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# Final Report to BioSpectra Inc. for Development of an Analytical Method for the Detection of Various Nitrosamines

## March 31, 2021

Issued by:

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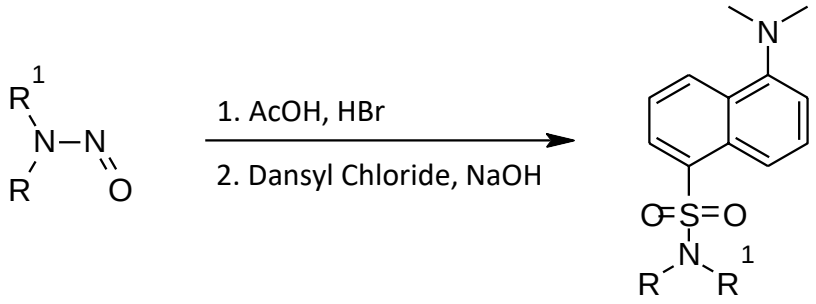
**Introduction.**

BioSpectra Inc. (BioSpectra) recently approached Kinentia Biosciences LLC (Kinentia) with regards to the development of a method to determine possible levels of Nitrosodimethylamine (NDMA), Nitrosodiethylamine (NDEA), Nitrososdiisopropylamine (N-DIPA), Nitrosoethylisopropylamine (NPEA) and Nitrosomethylphenylamine (NMPA) in Trisamine API. The method must be able to detect nitrosamine impurities in Trisamine at the low nanogram to picogram levels.

**Phase 1. Method Development.**

A survey of the literature suggests that a fluorescence-based pre-derivatization HPLC method may be utilized to detect nitrosamines at the picogram level. Wang *et. al*.[1] have reported a fluorescence HPLC method for the detection of NDMA and NDEA in tobacco with LOD values in the low picogram range. Residual nitrosamines were detected via de-nitrosation using hydrobromic acid in acetic acid then fluorescent tagging using Dansyl chloride (Figure 1).

**Figure 1**




R, $R^1$ = Me, NDMA
R, $R^1$ = Et, NDEA
R, $R^1$ = iPr, NDIPA
R = Et, $R^1$ = iPr, NPEA
R = Me, $R^1$ = Ph, NMPA

R, $R^1$ = Me, **1**
R, $R^1$ = Et, **2**
R, $R^1$ = iPr, **3**
R = Et, $R^1$ = iPr, **4**
R = Me, $R^1$ = Ph, **5**

Development of an Extraction Process for Nitrosamines from Trisamine

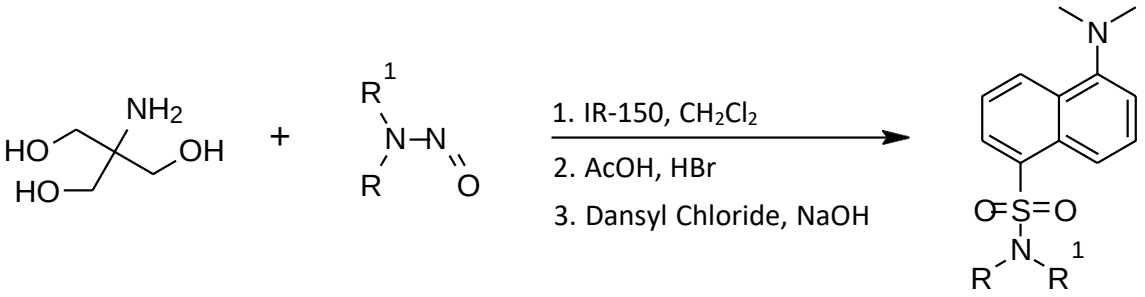
Initial efforts focused on developing an extractive process for nitrosamines from trisamine as this API will react with Dansyl Chloride. It was initially proposed that acidic aqueous solutions of trisamine could be extracted with methylene chloride and the extract further processed to afford Dansylated amines **1**-**5** as shown above. This extractive method has good precedent having been used to analyze levels of nitrosamines in beer samples and cigarette smoke.[2] Due to its ready availability, NDEA was used to optimize the extraction and derivatization process. Unfortunately, all initial attempts to replicate this method on spiked samples of trisamine did not afford any detectable levels of Dansylamine **2**. Several alternate approaches to isolating nitrosamines from trisamine using a range of solvents and acidic additives was to no avail. After significant experimentation it was discovered that pretreatment of trisamine with IR-150 ion exchange resin and copious washing of the resulting solids with methylene chloride did allow for the separation of nitrosamine spikes from trisamine. The resulting nitrosamine solutions could then be tagged as described above. This modified process for the extraction of nitrosamines from trisamine is shown in Figure 2 below.

Issues with NDIPA

Despite significant efforts to affect the formation of Dansylated amine **3** this material was not observed under any conditions. It was noted that the formation of authentic **3** from diiospropylamine and Dansyl chloride was significantly slower and poorer yielding than the other nitrosamines studied. It is believed that the considerable steric hindrance around the amino group prevents significant levels of Dansylation and given the already low levels of material in the trisamine sample the levels of **3** (if any) are below the limit of detection for the analytical method. Fortunately, as NDIPA or any reaction conditions / solvents that could generate NDIPA are not used in the manufacture of trisamine the formation of NDIPA in the manufacture of trisamine is highly unlikely.

**Figure 2**

**Modified Process for the Extraction of Nitrosamines from Trisamine**




R, $R^1$ = Me, NDMA

R, $R^1$ = Et, NDEA

R, $R^1$ = iPr, NDIPA

R = Et, $R^1$ = iPr, NIPEA

R = Me, $R^1$ = Ph, NMPA

R, $R^1$ = Me, **1**

R, $R^1$ = Et, **2**

R, $R^1$ = iPr, **3**

R = Et, $R^1$ = iPr, **4**

R = Me, $R^1$ = Ph, **5**

HPLC Method Development

In tandem with the extraction / tagging studies above the development of an efficient HPLC detection method for the nitrosamines of interest was developed. After authentic samples of the Dansylated amines **1-5** were generated from Dansyl chloride and the respective amine efforts were made to develop a HPLC method that would allow for the individual quantitation of these compounds. After some experimentation an efficient method for the analysis of Dansylated amines **1-5** was realized with a representative chromatograph being shown in Figure 3 below. As can be seen from Figure 3, the Dansylamine derived from NPEA and NPMA elute very closely to each other and often overlap. However, as the NMT Value / ADD for each nitrosamine is the same, as long as the observed nitrosamine level is below the given value then the material would pass specification with regards to nitrosamine content. Complete details of the analytical method can be found in the experimental section of this report.

Limits of Quantitation (LOQ)

Recently released guidelines from the FDA recommend an LOQ of <0.02ppm (20ng / mL). Given this, efforts were made to determine whether the fluorescence detection method described above could meet these criteria. In all cases the LOQ for each of the nitrosamine derived Dansylamines **1**,**2**,**4** and **5** were well below the target value. This data is shown in Table 1 below

**Table 1**
**LOQ Values of 1,2,4 and 5**

| Compound | LOQ |
|---|---|
| 1 | <0.02ppm |
| 2 | <0.02ppm |
| 3 | <0.02ppm |
| 4,5 | <0.02ppm |

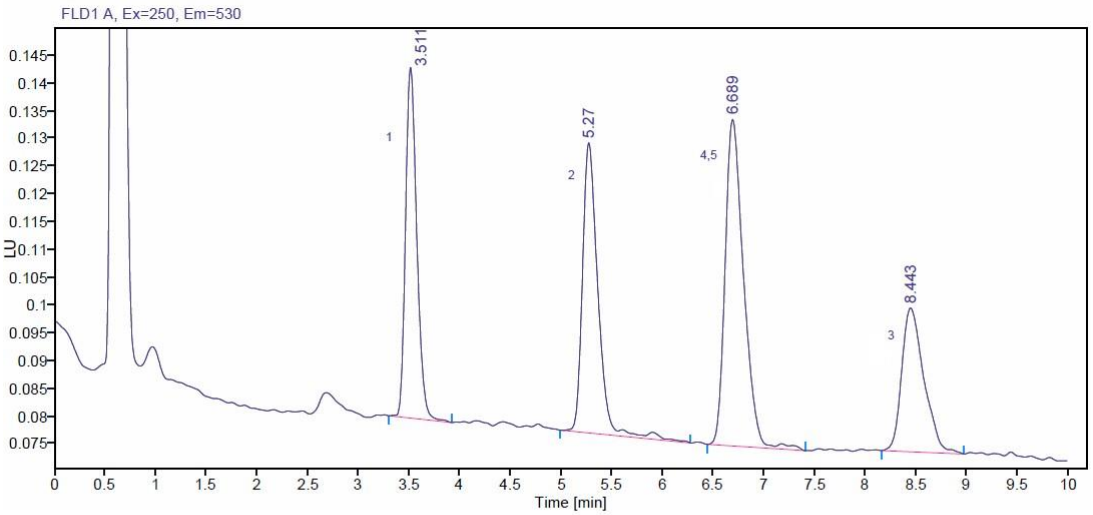
A chromatogram showing the detection of **1**,**2**,**3**,**4** and **5** at the 20ng / mL (0.02ppm) level is shown in Figure 3. As can be seen the signal to noise ratio is in the range of 160-65 so in effect the LOQ is at least another order of magnitude below the target value and in the 0.0012 -0.002 ppm range.

Targeted Levels of Nitrosamines in Trisamine

The FDA recommended No More Than (NMT) limits of nitrosamines in APIs are given in Table 2 below. These values are for anticipated daily dosage (ADD) of the API. For development work an ADD of 1g of trisamine was used. Based on this data, calibration curves were generated from trisamine / nitrosamine spiking studies in the range of 1-10000ng / mL were used.

**Figure 3**

**Detection of 1,2,3,4 and 5 at 0.02ppm level**




Signal Description FLD1 A, Ex=250, Em=530

| RT | Width | Tailing | S/N (USP) | Area | Area% | Amount | Name |
|---|---|---|---|---|---|---|---|
| 3.51 | 0.120 | 1.19 | 159.9 | 4.869618e-001 | 22.286 | 0.000 | |
| 5.27 | 0.159 | 1.25 | 132.3 | 5.563216e-001 | 25.460 | 0.000 | |
| 6.69 | 0.191 | 1.30 | 148.8 | 7.472413e-001 | 34.198 | 0.000 | |
| 8.44 | 0.229 | 1.27 | 65.5 | 3.945190e-001 | 18.055 | 0.000 | |

**Table 2**

**FDA Recommended NMT Limits of Nitrosamine Impurities / ADD**

| Compound | NMT Value / ADD |
|---|---|
| NMDA | 96 ng |
| NDEA | 26.5ng |
| NDIPA | 26.5ng |
| NPEA | 26.5ng |
| NMPA | 26.5ng |

**Part 2 Methods Validation.**

Efforts were then made to validate as much as possible the methods for each of the nitrosamines that could be analyzed. The following criteria were examined during this validation study:

- Selectivity/Specificity
- Precision / Accuracy
- Linearity / Range

Limits of Quantitation (LOQ) for 1,2,4and 5

The LOQ values for the fluorescence detection method developed are reported in Table 1 above. Per FDA guidelines, LOQ values of less than 0.02PPM were studied and the method readily gave baseline resolved peaks that could be readily quantified. Signal to noise rations for the observed peaks for **1**,**2**,**4** and **5** were in the range of 65-160 at the 0.02ppm level. Thus, the developed method readily achieves LOQ below the FDA mandated levels and are in fact 1-2orderers of magnitude lower.

Validation of the NDEA Method

*Selectivity / Specificity*

A sample chromatogram obtained from a 100ng spike of NDEA in 1g trisamine is shown in Figure 4 below. As can be seen from Figure 4, baseline resolution of the Dansylated amine derived from NDEA was observed. No overlap with any other peaks derived from starting materials or impurities from the extraction / Dansylation process were observed.

*Precision /Accuracy*

A calibration curve for the concentration range of 1-1000ng / mL levels of NDEA in trisamine was generated and are shown in Figure 5. Figure 6 shows the same calibration curve over the 1-100ng / ml range. Each data point was generated in triplicate and the standard deviation between each set of 3 datapoints was 0.008-0.049. This demonstrates an acceptable degree of linearity reproducibility and accuracy over the test range.

**Figure 4**

**HPLC Demonstrating Detection of 100ng Spike of NDEA in 1g of Trisamine**

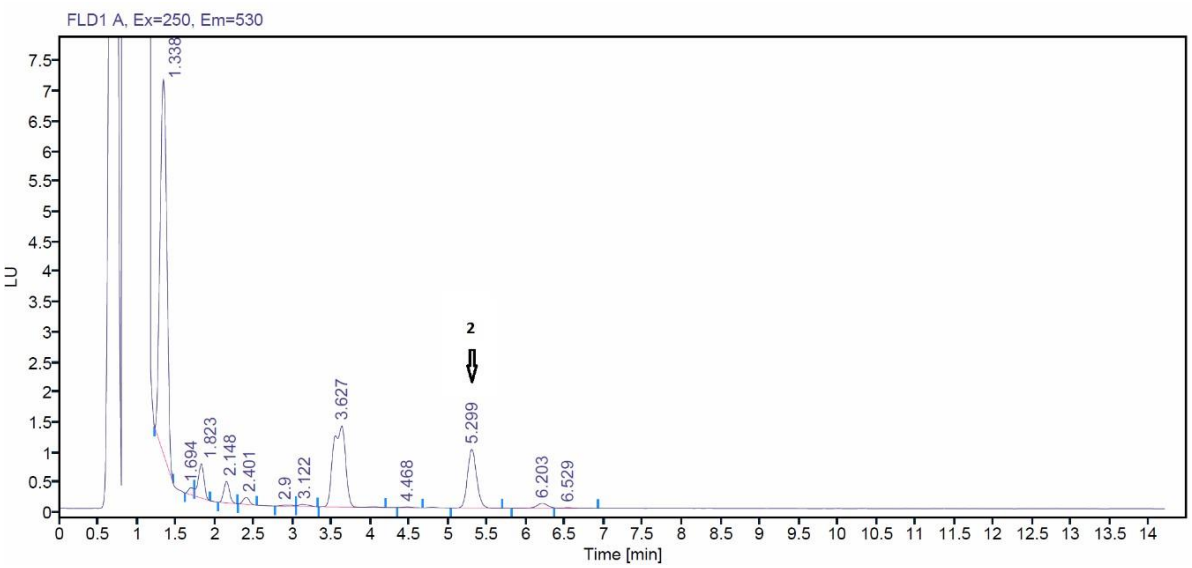



**Figure 5**

**NDEA / Trisamine Calibration Curve (1-1000ng / mL)**

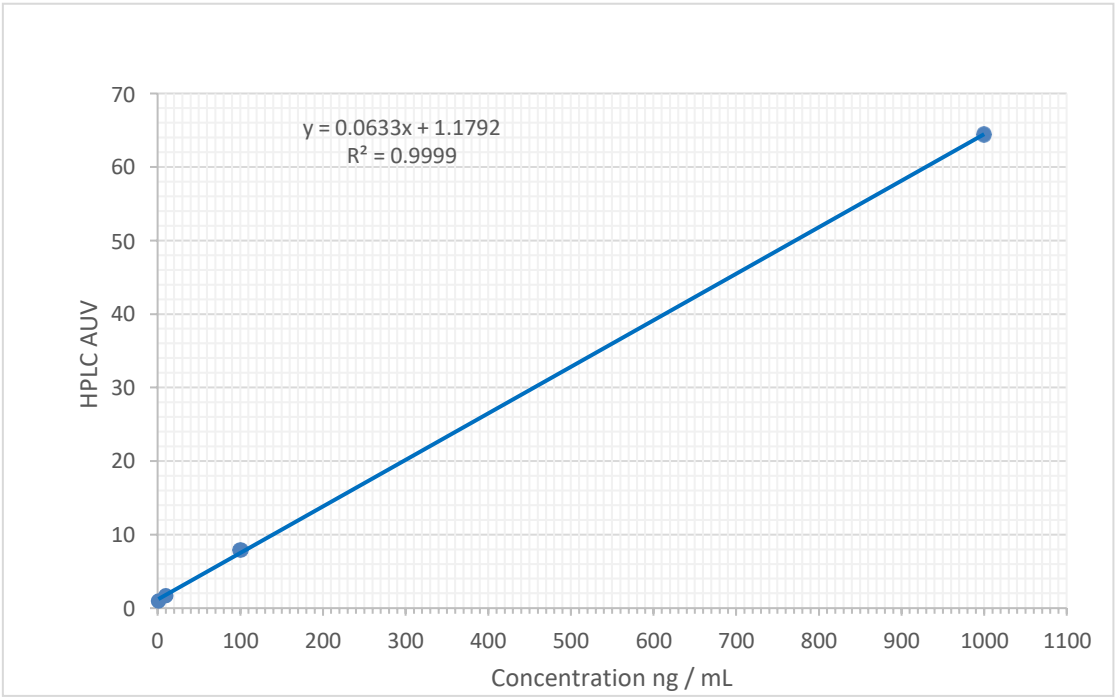
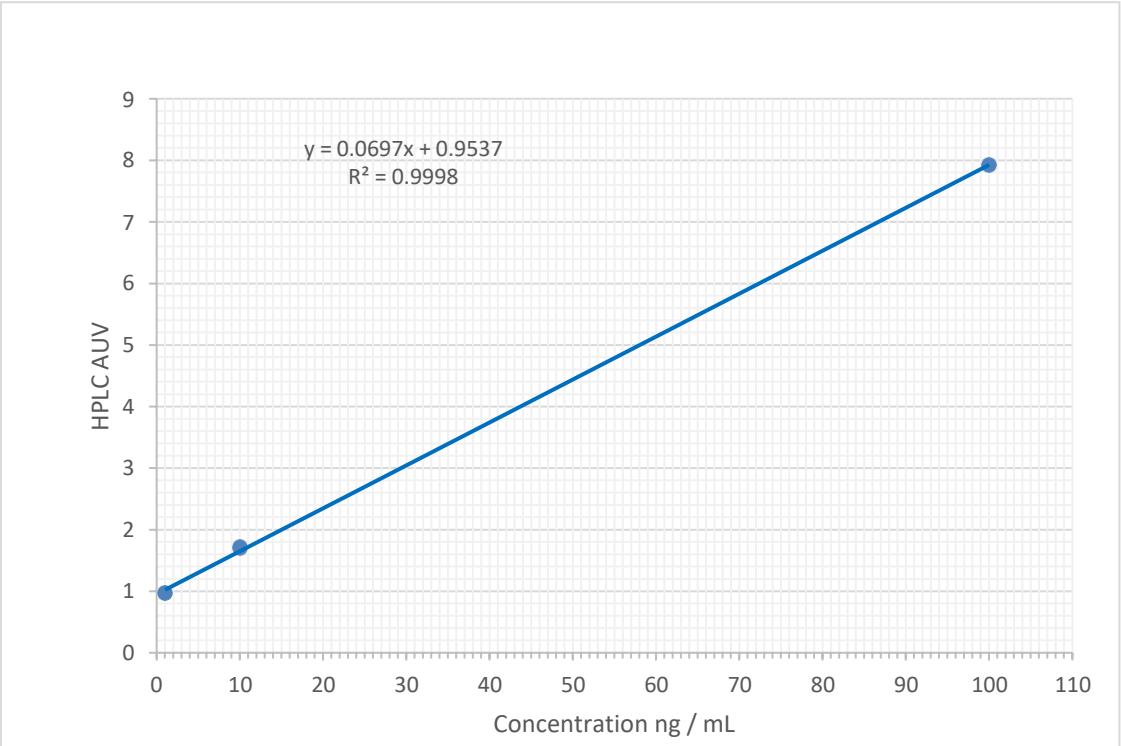


**Figure 6**
**NDEA / Trisamine Calibration Curve (1-100ng / mL)**




*Linearity / Range*

As can be seen from Figures 5 and 6 a high degree of linearity was observed in the data over the ranges examined ($R^2$ = 0.999). In addition, the daily FDA target NMT value of 26.5 ng is both well within the detection range studied the linear range of the calibration plot

Validation of the NPEA Method

*Selectivity / Specificity*

A sample chromatogram obtained from a 100ng spike of NPEA in 1g trisamine is shown in Figure 7 below. Baseline resolution of the Dansylated amine derived from NPEA was observed. No overlap with any other peaks derived from starting materials or impurities from the extraction / Dansylation process were observed.

*Precision /Accuracy*

A calibration curve for the concentration range of 10-10000ng / mL levels of NPEA in trisamine was generated and are shown in Figure 8. Figure 9 shows the same calibration curve over the 10-1000ng / ml range. Each data point was generated in triplicate and the standard deviation between each set of 4 datapoints was 0.03-0.4. This demonstrates an acceptable degree of reproducibility and accuracy over the test range.

**Figure 7**
**HPLC Demonstrating Detection of 100ng Spike of NPEA in 1.0g of Trisamine**

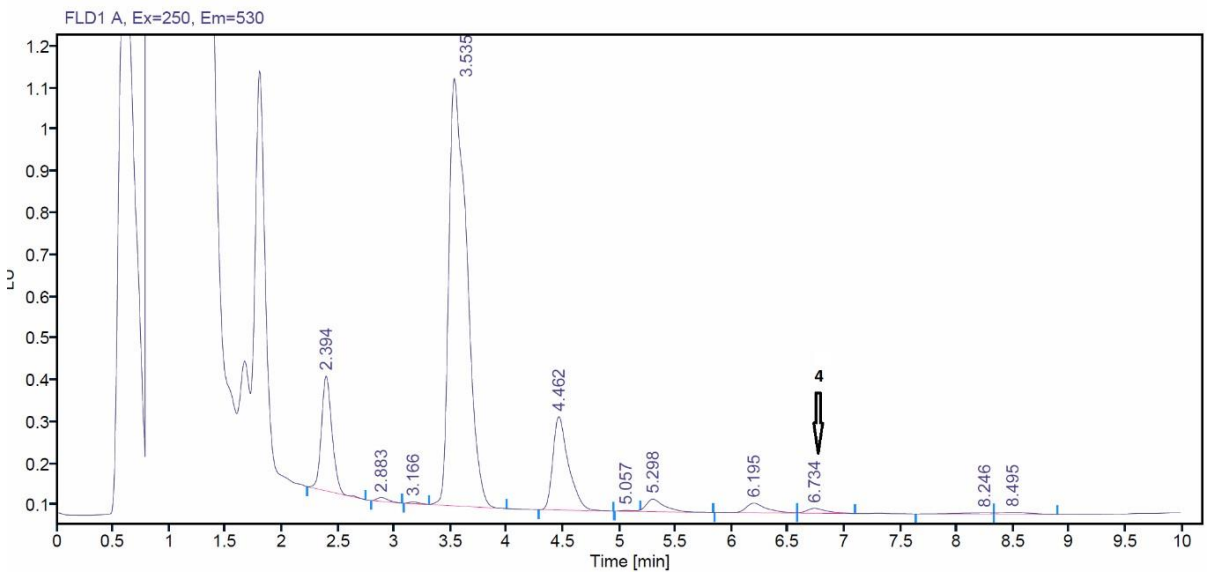



**Figure 8**
**NPEA / Trisamine Calibration Curve (10-10000ng / mL)**

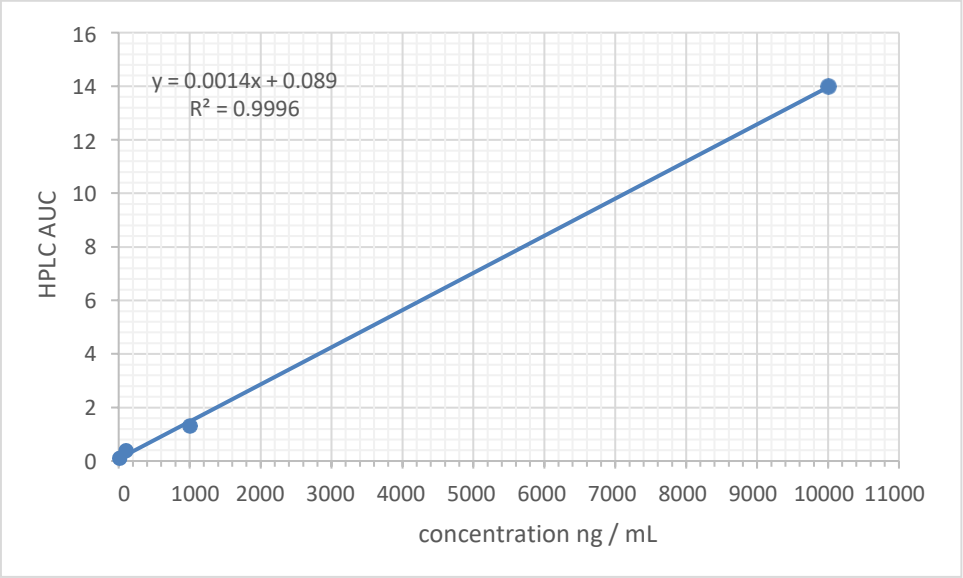
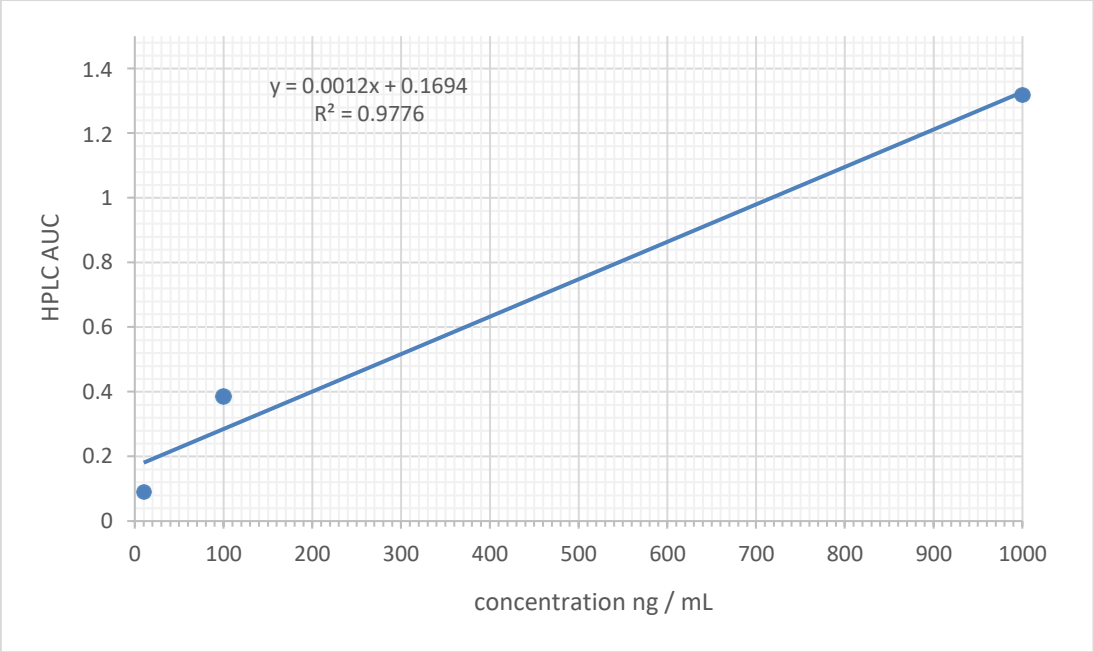


**Figure 9**
**NPEA / Trisamine Calibration Curve (1-1000ng / mL)**




Validation of the NMPA Method

*Selectivity / Specificity*

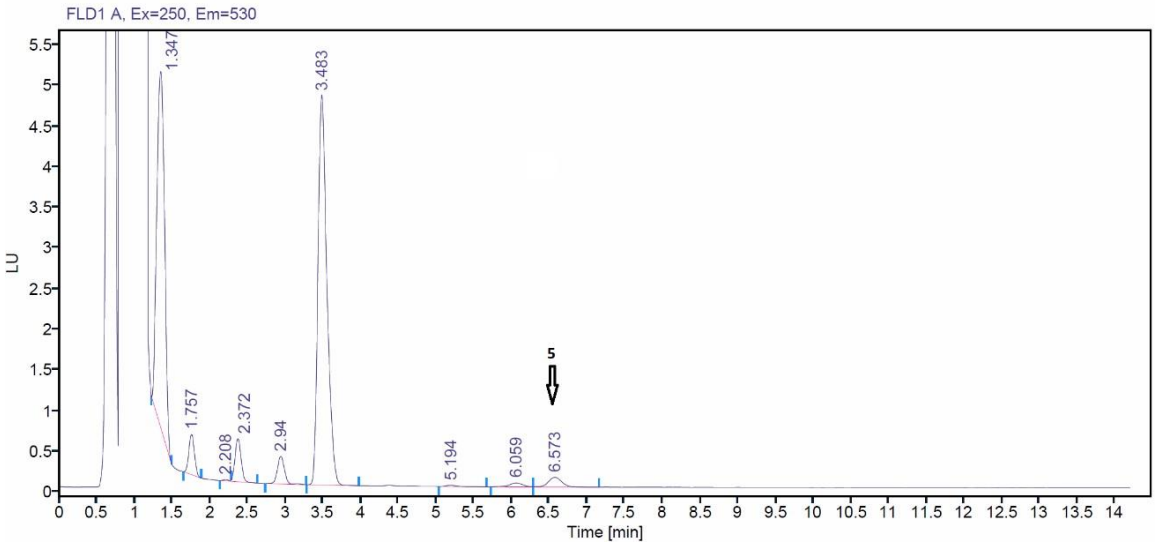
A sample chromatogram obtained from a 100ng spike of NMPA in 1g trisamine is shown in Figure 10 below. From Figure 10 baseline resolution of the Dansylated amine derived from NMPA can be observed. In addition, no overlap with any other peaks derived from starting materials or impurities from the extraction / Dansylation process were observed.

*Precision /Accuracy*

A calibration curve for various levels of NMPA in trisamine was generated and are shown in Figure 11. Each data point was generated in triplicate and the standard deviation between each set of 3 datapoints was 0.009 - 0.029. Figure 12 demonstrates the same data over the 1-100ng / mL range. In both concentration ranges demonstrates an acceptable degree of reproducibility and accuracy in the method.

**Figure 10**

**HPLC Demonstrating Detection of 100ng Spike of NMPA in 1g of Trisamine**




*Linearity / Range*

As can be seen from Figures 11 and 12 a high degree of linearity was observed in the data over the ranges examined ($R^2$ = 0.999). In addition, the daily FDA target NMT value of 26.5 ng is both well within the detection range studied the linear range of the calibration plot

**Figure 11**

**NMPA / Trisamine Calibration Curve (1-1000ng / mL)**

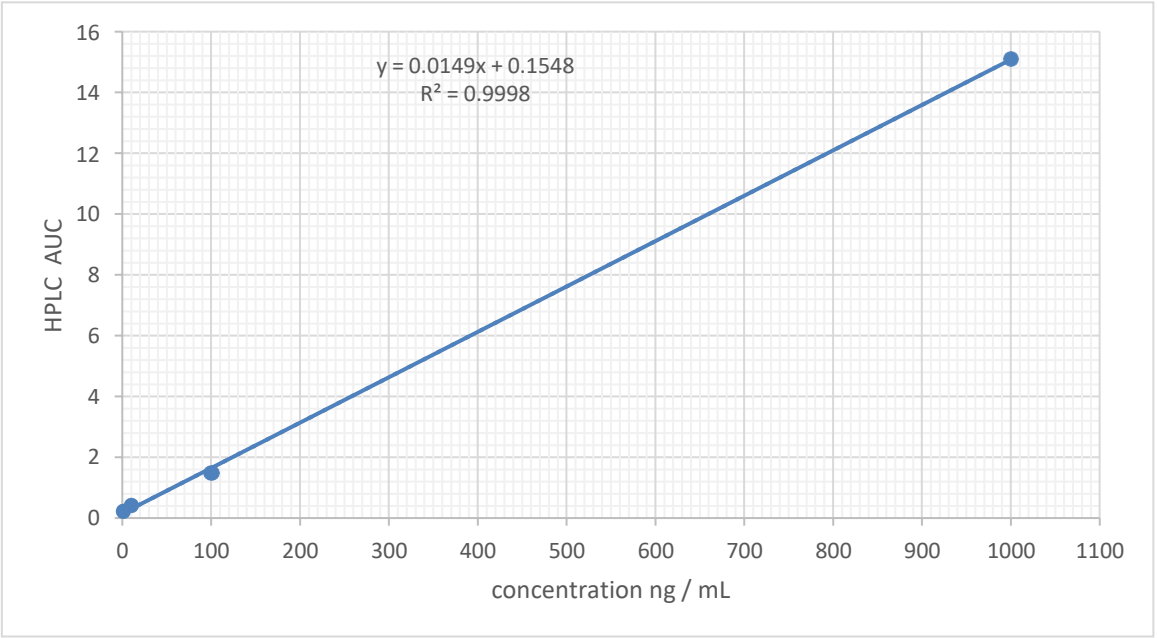
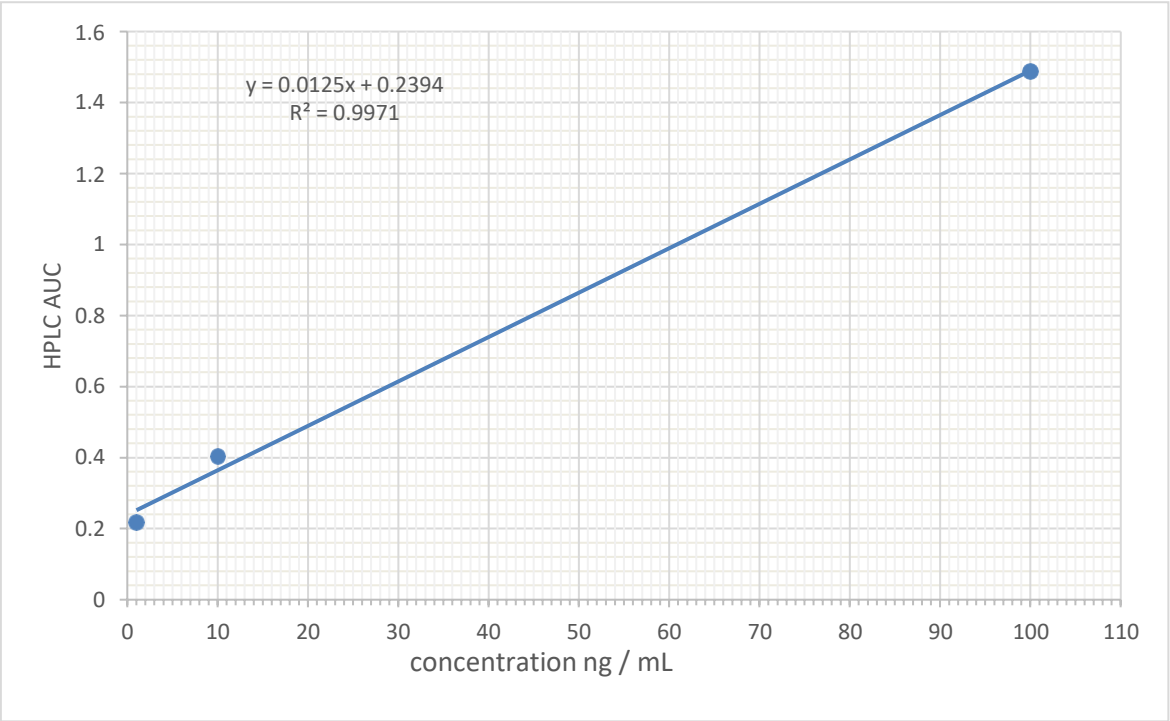


**Figure 12**
**NMPA / Trisamine Calibration Curve (1-100ng / mL)**




*Attempted Validation of the NDMA Method*

During the course of attempted validation of the NDMA method significant variation in the data was observed. After some investigative work into the reasons for this observation it was found that an impurity derived from Dansyl chloride was overlapping with the peak assigned to **1.** Figure 13 over page shows the chromatogram obtained from a sample of trisamine spiked with 100ng / g of NDMA and the corresponding chromatogram obtained without the spike. Unfortunately, the levels of impurity observed varied from run to run and did not allow for effective quantitation of NMDA levels.

Despite extensive efforts to optimize the process or HPLC method this impurity was observed and it always coeluted with **1**. The levels of Dansyl derived impurity observed from the standard tagging protocol were consistently in the range of 100 -500ng / mL which is above FDA daily no more than (NMT) value of 96ng / ADD. However, by adjusting the initial amount of Trisamine utilized to 10g but keeping the Dansyl chloride amount the same[1], the contribution of this impurity to the NMDA shifts below the NMT limit.

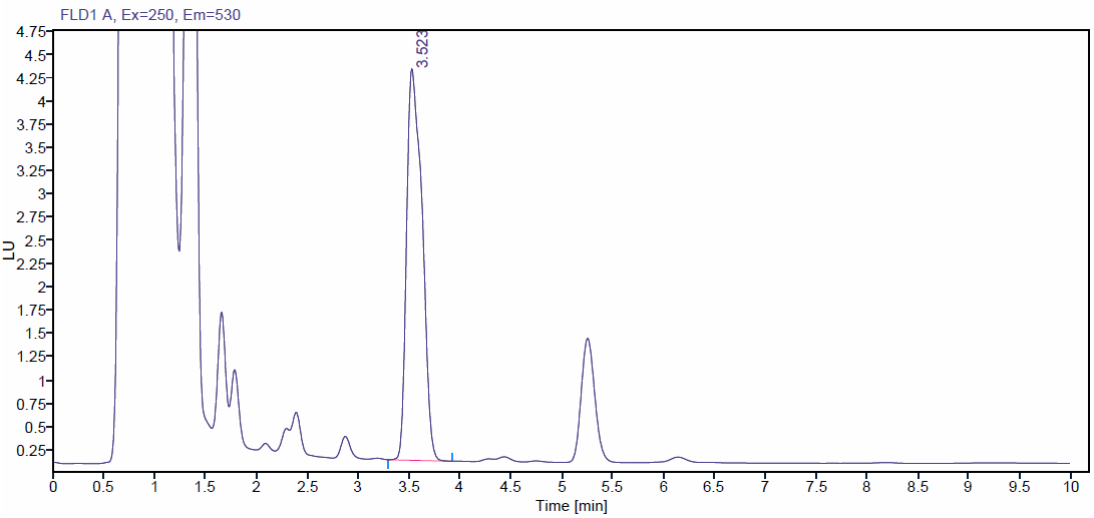
[1] Dansyl chloride was used in significant excess in all experiments typically 1000+ fold excess. Thus, sufficient Dansyl chloride would still be present to generate any Dansylated dimethylamine derived from NMDA impurities.

Thus, by increasing the sample size of API, the contribution of the Dansyl Chloride impurity to the NMDA value is below the NMT value. This conservative approach actually overestimates the levels of NMDA in Trisamine and if the NMT value is met then the levels of NMDA (if any) in Trisamine are extremely low and well below the NMT value.

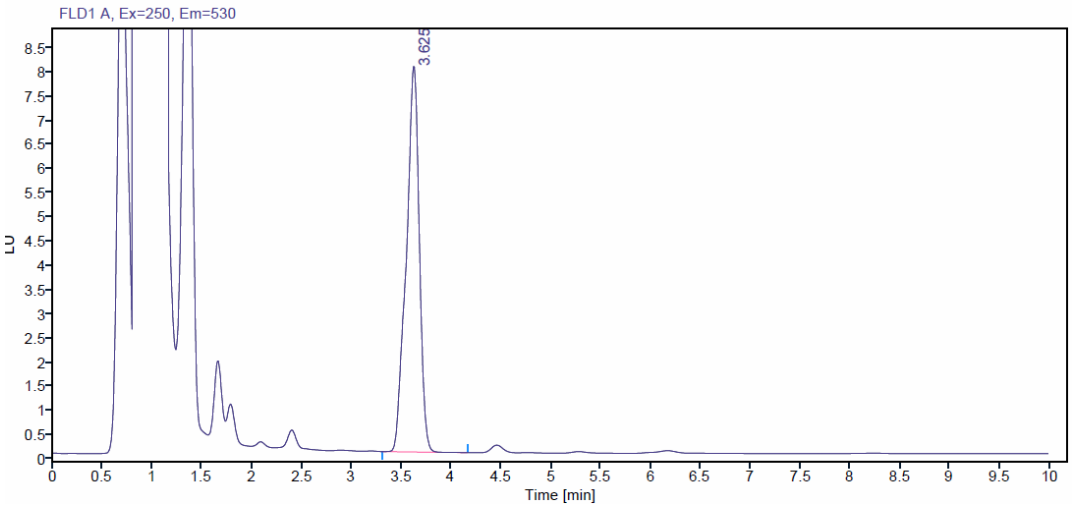
**Figure 13**

**HPLC Chromatograms Obtained With and Without 100ng Spike of NDMA**

With Spike




Without Spike



**Conclusions.**

In conclusion a method for the detection of several nitrosamines in trisamine API has been developed. The method involves Dansyl Chloride mediated fluorescent tagging of the de-nitrosated nitrosamine and detection / quantitation by HPLC utilizing fluorescence detection. The method is very sensitive and can quantify levels of NDEA, NPEA and NMPA at the low PPB level. A method for the detection of NMDA has also been developed, however due to interference of Dansyl Chloride derived impurities full quantitation can not be conducted. However, the presence of NMDA and a comparison to levels recommended in FDA guidelines can be made.

**Experimental.**

Standard Procedure for the Extraction and Tagging of NDEA, NPEA and NMPA

A 20 mL scintillation vial equipped with a Teflon coated magnetic stir bar was charged with trisamine-HCl (1.0 g) and IR120 ion-exchange resin[2] (1.0 g, $H^+$ form), and DCM (4 mL). The mixture was stirred for 60 min at RT. After this point the solids were removed by filtration through a small glass frit into a fresh scintillation vial. A solution of HBr in acetic acid (60μL) was added and the reaction capped and stirred at 45 °C for 1 hr. After this time the solvent was removed by rotary evaporation. The residue was treated with an aqueous solution of $NaHCO_3$ (20.0 mL of 0.2M solution), followed by a solution of NaOH (3 mL of 1 N solution) and Dansyl chloride (2.0 mL of 0.2 wt% in acetone). The reaction was capped and stirred at 50 °C for 1 hr. At this point, the reaction was cooled to rt and sampled for HPLC analysis.

Procedure for the Extraction and Tagging of NMDA

A 20 mL scintillation vial equipped with a Teflon coated magnetic stir bar was charged with trisamine-HCl (10.0 g) and IR120 ion-exchange resin[2] (10.0 g, $H^+$ form), and DCM (40 mL). The mixture was stirred for 60 min at RT. After this point the solids were removed by filtration through a small glass frit into a fresh scintillation vial. A solution of HBr in acetic acid (600μL) was added and the reaction capped and stirred at 45 °C for 1 hr. After this time the solvent was removed by rotary evaporation. The residue was treated with an aqueous solution of $NaHCO_3$ (20.0 mL of 0.2M solution), followed by a solution of NaOH (3 mL of 1 N solution) and Dansyl chloride (2.0 mL of 0.2 wt% in acetone). The reaction was capped and stirred at 50 °C for 1 hr. At this point, the reaction was cooled to rt and sampled for HPLC analysis.

HPLC Method with Fluorescence Detection

HPLC analyses were obtained on an Agilent 1100 HPLC system consisting of a G1322A degasser, a G1311A quaternary pump, a G1313A automatic liquid sampler, and a G1321B fluorescence detector controlled by an Agilent ChemStation chromatography data system. An injection volume of 10μL was utilized. The column was an XSelect 3.5μ CSH C18 150 × 4.6mm (Waters). Fluorescence excitation was conducted at 250 nm and emission monitored at 530 nm. An isocratic solvent program was used with a flow rate of 2 mL/minute and a mobile phase of 40:60 water : acetonitrile. No other solvent additives were used.