Conclusions

We reported a systematic approach to develop a simple and sensitive LC-MS method for the analysis of N-Nitrosodimethylamine (NDMA) in the presence of high concentrations of N,N-dimethylformamide (DMF) in complex pharmaceutical products. By optimizing the chromatographic separation using an Evosphere AQUA column, we successfully eliminated the interference of DMF at concentrations up to 1,000,000 times higher than NDMA. The limit of quantitation (LOQ) achieved is 3 ng/g, and the limit of detection (LOD) is 1 ng/g for metformin products, corresponding to 10% and 3% of the acceptable intake of NDMA in these products.

This method demonstrates robust performance even when utilizing a basic single quadrupole mass spectrometer (i.e., Waters QDa). For applications requiring enhanced sensitivity, coupling this method with a more sensitive mass spectrometer is feasible.

Given that DMF is a known risk factor for the formation of N-Nitrosodimethylamine (NDMA), confirmatory testing for NDMA is now a regulatory expectation whenever DMF is utilized in the synthesis process. This method provides a practical solution to meet this requirement, as it employs straightforward instrumentation and can be easily implemented in quality control laboratories for routine analysis.

5. Acknowledgement

The authors would like to thank Christine L. Radich for careful review of manuscript and insightful comments.

6. References

(1) Peto, R.; Gray, R.; Brantom, P.; Grasso, P. Dose and time relationships for tumor induction in the liver and esophagus of 4080 inbred rats by chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine. *Cancer Res* **1991**, *51* (23 Pt 2), 6452-6469.

(2) *IARC monographs on the identification of carcinogenic hazards to humans*. https://monographs.iarc.who.int/list-of-classifications (Accessed May 15, 2025).

(3) Walker, R. Nitrates, nitrites and N-nitrosocompounds: A review of the occurrence in food and diet and the toxicological implications. *Food Additives & Contaminants* **1990**, *7* (6), 717-768. DOI: 10.1080/02652039009373938.

(4) Food and Drug Administration, *FDA updates and press announcements on angiotensin II receptor blocker (ARB) recalls (Valsartan, Losartan, and Irbesartan)*. https://www.fda.gov/drugs/drug-safety-and-availability/fda-updates-and-press-announcements-angiotensin-ii-receptor-blocker-arb-recalls-valsartan-losartan (Accessed May 15, 2025).

(5) Food and Drug Administration, *FDA Updates and Press Announcements on NDMA in Metformin*. https://www.fda.gov/drugs/drug-safety-and-availability/fda-updates-and-press-announcements-ndma-metformin (Accessed May 15, 2025).

(6) Food and Drug Administration, *FDA updates and press announcements on NDMA in ranitidine (Zantac)*. (Accessed May 15, 2025).

(7) U.S. Pharmacopeia, General Chapter <1469> NITROSAMINE IMPURITIES. 2020 (Accessed May 15, 2025).

(8) European Pharmacopeia, Metformin Hydrochloride monograph. 2020. Link: https://pheur.edqm.eu/app/11-8/content/11-8/0931E.htm?

(9) Food and Drug Administration, Control of Nitrosamine Impurities in Human Drugs, Guidance for Industry. 2 ed.; 2024. (Accessed May 15, 2025).

(10) Ray, A.; Atal, S.; Sadasivam, B. Understanding the molecular-pharmaceutical basis of sartan recalls focusing on valsartan. *Glob Cardiol Sci Pract* **2020**, *2020* (2), e202025. DOI: 10.21542/gcsp.2020.25

(11) Yang, J.; Marzan, T. A.; Ye, W.; Sommers, C. D.; Rodriguez, J. D.; Keire, D. A. A Cautionary Tale: Quantitative LC-HRMS Analytical Procedures for the Analysis of N-Nitrosodimethylamine in Metformin. *Aaps j* **2020**, *22* (4), 89. DOI: 10.1208/s12248-020-00473-w.

(12) Ashworth, I. W.; Dirat, O.; Teasdale, A.; Whiting, M. Potential for the Formation of N-Nitrosamines during the Manufacture of Active Pharmaceutical Ingredients: An Assessment of the Risk Posed by Trace Nitrite in Water. *Organic Process Research & Development* **2020**, *24* (9), 1629-1646. DOI: 10.1021/acs.oprd.0c00224.

(13) Boetzel, R.; Schlingemann, J.; Hickert, S.; Korn, C.; Kocks, G.; Luck, B.; Blom, G.; Harrison, M.; François, M.; Allain, L.; et al. A Nitrite Excipient Database: A Useful Tool to Support N-Nitrosamine Risk Assessments for Drug Products. *Journal of Pharmaceutical Sciences* **2023**, *112* (6), 1615-1624. DOI: https://doi.org/10.1016/j.xphs.2022.04.016.

(14) Food and Drug Administration, Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) Method for the Determination of NDMA in Metformin Drug Substance and Drug Product. 2020. Link: FY20-058-DPA-S_Method-Determination-NDMA-Metformin-LC-HRMS_for-sharing-003_0.pdf. (Accessed May 15, 2025)

(15) Ripollés, C.; Pitarch, E.; Sancho, J. V.; López, F. J.; Hernández, F. Determination of eight nitrosamines in water at the ngL−1 levels by liquid chromatography coupled to atmospheric pressure chemical ionization tandem mass spectrometry. *Analytica Chimica Acta* **2011**, *702* (1), 62-71. DOI: https://doi.org/10.1016/j.aca.2011.06.024.

(16) Fritzsche, M.; Blom, G.; Keitel, J.; Goettsche, A.; Seegel, M.; Leicht, S.; Guessregen, B.; Hickert, S.; Reifenberg, P.; Cimelli, A.; et al. NDMA analytics in metformin products: Comparison of methods and pitfalls. *European Journal of Pharmaceutical Sciences* **2022**, *168*, 106026. DOI: https://doi.org/10.1016/j.ejps.2021.106026.

(17) Dong, H.; Guo, X.; Xian, Y.; Luo, H.; Wang, B.; Wu, Y. A salting out-acetonitrile homogeneous extraction coupled with gas chromatography–mass spectrometry method for the simultaneous determination of thirteen N-nitrosamines in skin care cosmetics. *Journal of Chromatography A* **2015**, *1422*, 82-88. DOI: https://doi.org/10.1016/j.chroma.2015.10.044.

(18) Zheng, J.; Kirkpatrick, C. L.; Lee, D.; Han, X.; Martinez, A. I.; Gallagher, K.; Evans, R. K.; Mudur, S. V.; Liang, X.; Drake, J.; et al. A Full Evaporation Static Headspace Gas Chromatography Method with Nitrogen Phosphorous Detection for Ultrasensitive Analysis of Semi-volatile Nitrosamines in Pharmaceutical Products. *Aaps j* **2022**, *24* (1), 23. DOI: 10.1208/s12248-021-00669-8.

(19) Grebel, J. E.; Mel Suffet, I. H. Nitrogen-phosphorus detection and nitrogen chemiluminescence detection of volatile nitrosamines in water matrices: optimization and performance comparison. *J Chromatogr A* **2007**, *1175* (1), 141-144. DOI: 10.1016/j.chroma.2007.09.073.

(20) Purnell, J. H. 256. The correlation of separating power and efficiency of gas-chromatographic columns. *Journal of the Chemical Society (Resumed)* **1960**, (0), 1268-1274, 10.1039/JR9600001268. DOI: 10.1039/JR9600001268.

(21) Shimadzu. *Got DMF? Chromatographic separation and identification of NDMA and DMF using LCMS-9030*; 2020.

https://www.ssi.shimadzu.com/sites/ssi.shimadzu.com/files/pim/pim_document_file/ssi/applications/application_note/23949/01%20-%202_LCMS-9030%20determination%20of%20DMF%20and%20NDMA%20ver%202.pdf.

(22) Nascimento, V.; Murgu, M.; Mullin, L., Waters Corporation application note 720007167, March 2021, Selective and Specific Quantitation of NDMA in Ranitidine with the Xevo G2-XS QTof. **2021**.

(23) Furey, A.; Moriarty, M.; Bane, V.; Kinsella, B.; Lehane, M. Ion suppression; A critical review on causes, evaluation, prevention and applications. *Talanta* **2013**, *115*, 104-122. DOI: https://doi.org/10.1016/j.talanta.2013.03.048.

(24) Zheng, J.; Radich, C. L.; Gong, X.; Liang, X.; Mowery, M. D. A practical HPLC-MS method for the analysis of nitrosamine drug substance related impurities using an inexpensive single quadrupole mass spectrometer. *Journal of Chromatography A* **2024**, *1736*, 465399. DOI: https://doi.org/10.1016/j.chroma.2024.465399.

(25) *ICH Harmonized Guideline, Validation of Analytical Procedures Q2(R2)*. ICH_Q2(R2)_Guideline_2023_1130.pdf (Accessed May 15, 2025).

Table 1. Summary of Method Validation Results

Validation Characteristic	**Targeted Acceptance Criteria**	**Validation Results**
Specificity	- No interference from blank injection - No interference from sample matrix	- No inteference with NDMA was found
Linearity	- R > 0.950	- R without NDMA-d6: 0.999 - R with NDMA-d6: 1.000
Accuracy Without NDMA-D6	- Mean %Recovery at each of three concentration leveld is 75-125%	- Spiked at 0.3, 3 and 10 ng/mL with triplicate preparations - % Recovery without NDMA-d6: 84-91% - % Recovery with NDMA-d6: 99-104%
Quantitation Limit	- S/N ratio ≥ 10	- S/N ratios: 17 – 22
Detection Limit	- S/N ratio ≥ 3	- S/N ratios: 3 – 5
Measurement Precision	- %RSD (n=6) ≤ 25%	%RSD without NDMA-d6: - 0.3 ng/mL: 5% - 1 ng/mL: 4% - 10 ng/mL: 3% %RSD with NDMA-d6: - 0.3 ng/mL: 9% - 1 ng/mL: 3% - 10 ng/mL: 2%
Repeatability	- %RSD of the %recovery from 9 spiked samples ≤ 25%	- %RSD without NDMA-d6: 6% - %RSD with NDMA-d6: 7%
Range	- Range is demonstrated from reporting Threshold to 330% of specification	- 0.3 ng/mL to 10 ng/mL, corresponding to 3 ng/g to 100 ng/g relative to metformin HCl, or 10% to 313% of specification limit of 32 ng/g

Table 1. Summary of Method Validation Results

Validation Characteristic	**Targeted Acceptance Criteria**	**Validation Results**
Specificity	• No interference from blank injection • No interference from sample matrix	• No inteference with NDMA was found
Linearity	• R > 0.950	• R without NDMA-d6: 0.999 • R with NDMA-d6: 1.000
Accuracy Without NDMA-D6	• Mean %Recovery at each of three concentration leveld is 75-125%	• Spiked at 0.3, 3 and 10 ng/mL with triplicate preparations • % Recovery without NDMA-d6: 84-91% • % Recovery with NDMA-d6: 99-104%
Quantitation Limit	• S/N ratio ≥ 10	• S/N ratios: 17 – 22
Detection Limit	• S/N ratio ≥ 3	• S/N ratios: 3 – 5
Measurement Precision	• %RSD (n=6) ≤ 25%	%RSD without NDMA-d6: • 0.3 ng/mL: 5% • 1 ng/mL: 4% • 10 ng/mL: 3% %RSD with NDMA-d6: • 0.3 ng/mL: 9% • 1 ng/mL: 3% • 10 ng/mL: 2%
Repeatability	• %RSD of the %recovery from 9 spiked samples ≤ 25%	• %RSD without NDMA-d6: 6% • %RSD with NDMA-d6: 7%
Range	• Range is demonstrated from reporting Threshold to 330% of specification	• 0.3 ng/mL to 10 ng/mL, corresponding to 3 ng/g to 100 ng/g relative to metformin HCl, or 10% to 313% of specification limit of 32 ng/g