

## THE CHALLENGE OF NITROSAMINES IN DRUG PRODUCTS AND API: HOW AUTOMATION CAN HELP (INITIAL RESULTS)

Camilla Liscio, Colin Hastie and Dan Carrier, Anatune Ltd., Girton, Cambridgeshire (UK).

### INTRODUCTION

In 2018, the group of potential carcinogens known as nitrosamines were found in several blood pressure medicines known as 'sartans'. This event triggered a wide-scale product recall and regulatory reviews which set stringent portfolio risk assessment requirements for the pharmaceutical industry. The testing mandated by the regulators brought up detection of nitrosamines contamination in additional medications other than the "sartans", increasing public concerns and inevitably escalating the pressure on stakeholders. The regulatory organisations worldwide are operating a zero-risk environment which poses an extremely challenging target for all the laboratories involved in the analysis of nitrosamines in active pharmaceutical ingredients and drug products.

In fact, whilst analysis of nitrosamines in solvent standards can achieve the very low limits of detection established by the regulations, it is the investigation of nitrosamines levels in the real samples which poses the hardest challenge to the analytical community. Sample preparation is a crucial part of any analytical workflow and its optimisation and control are essential in driving high quality data. Furthermore, automated sample preparation can significantly reduce contamination and the occurrence of false positives.

False positives are a key area that pharmaceutical companies have been constantly addressing and the need to automate the sample preparation has been a major focus for this sector. If a batch of active pharmaceutical ingredient showed a false positive result for nitrosamines contamination, it will take a significant amount of resources to disprove the result. This application note describes how false positives could be obtained if samples have been manually prepared.

Automation of sample preparation can help significantly in addressing some of the many challenges to be faced in the analysis of nitrosamines thanks to its inherent qualities: convenience, control, and consistency.

#### Convenience:

Automation delegates tasks to a robot. This is not only valuable from a health, safety, and time perspective, but it can be extremely beneficial in controlling and containing sources of contamination.

Automating the sample preparation workflow opens up the very useful ability of multitasking. A function known as "PrepAhead" allows the preparation and injection of every sample promptly, thereby staggering the sample preparation and analysis time in the most efficient way possible. Every sample is injected as soon as preparation is complete. A visualization of the operation mode of the PrepAhead function in comparison to the standard approach is shown in Figure 1. The PrepAhead option is particularly useful when sample stability is a concern.

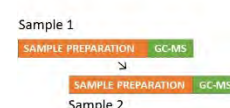
#### Control:

Automating the sample preparation allows the accurate control of the experimental variables involved such as timing, temperatures, and speeds (e.g. mixing, centrifuging and liquid handling). This control will have a strong effect on the extraction efficiency of the tested workflow.

#### STANDARD APPROACH



#### PrepAhead



**Figure 1:** Automation PrepAhead function in comparison to a standard approach

#### Consistency:

Automation can reduce biases since it will always perform a task in a consistent way. Method consistency will increase robustness and accuracy while minimising analytical variability - the main figures against which data quality is scored.

This application note will outline the approach taken by Anatune in tackling the nitrosamine challenge by harnessing the power of automation.

### INSTRUMENTATION

LC-MS/MS: Agilent 1260 Infinity Binary pump coupled to Agilent 6470 QqQ equipped with JetStream ESI source and APCI source.

Automation: GERSTEL Dual Head MPS Robotic configured with:

- Anatune CF200 Solvent Safe Robotic centrifuge
- GERSTEL QuickMix
- GERSTEL Filtration Unit with VT40-2mm filters tray
- Ultrasonication bath
- 180 mL Solvent Reservoirs
- VT12-10mL trays
- VT40-2mL trays
- Fast wash
- LC injection valve



**Figure 2:** Automated sample prep configuration for the analysis of nitrosamines in API and drug products

A video showcasing the modules used for the automation of this analytical workflow can be found [here](#):

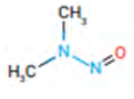
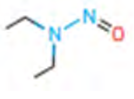
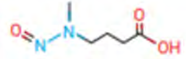
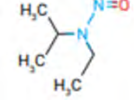


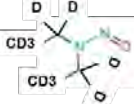
## MATERIALS AND METHODS

### Optimised LC-ESI- MS/MS Method

Chromatographic separation was carried out on an Agilent Poroshell HPH -C18 2.1x100mm 1.9um (PN 695675-702). Mobile phases were A: 0.2% Formic Acid in LC-MS grade water and B: LC-MS grade MeOH, respectively. Injection volume was 50 µL. The flow rate was 0.2 mL/min, column temperature was 40 °C and run gradient was as follows: 0 min 1%B, 1 min 1%B, 3.5 min 13% B, 7.0 min 80% B, 10 min 95% B, 12 min 95% B. ESI source conditions were optimised using design of experiment (Definitive Screening Design, 7 factors): Gas T 180 °C, Gas flow 4L/min, Nebuliser 10psi, Sheath Gas T 200°C, Sheath Gas Flow 12 L/min, Capillary 500V Nozzle Voltage 500V.

Table 1 and Table 2 summarise details for the ISTDs and target nitrosamines addressed in this study and Figure 3 shows the chromatography for the five target analytes.

**Table 1:** ISTDs and target nitrosamines name, abbreviation, and chemical structure

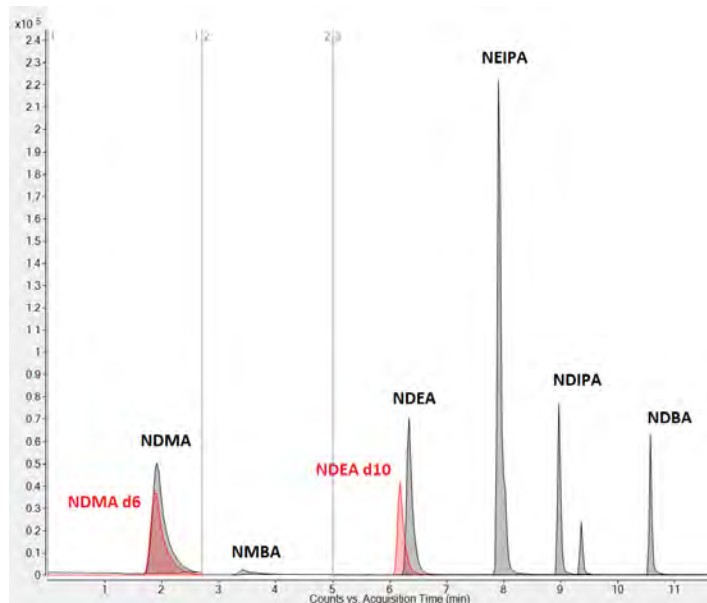
Name	Abbrev	Chemical Structure
N-nitrosodimethylamine	NDMA	
N-nitrosodiethylamine	NDEA	
N-nitroso-4-methyl-4-aminobutyric acid	NMBA	
N-nitrosoethylisopropylamine	NEIPA	
N-nitrosodiisopropylamine	NDIPA	
N-nitrosodimethylamine d6 (IS)	NDMA-d6	
N-nitrosodiethylamine d10 (IS)	NDEA-d10	

**Table 2:** Retention time and MRM transitions for the investigated nitrosamines

Analyte	Retention Time [min]	MRM Transitions
NDMA	2.15	Quant: 75.1>43.0 Qual: 75.1>58.0
NDEA	6.66	Quant: 103.1>75.1 Qual: 103.1>47.1
NMBA	4.55	Quant: 147.1>44.2 Qual: 147.1>87.2
NEIPA	7.74	Quant: 117.1>75.1 Qual: 117.1>47.1
NDIPA	8.36	Quant: 131.1>89.1 Qual: 131.1>43.1
NDMA-d6	2.12	Quant: 81.1>64.1 Qual: 81.1>46.1
NDEA-d10	6.53	Quant: 113.0>49.0 Qual: 113.0>81.0

### Sample Preparation

100 mg of API or a whole drug product tablet were manually weighed and transferred into a 10 mL screw cap glass vial. 4.5 mL of LC-MS grade water was added together with 250 µL spike of 1 µg/mL methanolic solutions of ISTD and target nitrosamines mix to achieve a final concentration in solution of 50 ng/mL. The sample was mixed for 5 min at 2000rpm, sonicated for 5 min and centrifuged at 4500rpm for 5 min to separate supernatant from residual excipients. An aliquot of 350 µL of the supernatant was filtered on a 0.45 µm PVDF 4 mm filter.



**Figure 3:** MRM Extracted Ion Chromatogram for the five target nitrosamines by LC-ESI-MS/MS

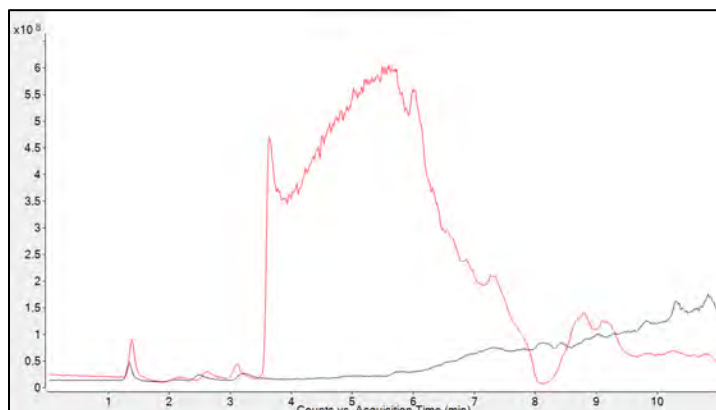
## RESULTS AND DISCUSSION

### Linearity and Signal to Noise

Linearity was assessed for all target analytes in both solvent and drug product extract in a concentration range 0-100 ng/mL. Linear regression coefficients R<sup>2</sup> ranged between 0.9991 and 0.9998. S/N above 10 were obtained for concentrations above 10 ng/mL.

## Matrix effect

Analysis of the target analytes in active pharmaceutical ingredient and drug product revealed a significant ion suppression due to the high concentration of API and excipients. The ion suppression mostly affected detection of NMBA which could only be detected in solvent standards. Some preliminary experiments were carried out using the APCI source to assess performances in relation to the ion suppression, but no improvement was observed. However, APCI source settings had not been fully optimised so additional work will be carried out in future work to address this. Figure 4 shows the Total Ion Chromatogram Scan for a Solvent Blank and the drug product extract in solvent.

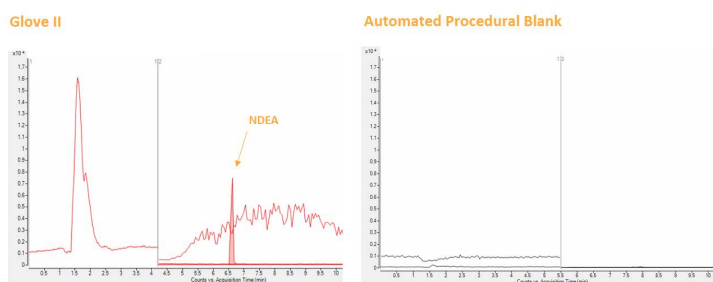


**Figure 4:** Total Ion Chromatogram Scan for Solvent Blank (black trace) and drug product extract (red trace)

It can be observed that most of the matrix signal elutes in a time range between 3.5 and 6 min which overlaps with the retention time of NMBA (4.554 min).

## Contamination

A preliminary assessment of the possible contamination sources involved with the manual preparation of samples for the analysis of nitrosamines was carried out. Two types of gloves and Pasteur pipette bulb were evaluated for nitrosamines contamination. The equivalent of 1 finger of glove/1 bulb was shredded and placed in a 10 mL vial containing 5 mL of MQ water. Sample was left overnight at ambient temperature before LC-MS/MS analysis. Traces of NDEA (approximately 3 ng/mL) were detected in one of the types of gloves. Figure 5 compares the MRM EIC traces for the glove extract and the automated procedural blank, respectively. The automated procedural blank did not reveal presence of any of the investigated nitrosamines.

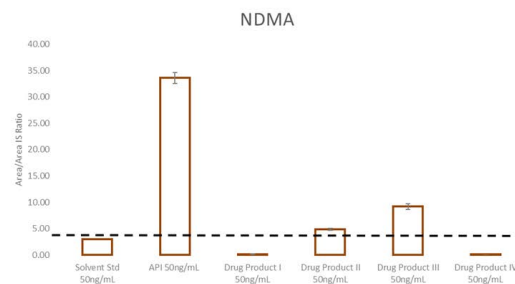


**Figure 5:** MRM Extracted Ion Chromatogram for glove extract (left hand-side, red trace) and automated procedural blank (right hand-side, black trace).

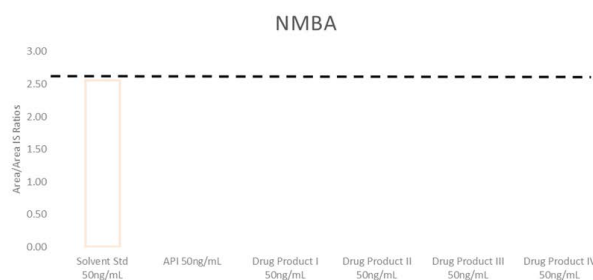
## Extraction efficiency

Procedural blank, solvent standard 50 ng/mL, API and four different drug products, all spiked at 50 ng/mL, were prepared in duplicate as described in the Sample Preparation section in the Methods. Figure 6, 7, 8, 9 and 10 compare the Area/IS Area ratios for the five target nitrosamines across the range of analysed samples. The dashed black line shows the reference ratio recorded for the solvent

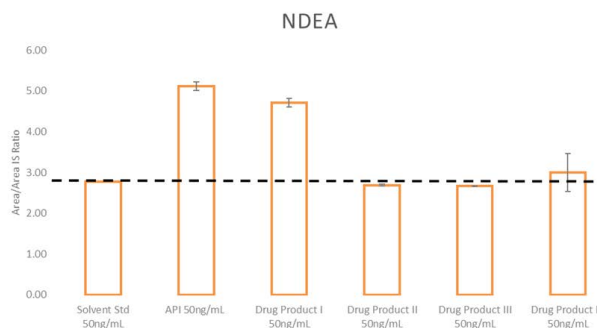
standard to help assessing recovery and presence of ion suppression, matrix enhancement or potential nitrosamines contamination.



**Figure 6:** NDMA area/IS area ratios for the solvent standard, API and four investigated drug products



**Figure 7:** NMBA area/IS area ratios for the solvent standard, API and four investigated drug products



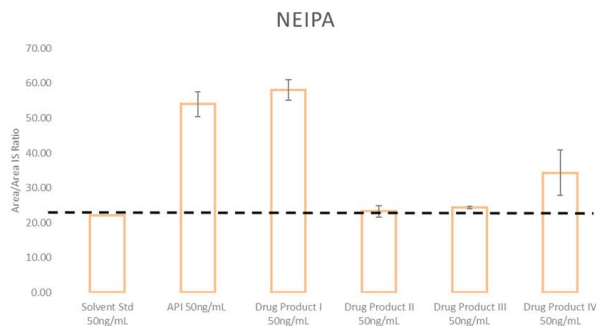
**Figure 8:** NDEA area/IS area ratios for the solvent standard, API and four investigated drug products

As summarised by the bar graphs, the investigated API highlighted high levels of nitrosamines when compared to the tested to the solvent standard for 50 ng/ml spike. This trend has been confirmed by analysis done by collaborators on the same material. NMBA detection was heavily affected by ion suppression in API and drug product. Otherwise, good recoveries were observed across the range of different analytes and tested matrices.

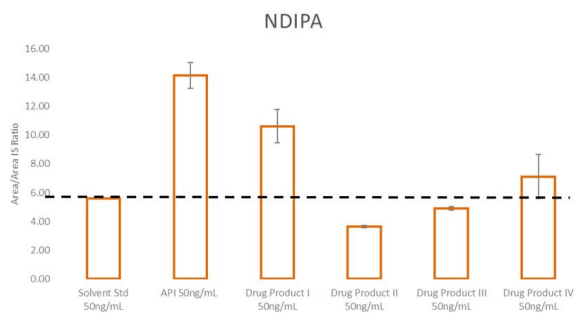
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**Figure 9:** NEIPA area/IS area ratios for the solvent standard, API and four investigated drug products



**Figure 10:** NDIPA area/IS area ratios for the solvent standard, API and four investigated drug products

Table 3 lists the procedural RSD% obtained across the range of analysed matrices (solvent, API and drug products) which were, below 10 % with the exception of Drug Product IV which showed the highest variability (15-21 %).

**Table 3:** Procedural Relative Standard Deviations (RSD%) for the five target nitrosamines

Sample Type	NDMA	NMBA	NDEA	NEIPA	NDIPA
API	3	NA	2	7	6
Drug Product I	NA	NA	2	5	11
Drug Product II	5	NA	1	7	2
Drug Product III	6	NA	0.4	2	3
Drug Product IV	18	NA	15	19	21

## CONCLUSIONS

This application note outlined the preliminary approach taken here at Anatune to address the challenging detection of nitrosamines in API and drug products. An LC-ESI-MS/MS method was developed and optimised for the analysis of the 5 main target nitrosamines. An exhaustive sample preparation workflow was developed and applied to a type of active pharmaceutical ingredient and four different drug products containing the same API. Good RSD% were obtained for the sample preparation across the whole range of matrices. Ion suppression issues were observed for all analytes in drug products, but more specifically for NMBA. Future work will be focused on the optimisation of an alternative LC-APCI-MS/MS method and investigation of potential clean-up methods to reduce the matrix load such as liquid-liquid extraction (LLE) and solid phase extraction (SPE).