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Quantitation of N-nitrosamines in dietary supplement by using LC-MS/MS

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Introduction

Proteins are essential for building and maintaining muscle, bone strength, and numerous body functions. It has been observed in considerable population that due to consumption of inappropriate diet, daily protein needs are not met. Proteins as powdered formulations (Figure 1) are used by many to fulfill their daily need of protein intake. These formulations generally originate from plants such as soybeans, peas, rice, potatoes, hemp or some time come from animal origin like eggs, or milk (casein or whey protein). The powders may include other ingredients such as added sugars, artificial flavoring, thickeners, vitamins, and minerals. Food regulators ensures that the manufacturers evaluate and control the safety and labeling of products. Recent research in this field shows presence of numerous toxins in protein powders and related formulations. Hence, there is a dire need for such formulation to be not only tested for their efficacy but also to be tested for presence of harmful contaminants.

Figure 1. Protein Powder samples

One such type of contaminants are N-nitrosamines (Figure 2). N-nitrosamines, or more correctly N-nitrosamines, refer to any molecule containing the nitroso functional group. According to World health organization (WHO) formation of N-nitrosamines is only possible when secondary or tertiary amines react with nitrous acid. Nitrous acid itself is unstable but can be formed in situ from nitrites (NO_2) under acid conditions. N-Nitrosodimethylamine is the most commonly found N-nitrosamines in water, food and pharmaceuticals. NDMA belong to the so-called "cohort of concern", which is a group of highly potent mutagenic carcinogens that have been classified by the WHO's International Agency for Research on Cancer as probably human carcinogens. Despite the potency of these impurities, there is still a very low risk that N-nitrosamine impurities at the levels found could cause cancer in humans. Higher daily dose of protein powder and highly potent nature of impurities demands for testing. This study demonstrates a quick, simple accurate and high throuput analytical method for the low level quantitation of 4 N-nitrosmaines namely N-nitrosodimethylamine (NDMA), N-nitrosoethylisopropylamine (NEIPA), N-nitrosodipropylamine (NDPA) and N-nitrosodiisopropylamine (NDIPA) in protein powder formulation sample using LCMS-8060NX triple quadrupole system. (Figure 2)

Materials and method

Sample preparation

- **Preparation of calibration curve standards and quality control (QC) samples**

The N-nitrosamine standards were procured from local vendor and standard solutions were prepared. From these standard solutions, 1000 ppb standard stock solution was prepared in Methanol for LC-MS analysis using the instrumental parameters described in table 1. Extracted protein powder matrix blank was used to prepare 50.0 ppb working stock which was further diluted to make matrix match linearity from 0.1 ppb to 5.0 ppb.

- **Sample extraction procedure**

Commercially available protein powder sample was procured from local vendor. This powdered protein sample was weighed about 0.4 g in 15 mL centrifuge tube. The sample was then spiked with N-nitrosamine standard solution to have spiking concentration of 10 ppb. 5 mL LC-MS grade water was added to the sample tubes to make a fine slurry. Further, 4 mL of extraction solvent was added to the same tube and was vortexed for 2 min. Added salts to maximize the extraction efficiency & the sample tube was again vortexed for 2 min. The sample tubes were centrifuged at 7000 rpm at 4 °C for 10min. The supernatant was collected and filtered through 0.45 µ nylon syringe filter in auto-sampler vial and injected onto LCMS-8060 NX.

Figure 2. Shimadzu LCMS-8060NX triple quadrupole with Nexera X3 liquid chromatography

LC-MS/MS analysis

Table 1. LC-MS/MS Instrument parameters

UHPLC condition (Nexera X3)

Column	: Shim-pack Scepter C8-120, 5 µm, 3.0 x 150 mm (P/N: 227-31039-04)
Mobile phase	: A: 0.1% formic acid in LC-MS grade water B: 0.1% formic acid in LC-MS grade methanol
Flow rate	: 0.5 mL/min
Column temperature	: 40 °C
Elution mode	: Gradient B concentration in % : 0-1.5 min 10%, → 1.5-9 min 95%, → 9-10 min 95%, 10-10.2 min 10% → 10.2-13 min 10%.

Mass Spectrometry parameters

MS interface	: Atmospheric pressure chemical ionization (APCI)
Desolvation line temperature	: 180 °C
Heating block temperature	: 200 °C
Interface temperature	: 300 °C
Nebulizing gas flow	: 4 L/min
Drying gas flow	: 5 L/min

MRM and their CEs :

Comp.	Precursor	Product	CE
NDMA	75.30	58.3	-11
NEIPA	117.00	75.10	-13
NDPA	131.00	89.05	-12
NDIPA	131.00	42.90	-17

Results

Linearity

A matrix match standard linearity ranging from 0.1 ppb to 5.0 ppb was plotted. All calibration levels showed linear response with the accuracy ranging from 80 to 120%. (Figure 3)

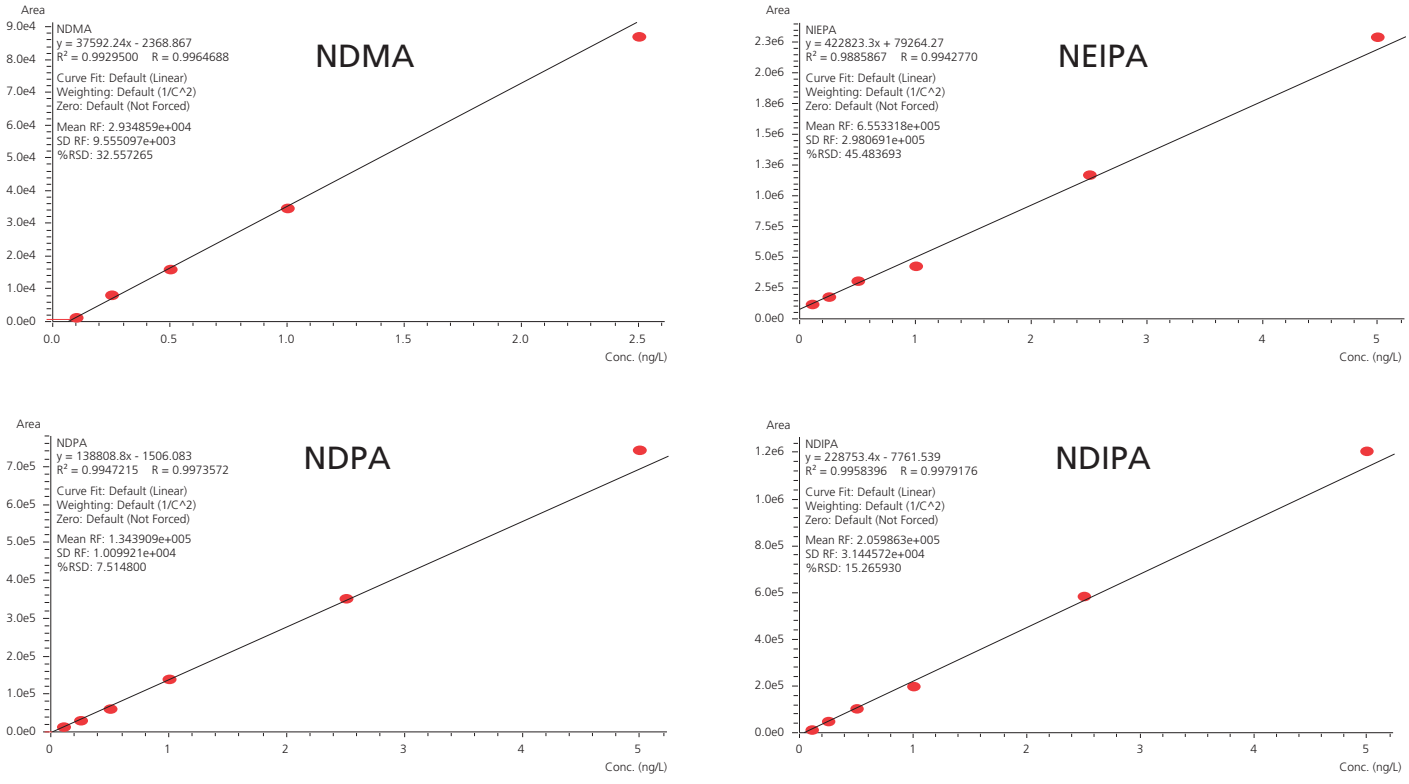

Figure 3. Calibration curve for NDMA , NEIPA, NDPA & NDIPA

Recovery

Recovery was evaluated by analyzing three pre-spiked samples at 10.0 ppb against matrix match calibration curve mentioned in section 3-2. Average recovery values of N-nitrosamines were found to be within acceptance criteria of 70-120 % as per SANTE guidelines (Figure 4 & 5).

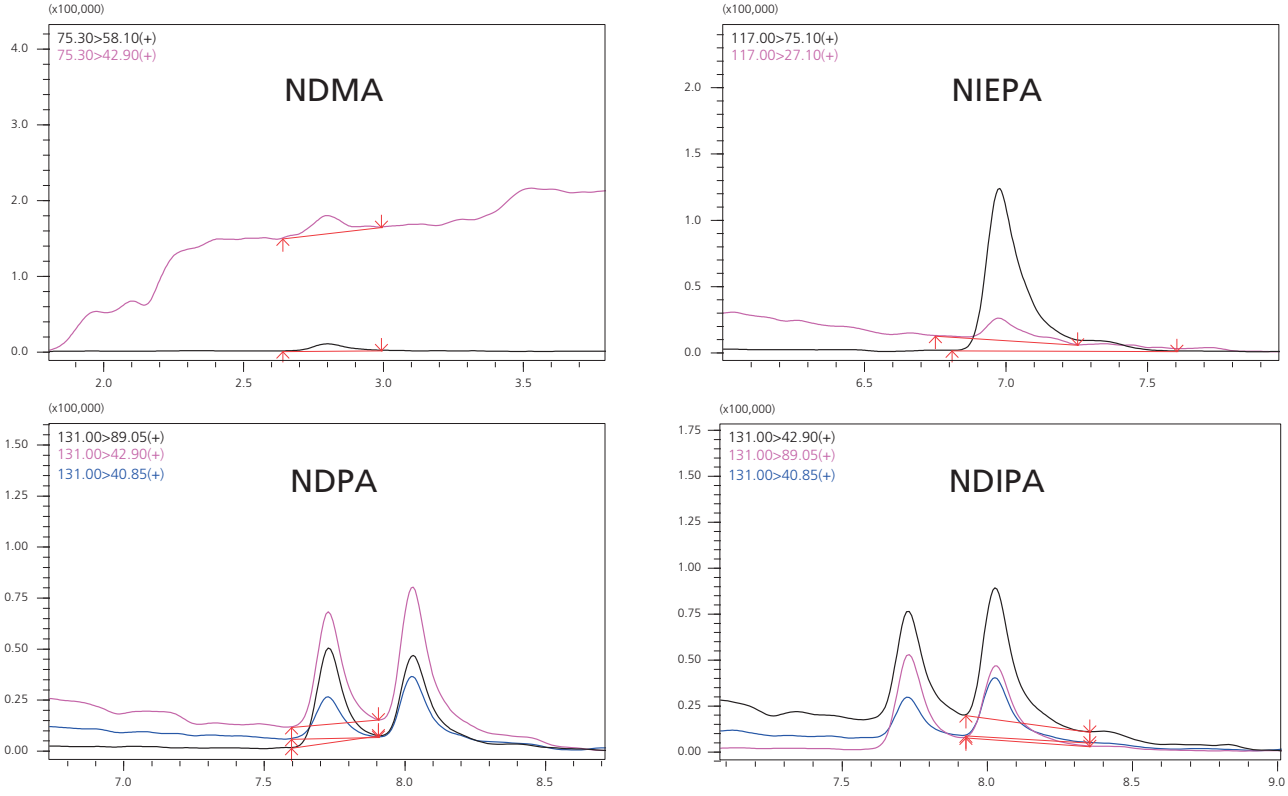

Figure 4. Chromatograms for NDMA, NEIPA, NDPA & NDIPA of pre-spiked protein powder sample

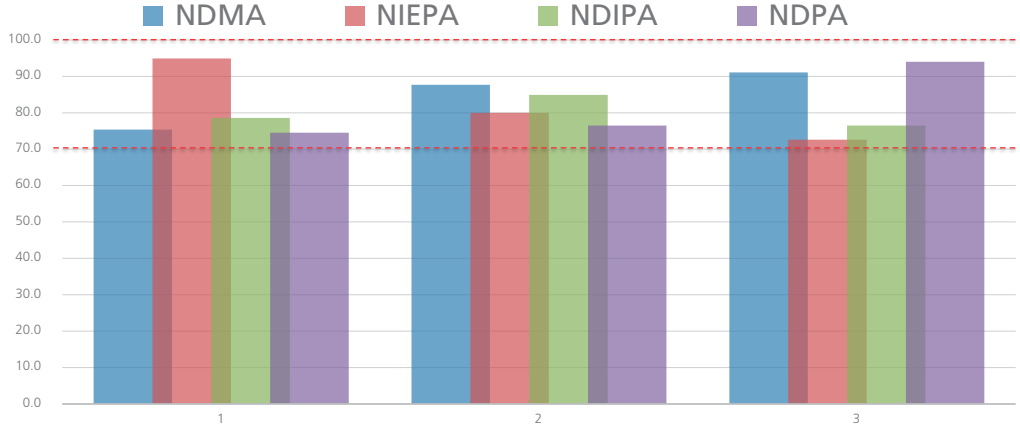

Figure 5. Graphical representation of N-nitrosamine recoveries in pre-spiked protein powder sample

Reproducibility (RSD)

Three recovery samples, at 10 ppb level were injected and checked for reproducibility. LC-MS/MS N-nitrosamines showed good precision (RSDr) with less than 20% RSD which is within acceptance criteria as per SANTE guidelines (figure 6)

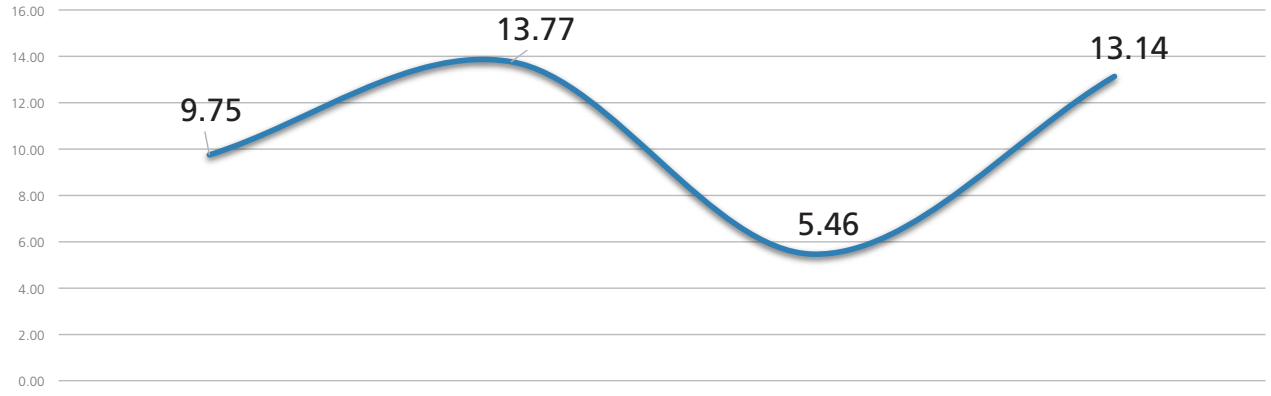

Figure 6. RSD of pre-spike samples analysed on LCMS-8060NX

Conclusion

- Average recovery values for N-nitrosamines in protein powder sample were found to be within the acceptance criteria as per the SANTE/12682/2019 guidelines.
- The results obtained at 10.0 ppb concentration level were accurate, repeatable and reproducible with RSD less than 20%.
- A simple liquid-liquid extraction method has been successfully developed and validated for the simultaneous quantification of 4 N-nitrosamines in a single run.

References

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3. https://www.health.harvard.edu/staying-healthy/the-hidden-dangers-of-protein-powders

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