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Development of a new analytical tool for assessing the mutagen 2-methyl-1, 4-dinitro-pyrrole in meat products by LC-ESI-MS/MS

Luciano Molognoni[a,b,c], Heitor Daguer[a], Leandro Antunes de Sá Ploêncio[a,d], Suzana Eri Yotsuyanagi[e], Ana Lucia da Silva Correa Lemos[e], Antonio Carlos Joussef[d], Juliano De Dea Lindner[c,*]

[a] Ministério da Agricultura, Pecuária e Abastecimento, Laboratório Nacional Agropecuário, Seção Laboratorial Avançada de São José (SLAV/SC/LANAGRO/RS), São José, SC 88102-600, Brazil
[b] Instituto Catarinense de Sanidade Agropecuária (ICASA), Florianópolis, SC 88034-000, Brazil
[c] Universidade Federal de Santa Catarina (UFSC), Centro de Ciências Agrárias, Departamento de Ciência e Tecnologia de Alimentos, Florianópolis, SC 88034-000, Brazil
[d] UFSC, Centro de Ciências Físicas e Matemáticas, Departamento de Química, Florianópolis, SC 88040-900, Brazil
[e] Secretaria da Agricultura e do Abastecimento do Estado de São Paulo, Instituto de Tecnologia de Alimentos, Centro de Tecnologia de Carnes, Campinas, SP 13073-001, Brazil

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ABSTRACT

The use of sorbate and nitrite in meat processing may lead to the formation of 2-methyl-1,4-dinitro-pyrrole (DNMP), a mutagenic compound. This work was aimed at developing and validating an analytical method for the quantitation of DNMP by liquid chromatography-tandem mass spectrometry. Full validation was performed in accordance to Commission Decision 2002/657/EC and method applicability was checked in several samples of meat products. A simple procedure, with low temperature partitioning solid-liquid extraction, was developed. The nitrosation during the extraction was monitored by the *N*-nitroso-DL-pipecolic acid content. Chromatographic separation was achieved in 8 min with di-isopropyl-3-aminopropyl silane bound to hydroxylated silica as stationary phase. Samples of bacon and cooked sausage yielded the highest concentrations of DNMP (68 ± 3 and 50 ± 3 $\mu g\,kg^{-1}$, respectively). The developed method proved to be a reliable, selective, and sensitive tool for DNMP measurements in meat products.

1. Introduction

Cancer is one of the most feared diseases by the world population. In fact, after the cardiovascular diseases, cancer is the main cause of human death [1]. Since it involves genetic and environmental factors, cancer has been considered a multi factorial disease. Despite this, the risk of developing many types of cancers may be associated with diet [2]. According to the International Agency for Research on Cancer (IARC), some compounds may be classified as carcinogens for humans (group 1) and may be present in some foodstuffs, such as meat products. During meat processing, the curing and smoking, among other technologies, may result in the formation of carcinogenic compounds, such as the *N*-nitroso-compounds, polycyclic aromatic hydrocarbons, and heterocyclic aromatic amines [3]. Furthermore, the irresponsible use of preservatives and other additives, and the formation of substances due to changes in raw materials, are among the major chemical hazards in foodstuffs [4].

Some studies have evidenced the positive effects of the combined use of preservatives sorbate (SOR) and nitrite (NIT) on the yield of meat products [5,6]. The increase of their useful life, due to the growth delay of deteriorating microorganisms and the inhibition of pathogens, such as *Clostridium botulinum*, has been evidenced. Since then, the decrease of nitrite content in the formulation of meat products has been encouraged, to avoid the formation of nitrosamines [5–8]. However, studies by Kito, Namiki, and Tsuji [9] and Namiki et al. [10] demonstrated that the combined use of those additives may lead to the formation of mutagen compounds, such as the 2-methyl-1,4-dinitro-pyrrole (DNMP). Binstok et al. [11] evaluated the main conditions for the *in vitro* formation of DNMP (pH, temperature, proportion of each preservative, among other factors), under similar conditions to those found in meat matrices. Subsequently, features about the DNMP kinetics, its mutagenic activity and decomposition, among other relevant chemical aspects, were further investigated [12–14].

* Correspondence to: Rodovia Admar Gonzaga, 1346, Itacorubi, Florianópolis, SC 88034-001, Brazil.
E-mail address: juliano.lindner@ufsc.br (J. De Dea Lindner).

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Due to the need to synthesize the DNMP for the development of analytical methods, data from several research papers, aimed at the characterization of this compound, are available in the literature. Analytical techniques, such as thin-layer chromatography [9], nuclear magnetic resonance spectroscopy [9,11,14], molecular absorption UV–Vis spectrometry [12–14], infrared spectroscopy [11], mass spectrometry [11,12,14], and high performance liquid chromatography with a UV–Vis detector [15], have been used for the identification and quantitation of DNMP. However, the difficulty of measuring the DNMP at low concentrations (μg kg^{-1}) in complex matrices such as foodstuffs, as well as the current lack of commercial standards, justify the scarcity of analytical methods with enough sensitivity for its quantitation in meat products.

The evolution of food analytical methods shall follow the sophistication degree of possible adulterations and major hazards to consumers' health [4]. Recent studies with modern measurement tools have shown that the use of preservatives in food of animal origin is a widely disseminated industrial practice, even if forbidden for some categories of processed products [4,16–18]. Although the abuse of preservatives in meat products has been evident, compounds such as DNMP may be formed in meat products by the synergism between some chemical additives, such as nitrite and sorbate. Thus, the use of new control methods, combining the separation efficiency of the analytes by liquid chromatography to high sensitivity and unequivocal detection provided by modern analytical techniques such as tandem mass spectrometry (LC-MS/MS), becomes an indispensable tool for effective food control. In this context, this work was aimed at developing and validating a sensitive analytical method for the quantitation of DNMP in meat products by LC-MS/MS. Method applicability was checked in commercial samples of meat products, which results were non-compliant for both preservatives (NIT and SOR), was also performed.

2. Materials and methods

2.1. Standards, reagents, and instrumentation

All chemicals were in analytical grade. All water was purified by the Integral 10 Milli-Q system (Millipore SAS, Molsheim, France).

All commercial standards had 98% minimum purity. Sodium nitrite (NIT) (CAS no. 7632-000), sodium nitrate (NAT) (CAS no. 7631-99-4), potassium sorbate (SOR) (CAS no. 110-44-1), DL-pipecolic acid (PIC) (CAS no. 535-75-1), 1-methyl-imidazole (1-MEI) (CAS no. 616-47-7), and tetramethylsilane (TMS) (CAS no. 75-76–3) were supplied by Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

All solvents were in chromatographic grade. Methanol (MeOH), acetonitrile (ACN), ethyl acetate, hexane, deuterated chloroform ($CDCl_3$), and dichloromethane were supplied by Merck KGaA (Darmstadt, Germany). Formic acid, sulfuric acid, and hydrochloric acid were supplied by J.T. Baker Chemical Co. (Phillipsburg, USA).

Stock standard solutions were prepared at 1000 mg L^{-1} by dissolving their solutes in methanol with the aid of an ultrasound. The spiking solutions were prepared from stock solutions in acetonitrile at the following concentrations: PIC, 100 mg L^{-1}; 1-MEI and 2-methyl-1,4-dinitro-pyrrole (DNMP), 10 mg L^{-1}. All solutions were stored at −30 ± 10 °C.

The LC-MS/MS analyzes were carried out in the 5500 QTrap hybrid triple quadrupole-linear ion trap-mass spectrometer from Sciex (Framingham, USA), equipped with electrospray ionization and ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization) sources. The mass spectrometer was coupled to the 1290 Infinity high performance liquid chromatography binary pump from Agilent Technologies, Deutschland GmbH (Waldbronn, Germany). Capillary zone electrophoresis was performed in the 7100 system by Agilent Technologies, Deutschland GmbH (Waldbronn, Germany), equipped with a diode array detector.

2.2. Synthesis of 2-methyl-1,4-dinitro-pyrrole (DNMP) and N-nitroso-DL-pipecolic acid (NPIC)

The DNMP synthesis was conducted in accordance to Kito et al. [9] and Namiki et al. [10]. Two solutions were prepared by dissolving 16.5 g of NIT and 4.41 g of SOR in 64 mL and 1300 mL of water, respectively. The two solutions were mixed and heated at 60 °C for two hours. The pH was kept at 3.5 by direct drip of sulfuric acid (2 mol L^{-1}). Partitioning with dichloromethane was carried out in a separator funnel, by adding five volumes of 75 mL. The precipitated, yellow-colored fraction (organic phase), was washed with two portions (100 mL) of saturated sodium bicarbonate solution. It was then dried over anhydrous sodium sulfate and activated charcoal, filtered, and evaporated in a rotary evaporator.

NPIC was synthesized in accordance to Lijinsky, Keefer, and Loo [19], by dissolving 3 g of racemic PIC in 30 mL of hydrochloric acid in an ice bath. Subsequently, 2.2 g of $NaNO_2$ were slowly dissolved in the solution and the mixture was transferred to a separator funnel. The partition was carried out with five volumes of 75 mL of dichloromethane. The organic phase was dried over activated charcoal and anhydrous sodium sulfate, transferred to a round-bottomed flask and completely dried under argon flow.

The synthesized compounds were stored in tightly closed flasks with an inert atmosphere (argon) at −80 °C in an ultra-freezer (Panasonic Biomedical Co., Wood Dale, USA).

The characterization of the compounds was performed by UV–Vis molecular absorption spectrum, nuclear magnetic resonance spectroscopy (^{1}H NMR), and mass spectrometry. The results were compared to the literature [9,12,14,19,20].

The molecular absorption spectrum was obtained after reverse phase chromatographic separation with Venusil XPB C_{18} as stationary phase 150 mm × 4.6 mm i.d, 5 μm particle size (Bonna-Agela Technologies Inc., Wilmington, USA) and methanol 70% as mobile phase, in isocratic elution mode. Scanning was carried out in the range from 200 to 400 nm wavelengths on the Alliance 2695 HPLC-DAD system (Waters Corporation, Milford, USA).

The ^{1}H NMR spectrum was obtained on the Avance 200 spectrometer (Bruker GmbH, Rheinstetten, Germany), operating at 200 MHz. About 20 mg of DNMP were dissolved in $CDCl_3$. TMS was used as internal standard. Chemical shifts (δ) were reported in ppm, referenced to the solvent peak of $CDCl_3$ or TMS as reference. Multiplicity, coupling constant (J) in Hertz, and integrated intensity were also reported.

For the NPIC characterization (exact mass 158.07 g mol^{-1}, $C_6H_{10}N_2O_3$), the precursor 159 *m/z* and the fragments 128 *m/z* (loss of NO), 113 *m/z* (loss of COOH) and 83 *m/z* (loss of COOH and NO) were monitored. Fragmentation mass spectra of the DNMP molecule were obtained during method development. NPIC and DNMP were characterized by direct infusion of the compounds onto the 5500 QTrap hybrid triple quadrupole-linear ion trap-mass spectrometer. The compounds were dissolved in MeOH 50% solution with 0.1% formic acid and separately infused through a continuous flow of 7 μL min^{-1}.

2.3. Samples for method development/validation

2.3.1. Commercial samples

In total, 54 commercial samples of fermented, cured, dried, and cooked meat products, were assessed for method development/validation. All samples were processed by Brazilian factories under federal inspection. All samples were frozen, ground, and homogenized using an automatic disk mill, stored in plastic bags and kept at −80 ± 10 °C until analysis.

2.3.2. In-house processed samples

For the extraction method development and for the DNMP's extract stability test, samples of mortadella were processed in a pilot plant under the best conditions for the analyte formation, according to

available data by Hartman [21], Namiki et al. [10], and Binstok et al. [11]. With this purpose, proportions of 8:1 (0.3%:0.0375%) and 2:1 (0.3%:0.15%) of NIT and SOR, respectively, were used. Glucono-delta-lactone (1.5%) and food grade phosphoric acid (0.25%) were used to keep the meat emulsion pH around 5.0 ± 0.5. The mortadella composition consisted of mechanically deboned poultry (60%), pork (26%), texturized soy protein (3.5%), cassava starch (5%), sodium chloride (3.5%), sucrose (0.6%), sodium pyrophosphate (0.3%), and sodium erythorbate (0.1%).

2.4. DNMP method optimization

2.4.1. Extraction method

For the development of the low temperature partitioning solid-liquid extraction procedure (SLE-LTP), the in-house processed mortadella samples (item 2.3.2) were used. Briefly, 2.0 ± 0.1 g of sample were directly weighed into 50 mL polypropylene tubes. Then, 5 mL of cooled (4 ± 2 °C) extraction solution (ACN 90% acidified with 0.1% formic acid) and homogenization ceramics were added to each tube. Orbital agitation was carried out for 20 min. The tubes were then centrifuged at 3488 g-force for 10 min at 4 ± 1 °C. The supernatant was transferred to polypropylene tubes and frozen at − 30 ± 10 °C for one hour. Centrifugation was carried out once again and the whole content was dried at 25 ± 2 °C, under nitrogen flow. The extracts were re-ssuspended with ACN 70% acidified with 0.1% formic acid (1 mL) and ethyl acetate (0.2 mL). After orbital agitation for 5 min, the extracts were transferred to polypropylene microtubes and centrifugated at 17,300 g-force for 5 min at 4 °C. Finally, aliquots of 0.6 mL were placed on glass vials and injected onto the liquid chromatograph for LC-MS/MS analysis.

A Plackett-Burman-type saturated fractional factorial design [22] was used to visualize the main effects of the extraction variables: (1) sample mass (1 and 2 g), (2) extraction solvent (pure acetonitrile and 90% acetonitrile in water), (3) volume of extraction solution (5 and 10 mL), (4) extraction acid (0.1% acetic acid and 0.1% formic acid), (5) drying temperature of the extract (25 and 50 °C), (6) clean-up with apolar solvent (hexane and ethyl acetate), (7) amount of organic solvent in the extract reconstitution (10% and 30% ACN), (8) reconstitution of the extract (ultrasound and orbital shaker). The experiment without replicates was conducted according to the matrix designed according to the following equation:

$$Y = \beta_0 + \sum \beta_i X_i \quad (i = 1, \quad ..., \quad k) \tag{1}$$

Where Y: estimated target function; β_0: constant; β_i: regression coefficient; X_i: independent variable; k: number of variables.

The Lenth's method was used to estimate the main effects of the variables [23]. For this purpose, the contrasts margin of error (ME), the pseudo standard error (PSE), and the simultaneous contrasts margin of error (SME) were calculated with 95% confidence, using the Microsoft Excel software.

The nitrosation during the extraction was assessed, as recommended by Herrmann, Duedahl-Olesen, and Granby [20], by adding PIC to each tube at 500 μg L^{-1} before analysis. The formation of NPIC was thus monitored.

2.4.2. LC-MS/MS method

Mass spectrometry was optimized by the multiple reaction monitoring mode (MRM). The compounds were dissolved at concentrations between 10 and 200 μg L^{-1} in MeOH 50% solution with 0.1% formic acid and separately infused through a continuous flow of 10 μL min^{-1} in the mass spectrometer. Ionization forms were tested by using both ESI and APCI sources, working in positive and negative modes. The chromatographic optimization was performed by considering several stationary phases in reverse (C_{18}, C_8, and CN) and HILIC (Hypersil Gold®) modes. Mobile phase, buffers, injection volume, column temperature, and elution gradients were univariately tested. Best chromatographic separation was achieved by using the Zorbax 300 SB-CN column (150 mm x 4.6 mm i.d., 5 μm particle size, 300 Å), with diisopropyl-3-aminopropyl silane bound to hydroxylated silica as stationary phase (Agilent Technologies, Inc., Santa Clara, USA). Gradient elution and instrument parameters were set as follows: 95% A (0–3 min), 70% A (3–5 min), 10% A (5–6 min), 50% A (6–8 min), and 95% A (2 min for self-equilibrate); eluents: aqueous formic acid 0.1% (mobile phase A); formic acid (0.1%) in ACN (mobile phase B); column heater: 40 °C; flow: 500 μL min^{-1}; injection volume: 15 μL.

2.5. Quantitation of nitrate, nitrite, and sorbate

Samples of item 2.3 were also submitted to quantitation of NIT and NAT by capillary zone electrophoresis with diode array detection (CZE-DAD), as described by Della Betta et al. [24]. SOR was quantitated by the LC-MS/MS method proposed by Molognoni et al. [4], with the ESI source. All results were expressed in mg kg^{-1}. Both methods were previously in-house validated and the following limits of quantitation were achieved: 5.00 mg kg^{-1} (NIT), 5.00 mg kg^{-1} (NAT), and 1.00 mg kg^{-1} (SOR).

2.6. Method validation

Method validation was conducted as the protocol proposed by the Commission Decision 2002/657/EC [25], in terms of selectivity, analytical curves, precision, recovery, decision capability (CC_α), detection capability (CC_β), and stability.

The selectivity was verified by the analysis of 20 commercial blank samples, for the evaluation of the possible interferences from matrices, transitions, and contaminations between runs. The presence of interfering peaks around the retention times of the analytes was considered, by comparing the chromatograms of blank samples, before and after the spiking procedure.

Analytical curves were prepared with internal standardization (1-MEI) and six concentration levels (including zero) in triplicate points. A linear (unweighted) functional relation of the concentration ratio (x-axis) *versus* the peak-area ratio (y-axis) was used. The working range comprised 10.0 and 400 μg kg^{-1} of DNMP. Reproducibility was evaluated by preparing curves with three replicates per level, on three distinct days, using blank sample. The acceptance criterion was the mean of the regression coefficients (R^2), which should be greater than 0.98. In addition, analysis of variance (ANOVA single factor with 95% probability) was performed with the Microsoft Excel software in order to verify if there was a significant difference between the derivatives obtained on different days.

The matrix effect was assessed by the increase or suppression of the signal in the ionization sources. The procedure was based on the analysis of three types of analytical curves. Curve I was prepared in solvent by diluting the standard solutions in the initial mobile phase. Curve II was prepared in a fortified blank matrix before the extraction procedure. Finally, curve III was prepared in blank matrix extract fortified after the extraction procedure. The derivatives of the lines obtained in matrix and solvent were compared [26,27].

Recovery and precision (in terms of repeatability and in-house reproducibility) were determined by spiking blank samples and commercial samples. Three groups of six aliquots fortified in the first three levels of response were assessed, after the minimum required performance limit (MRPL) was established. Recovery and precision in terms of in-house reproducibility were evaluated by considering the day of analysis (n = 3) as the measurement variable. The results were obtained from matrix-matched analytical curves prepared with triplicate points, on the same day of the experiment for each matrix. The recovery rate should be among − 20% to + 10% to be considered acceptable.

The extract stability was evaluated for 15 days of storage under controlled temperature (− 30 ± 10 °C). This procedure was used to

simulate the treatment that the extracts could be submitted during analytical routine in a control laboratory. The extracts (n = 21) were analyzed under reproducibility conditions of preparation and in-house reproducibility.

For each period of 1, 3, 5, 7, 10, and 15 days, the extracts were analyzed. The variability of this procedure was evaluated in control charts of standard deviation and central tendency. The mean of the deviations (n = 21) was considered as the center line. The upper and lower limits were obtained by multiplying the mean standard deviation by the constants 2.57 and 0, respectively, as a function of the number of replicates to calculate each standard deviation of the chart (n = 3). The stability of the DNMP stock solution at 1000 mg L^{-1} was also tested following these same criteria. However, storage has been tested with and without the use of amber glass. The procedure was carried out under conditions of in-house reproducibility of preparation, since dilution procedures were necessary before analysis. Finally, ANOVA (single factor) at the 95% probability level has been applied to evaluate if there was a significant difference between the concentrations obtained by the experiment. If so, the t-test (in pair for averages) was used to determine on which day it differed significantly (at the 95% probability level) of the reference (day zero).

2.7. Study of errors and analytical measurement uncertainty

CC_α, CC_β, and standard uncertainties were calculated by considering the analysis of blank samples spiked at the MRPL, in equidistant steps (1, 1.5, and 2 times the MRPL). The α-error was obtained by multiplying the standard deviation of the mean of the spikes performed during method validation by the factor 2.33 and weighted in the overall mean. The β-error was obtained by multiplying the standard deviation of the mean by the factor 2.33 and adding this value to the CC_α. In order to identify the uncertainty contributions to the analyte determination procedure, mathematical modeling was used in accordance to the Guide for the Expression of Uncertainty in Measurement [28], as follows:

$$C(\mu g kg^{-1} DNMP) = \frac{\left(\frac{A_{analyte}}{A_{IS}} - b\right).\ C_{IS}.V}{a.\ m} \quad (2)$$

Where $C_{\mu g\,kg^{-1}}$: analyte concentration in stock solution (μg kg^{-1}); m: sample mass (g); $A_{analyte}$: analyte area (a.u.); A_{IS}: internal standard area (a.u.); C_{IS}: concentration of internal standard at the extraction solution (μg kg^{-1}); V: extraction volume (mL); a: angular coefficient (a.u.); b: linear coefficient (a.u $mg^{-1}\,kg$).

The combined standard uncertainty (u_c) of the overall model was calculated according to the following equation:

$$\begin{aligned} u^2(f(A_{analyte},\ A_{IS},\ C_{IS}, a, b, m, V)) &= \left(\frac{\partial f}{\partial A_{anayite}}\right)^2.(\sigma A_{analyte})^2 + \left(\frac{\partial f}{\partial A_{IS}}\right)^2.(\sigma A_{IS})^2 \\ &+ \left(\frac{\partial f}{\partial C_{IS}}\right)^2.(\sigma C_{IS})^2 + \left(\frac{\partial f}{\partial a}\right)^2.(\sigma a)^2 + \left(\frac{\partial f}{\partial b}\right)^2.(\sigma b)^2 \\ &+ \left(\frac{\partial f}{\partial m}\right)^2.(\sigma m)^2 + \left(\frac{\partial f}{\partial V}\right)^2.(\sigma V)^2 + 2.\left(\frac{\partial f}{\partial A_{analyte}}\right) \\ &. (\sigma A_{analyte}).\left(\frac{\partial f}{\partial A_{IS}}\right).(\sigma A_{IS}).r_{A_{analyte},\ A_{IS}} \\ &+ 2.\left(\frac{\partial f}{\partial a}\right).(\sigma a).\left(\frac{\partial f}{\partial b}\right).(\sigma b).r_{a,b} \end{aligned} \quad (3)$$

Where u^2: combined standard uncertainty; $A_{analyte}$, A_{IS}, C_{IS}, a, b, m, V: measurement variables of the overall model in a.u., a.u., μg kg^{-1}, a.u. μg^{-1} kg, a.u., g, and mL, respectively; $(\sigma x_i)^2$: output standard uncertainty; $\frac{\partial f}{\partial x_i}^2$, $\frac{\partial f}{\partial x_j}$: sensitivity coefficients; $r_{i,j}$: estimated correlation coefficient.

The expanded standard uncertainty (U) was obtained by multiplying the combined standard uncertainty by the coverage factor (k), considering the t-Student distribution (95%). Statistical analysis of random uncertainties due to method validation was also performed. With this aim, gross errors were deleted by applying the Dixon test (95%) and the occurrence of systematic errors was checked on dispersion charts. Finally, after the estimation of the main sources of errors and uncertainties, the output standard uncertainties were checked by considering the measurand level and measurement readability.

2.8. Method applicability

All the 54 samples of meat products which yielded quantifiable results for NIT, NAT, and SOR, were submitted to quantitation of DNMP. Quantitative DNMP results, expressed as μg kg^{-1}, included the probability of hitting (k = 2). Results were considered positive based on the β-error. Recovery and precision (conducted as described on item 2.6) were also checked with commercial samples. Matrix effects and the respective control charts were evaluated in accordance to Hoff et al. [27].

3. Results and discussion

3.1. Organic synthesis and characterization of the compounds

The analytical data obtained in the characterization of the compounds by different techniques, such as TLC, HPLC-DAD NMR, and MS, corresponded to the available literature [9,12,14,19,20]. A table with ^{1}H NMR chemical shifts, multiplicity parameters, and coupling constants was provided as Supplementary material (Table S1). The fragmentation of the DNMP molecule by LC-MS/MS provided high selectivity mass accuracy data. Since no quantitative methods for the analysis of DNMP by LC-MS/MS can be found in current literature, data on DNMP MRM reactions were not readily available. In this way, the main breaks of the DNMP molecule were obtained during method development. Fig. 1 (part A) depicts a chromatogram with three transitions used for analysis. In positive mode, the fragmentation spectrum of the precursor 172 *m/z* includes the 126 *m/z* (loss of NO_2), 109 *m/z* (loss of NO_2, CH_3), and 62 *m/z* (loss of NO_2, NO_2, and CH_3) fragments (Fig. 1, part B). Those were reasonable findings, since the loss of the nitro groups and methyl from the pyrrole-ring was already expected.

3.2. Extraction method

Meat products usually have large proportions of macro-constituents, such as proteins, fats, dyes and additives. When dealing with such complex matrices, the SLE-LTP technique may be difficultly adapted [29]. However, this procedure properly extracted the DNMP from meat products. The main factors that influenced the extraction procedure were: (F2) extraction solvent, (F4) acid type in extraction, (F5) extract drying temperature, (F6) clean-up using apolar solvent, and (F7) % of organic solvent (acetonitrile) in the extract reconstitution solution (Fig. 2). When a low proportion of water was used, the acetonitrile extraction solution favored the analyte extraction, besides it promoted the precipitation of proteins and other concomitants. Acetonitrile is used to extract a wide range of analytes and co-extractive interfering components from matrices, including lipids [30]. The pH around 4.3 ± 0.5, obtained with the addition of 0.1% formic acid, led many proteins to their isoelectric point and changes in their conformation. When they were subjected to low temperatures (−30 °C) and refrigerated centrifugation, large protein conglomerates and fat micelles could be removed. Ethyl acetate played a better role over hexane in the extract clean-up, since it removed several miscible compounds (such as dyes and other additives), as well as remaining fats from previous procedures. The lack of NPIC peaks of and the stability of the PIC signal, indicated that no nitrosation reaction occurred during the DNMP extraction. This was in part due to the control in the extraction

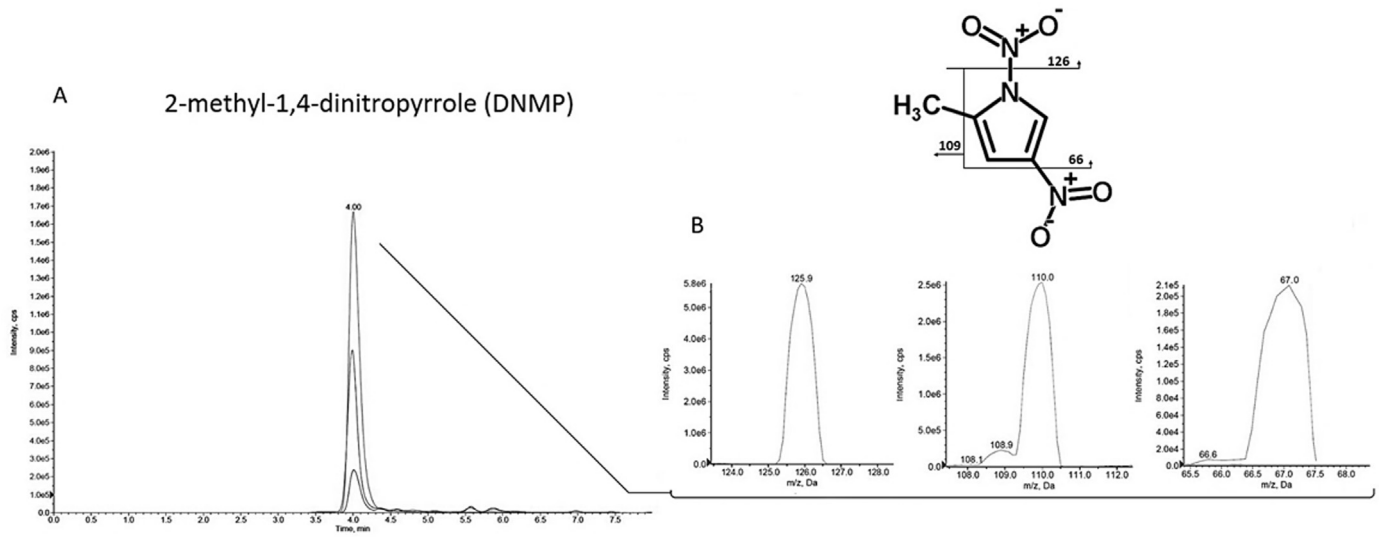

Fig. 1. Analysis of DNMP by LC-MS/MS, where A: reverse phase chromatogram of three transitions employing cyanopropyl as stationary phase; B: mass spectra of the DNMP (exact mass: 171 g mol^{-1}), parent ion: 172 *m/z*, fragments: 126 *m/z* (loss of NO_2), 109 *m/z* (loss of NO_2, CH_3), and 62 *m/z* (loss of NO_2, NO_2, CH_3).

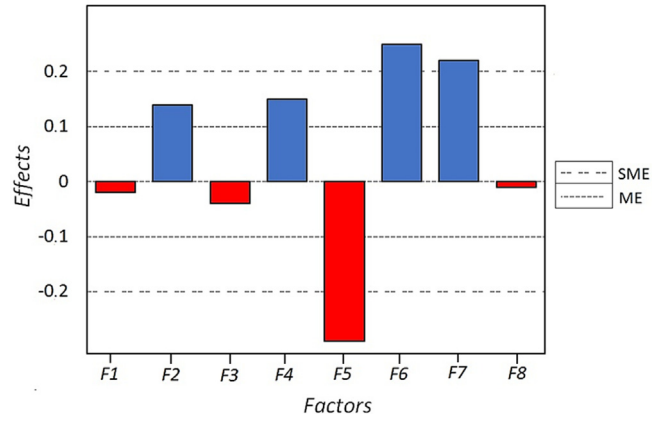

Fig. 2. Lenth's plot with the main effects of the variables applied to the DNMP extraction procedure by solid-liquid extraction with low temperature partition for meat products (SLE-LTP).

temperature (25 °C), in addition to the acid medium, which prevented the decomposition of DNMP in Meisenheimer-type adduct by the action of nucleophiles (OH^- ions) from the medium [12,14]. In a similar study, the extraction of volatile nitrosamines (molecules with similar chemical properties) by SLE-LTP and their quantitation by LC-MS/MS, the formation of NPIC was not reported, since similar care was taken [20]. Finally, the best sensitivity was achieved by the extract reconstitution with a 30% of organic (acetonitrile) solution, which favored better recovery rates.

3.3. LC-MS/MS analysis

Some difficulties faced during method development were the lack of a commercial DNMP standard, the previous knowledge about the analyte volatility and instability, very low expected concentrations (in the order of μg kg^{-1}), as well as the matrix complexity. Nevertheless, the developed method proved to be a reliable, selective, and sensitive tool for DNMP measurements in meat products. To the best of our knowledge, for the first time a method capable of determining DNMP traces by LC-MS/MS is described. With this measurement tool, a new outlook on the concentration of DNMP in foodstuffs may arise, strengthening inspection and control actions aimed at the insurance of public health.

The mass spectrometer was optimized to achieve maximum sensitivity in the quadrupoles. According to Table 1, a precursor ion and three second generation ions were required to identify the analyte. The most intense fragment (126 *m/z* - loss of NO_2) was used for DNMP quantitation. The fragments 109 *m/z* (loss of NO_2, CH_3) and 62 *m/z* (loss of NO_2, NO_2, CH_3) were used for identification (Fig. 1). The relative intensities of the transitions were all above 30%. Thus, the criterion for the identification of banned substances in foodstuffs was met, according to Commission Decision 2002/657/EC [25]. It was possible to analyze DNMP by using both ESI and APCI sources, both in positive mode. The standards in solvent yielded similar results and analyte intensity by both sources. However, a better sensitivity was achieved for the NPIC by employing the APCI source. This was a reasonable finding, since it has already been reported that better results for the LC-MS/MS analysis of some volatile nitrosamines could be achieved with the APCI source than the ESI one [20]. However, when analyzing spiked

Table 1
Values of the optimized multiple reaction monitoring (MRM) parameters using electrospray ionization in positive mode (ESI^+) for the determination of 2-methyl-1, 4-dinitro-pyrrole (DNMP) in meat products by LC-MS/MS.

Compound	Molecular formule	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)	CE (V)
2-methyl-1,4-dinitro-pyrrole (DNMP)	$C_5H_5N_3$	4.09	171.9	125.1 (Q)	61	17
				67.0 (I)	61	23
				109.0 (I)	61	17
1-methyl-imidazole (I-MEI) (IS)	$C_4H_6N_2$	3.28	83.0	56.0 (Q)	25	10
DL-Pipecolic acid (PIC)	$C_6H_{11}NO_2$	3.36	130.0	66.9 (Q)	71	35
				64.8 (I)	71	43
N-Nitroso-DL-Pipecolic acid (N-PIC)	$C_6H_{10}N$	4.76	159.1	84.0 (Q)	56	21
				128.0 (I)	56	9

CE: collision energy; DP: declustering potential; IS: internal standard; Q: quantitation ion; I: compound identification ion.

matrices, a greater signal suppression was verified with the APCI source. In addition, many peaks eluted near the retention time of DNMP, unlike the ESI source, which led the assays to proper selectivity and reproducibility. Thus, the optimized parameters in the ESI source consisted of 4500 V (ionspray voltage), 600 °C (source temperature), 55 psi (auxiliary and drying gases pressures), and 20 psi (curtain gas pressure).

Reverse phase chromatography with cyanopropyl (CN) as the stationary phase and acetonitrile and water as mobile phases was suitable for the separation of all compounds studied by this method. On the other hand, a high percentage of organic solvent at gradient's end, avoids carry-over phenomena [29]. The use of formic acid in 0.1% as a mobile phase additive improved the chromatography, sparing the use of volatile buffer salts. The stationary CN phase was the best established condition between run time, flow, and retention of the analytes (DNMP, N-PIC, PIC, and 1-MEI), compared to other stationary phases (C_{18}, C_8, and Hypersil Gold®). When the standards were analyzed in solvent, the sensitivity of the different stationary phases varied (C_{18} > CN > Hypersil > C_8). During method development, we tried to retain the analyte until the middle of the chromatographic run. A high proportion of water (95%) at the beginning of the elution gradient was an important strategy for the analysis of food matrices, since it promoted the elution of interfering hydrophilic substances, such as aldoses, ketoses, and electrolytes, in the first few minutes of running. On the other hand, by avoiding that the analyte was much retained by the stationary phase, shorter runs could be obtained, reducing the equilibration time. Thus, the CN phase better retained the analyte (for four minutes). The MS by-pass valve was be used in the first three minutes of each run, getting rid of undesirable compounds extracted from the matrix, and a small equilibration time was established. This procedure reduced soiling and oxidation at the ionization source. A total running time of eight minutes was performed, with two minutes for self-equilibrate. This was enough to ensure the cleanliness of the column and the ionization source, since contaminations between runs were no longer observed during the selectivity evaluation.

3.4. Method validation

Analytical validation led to conclusion that the developed method has metrological reliability. All results were in accordance to the Commission Decision 2002/657/EC [25] (Table 2).

Selectivity was proven, since no interfering transition was observed around the retention time of the analyte and the other compounds assessed by this method (1-MEI, PIC, and NPIC). This parameter is fundamental for the reliability of the results, because analytes with low molecular mass are susceptible to interfering transitions from substances contained in foods during LC-MS/MS analysis [4].

The matrix-matched analytical curves were prepared with cooked and fermented meat products, in the range of 12.5–400.0 μg kg^{-1}. For bacon samples, the analytical range was among 10.0 and 400 μg kg^{-1} of DNMP. Reproducibility was satisfactory and the mean regression coefficients were all higher than 0.98, even if a measurement variable was applied. On the other hand, matrix effect decreased the analytical signal by more than 70% (Table 2). All matrices showed a similar profile in signal suppression. In cooked and fermented meat products, the suppression was higher than in bacon samples. This may be due to the fact that bacon has a higher proportion of fat in its interfering composition. This component may have been effectively removed by our extraction protocol compared to other constituents. Proteins, endogenous constituents of foods of animal origin, are among the main interfering agents responsible for causing signal suppression in ESI sources [27]. In addition to the chemical structure of the analyte, its physical and chemical evolution during processing methods, such as curing, cooking, and fermentation, may be related to the matrix effect [31]. Thus, processed meats may present different physicochemical behaviors for LC-MS/MS analysis. The LMDR in solvent was readily set to 1.5 μg kg^{-1} of DNMP. However, in the presence of the matrices, this value has risen to 10 μg kg^{-1} for bacon and 12.5 μg kg^{-1} for the other kinds of samples. Once matrix effect was verified, matrix-matched analytical curves were adopted for DNMP analysis in order to assess method applicability. Both precision and recovery yielded acceptable values for all matrices (Fig. 2). Accuracy in terms of repeatability and in-house reproducibility yielded CV values below 20%. Recovery rates were among 80% and 110%.

It can be inferred from the stability assessment that DNMP remains stable on extracts until the 15th day, if they are stored at −30 ± 10 °C. In contrast, stock solutions showed significant differences between the stability results in the presence and absence of light. In all cases, the concentration of DNMP significantly differed from the seventh day ($P < 0.05$). This parameter confers high reliability to the results due its low uncertainties of 3.7% (extract, k = 2) and 12% (standard solution, k = 2), respectively. The low uncertainties corroborate good precision of the developed method.

The uncertainty due to the stability assessment of the standard solutions was higher than that obtained for the extracts, because random uncertainties generated by the analytical instrumentation were smaller than those from routine preparation procedures performed by analysts, when both parameters are compared under conditions of in-house reproducibility. Assessing this parameter is very important to avoid confusion among stability results and random errors.

3.5. Errors, measurement uncertainty, and expression of results

The results of the standard output uncertainties u (yi) and the analytical errors (α- and β-errors) are presented in Fig. 3. The greatest source (24%) of type A uncertainty (obtained experimentally) was related to precision in terms of in-house reproducibility, u(R&R). The uncertainty due to recovery, u(bias), has been the second largest uncertainty source, contributing with 18% for the overall uncertainty. Although CVs and recovery rates were all satisfactory, the parameters variability was responsible for expressive random uncertainties. The standard uncertainty due to the slope of analytical curves, u(a), was

Table 2
Validation results of the LC-MS/MS method for the analysis of 2-methyl-1,4-dinitro-pyrrole (DNMP) in meat products.

Kind of matrix	Spiking level (μg kg^{-1})	Recovery (%)	Repeatability (CV%)	In-house reproducibility (CV%)	Linearity (R^2)	[a]ME (%)	CC_α (μg kg^{-1})	CC_β (μg kg^{-1})
Cooked sausages	12.5	82	18	19	0.98	−87	14.3	16.2
	25.0	90	12	14				
	50.0	98	6.4	7.1				
Fermented products	12.5	93	10	15	0.98	−81	14.8	17.3
	25.0	105	7.9	8.2				
	50.0	109	4.7	6.8				
Bacon	10.0	98	7.1	15	0.99	−74	11.6	13.1
	15.0	102	5.4	6.2				
	20.0	99	4.5	5.4				

[a] ME – matrix effects calculation in %. Minimum required performance limit (MRPL) in μg kg^{-1} corresponds to first spiking level.

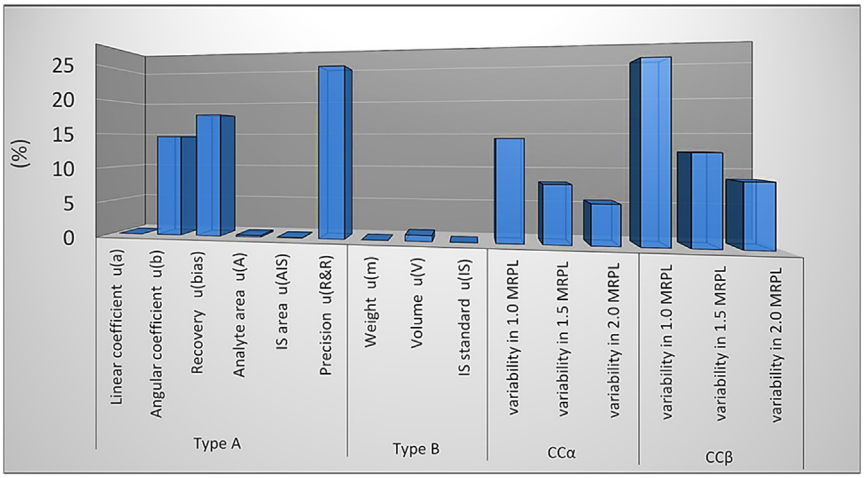

Fig. 3. Uncertainties sources and errors of the analytical method for the quantitation of DNMP in meat products by LC-MS/MS.

considered an expressive source (15% contribution), due to the reproducibility of matrix-matched analytical curves. Among the uncertainties from the analytical signals, the area of analyte, u(A), represented 0.2% of the uncertainty contributions, against just 0.07% due to the internal standard ($u(A_{IS})$). This was due to the DNMP's lower purity (around 90%) in relation to the commercial internal standard 1-MEI (with 99% purity). These sources of uncertainty have to be considered by the mathematical modeling as uncertainties arising from the impurity of the standards. However, both sources were insignificant if the order of magnitude of the other type A uncertainties were compared.

On the other hand, the type B uncertainties were negligible in relation to the uncertainties arising from the random errors due to method validation. The largest contribution (0.8%) was related to the dilution volume of the extract, u(V), which combines uncertainties, errors, and performance data inherited from volumetric instruments. However, as shown in this study, experimentally obtained uncertainties seem to be a good estimator of the overall uncertainty for chemical measurements, since type B uncertainties are usually insignificant compared to the uncertainties due to equipment and analysts [28].

The detection capability (CC_β) and decision limit (CC_α) are experimental approaches defined by Commission Decision 657 to measure the performance of the analytical procedure and assist in the results interpretation in interest levels [25]. In this work, the β-error was successfully used to increase the reliability of positive results, considering that DNMP is a banned analyte for foodstuffs. Samples which yielded results above their respective CC_β values were considered positive (Table 2). The values in % of the errors were similar to the sources of standard output uncertainties u(R&R), u(bias), and u(b), since the same accuracy, recovery, and residues data from analytical curve were considered for calculation. On the other hand, errors consider a greater coverage coefficient in relation to the expanded uncertainty, since decisions must be taken even if very low concentrations (near the MRPL) of the measurand are found and uncertainty is highly propagated. This is demonstrated by Fig. 3, where the sources of random errors increase with the decrease of the measurand concentration (1.0 LMDR), considering a limited number of measures.

Finally, the expanded standard uncertainty (33%) propagated all expressive sources of uncertainty: u(a), u(bias), u(A), u(R&R), and u(V). Thus, by considering the measuring range of the measurand, an acceptable probability of hitting set was provided, in accordance to Guidelines on Measurement Uncertainty [32]. A calculation memorial on how uncertainty was computed for this method was provided as Supplementary materials (Table S2).

3.6. Method applicability

Newly obtained analytical performance parameters can be compromised when new matrices are faced in analytical routine, especially if they differ from those used during method validation, when a limited number of observations were considered. However, we have already reported that it seems to be a common pitfall for food control laboratories, since the physicochemical profiles of foodstuffs are among the most diversified material for the Analytical Chemistry [4].

On the other hand, our analytical method could be properly simulated in an analytical routine, increasing the reliability of the results. Selectivity, precision, and accuracy were maintained even when different commercial samples (Table 3) were considered for analysis. Thus, a huge variety of possible samples was assessed.

The recovery rates of the spiked samples were among 20% and 10% and the repeatability (n = 6) CVs were lower than 20%. Furthermore, the results of the matrix effect achieved by method validation with representative matrices (bacon, fermented, and cooked meat products) at Table 2 presented a good approximation with the results achieved by the applicability assessment (Table 3).

Food safety is a major public health concern for control agencies. The insurance of safe food supply is among the major challenges faced by food regulators. In October 2015, the International Agency for Research on Cancer (IARC) issued a press release about the carcinogenicity of some meat products, which raised the rating of those foodstuffs to group 1, corresponding as carcinogenic to humans [3]. However, further data on the compounds, mechanisms of action, types of cancers arising from the consumption of red meats, as well as other meats and processed meats, the influence of cooking, among other points, are required to support this decision. Thus, it would be highly recommendable to carry out new epidemiological studies to support this classification [33].

From the total of 54 analyzed samples of meat products, 22 samples contained the three preservatives (NIT, NAT, and SOR), from which six quantifiable results for DNMP were yielded. With the exception of one sample, the other five samples were non-compliant for the maximum residue level settled for NIT and NAT ($150\ mg\ kg^{-1}$) by Brazilian regulation [34]. In addition, the irregular addition of sorbate were also observed, since this is a forbidden preservative to those kind of samples, according to Brazilian current regulation. On the other hand, all samples which were negative for sorbate, were also negative for DNMP. Usually, products to which formulation SOR can be added are not subjected to thermal processing, which is one of the factors for the formation of DNMP. Thus, the use of sorbate should be controlled and restricted, once our results confirm the formation of DNMP in products that have both preservatives (nitrite and sorbate). Samples of bacon and

Table 3
Applicability results of the analytical method for quantitation of 2-methyl-1,4-dinitro-pyrrole (DNMP) in meat products by LC-MS/MS.

Results	Samples of meat products							
	Cooked sausages	Mortadella	Cooked ham	Bacon	Cooked sausages (Calabrese-like)	Cooked loin	Mortadella (8:1)[a]	Mortadella (2:1)[b]
Sorbic acid (SOR), in mg kg^{-1}	361.7	320.6	0.300	210.0	307.9	175.0	5.830	128.5
Sodium nitrite (NIT), in mg kg^{-1}	91.87	117.1	183.2	97.20	122.8	54.70	NA	NA
Sodium nitrate (NAT), in mg kg^{-1}	68.76	39.01	50.46	30.40	61.09	66.70	NA	NA
DNMP (μg kg^{-1})	50.06	18.47	19.25	67.76	22.64	13.20	59.59	15.2
	Spiked samples at minimum required performance limit (MRPL) (n = number of independent samples)							
Parameters	Cooked sausages	Mortadella	Cooked ham	Corned beef	Bacon	Calabrese-like cooked sausages	Salami	Pepperoni
	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
Recovery (%)*	97.6	103	107	101	101	109.0	88.0	81
Repeatability (CV%)	2.99	12.30	8.13	9.91	9.91	12.5	11,5	1.8
Matrix effect (%)	81	80	87	85	69	88	81	87

[a,b] In-house processed samples with 8:1 and 2:1 ratios of sodium nitrite and potassium sorbate, respectively.
Minimum required performance limit (MRPL) corresponds to 12.5 μg kg^{-1}, excepting bacon (MRPL = 10 μg kg^{-1}).
* Recovery rates of spiked samples, interpolated in matrix-matched analytical curves in triplicate. Expanded standard uncertainties in percentages: sorbic acid (SOR) = 9.1%, sodium nitrite (NIT) = 10%, sodium nitrate (NAT) = 5%, and 2-methyl-1,4-dinitro-pyrrole (DNMP) = 33%.

cooked sausage yielded the highest concentrations of DNMP (68 ± 3 and 50 ± 3 μg kg^{-1}, respectively). Samples of mortadellas which were specifically processed for this research, also yielded quantifiable results. The formulations with 8:1 ratio of NIT and NAT yielded higher DNMP concentrations than the 2:1 ratio (53 ± 3 and 18 ± 3 μg kg^{-1}, respectively). These results were in agreement with the literature data. It has been demonstrated that the *in vitro* formation of DNMP is optimized when nitrite and sorbate are combined in the 8:1 ratio. However, the formation of the mutagen still can be detected, even if this ratio falls to 2:1 (Hartman, 1983). However, the profile of applicability samples showed a higher proportion of SOR in relation to nitrite. Curiously, samples assessed during method applicability of our research yielded higher amounts of SOR in relation to NIT. One possible explanation is that nitrite concentration tends to be quite instable in meat products. Nitrite tends to be reduced to nitric oxide, especially if acidifying agents, such as the erythorbate, were used in the formulation [35].

Even considering the sum of NIT and NAT results, sorbate remained in higher proportion. Most of the results presented a ratio of SOR and NIT + NAT close to 2:1. Considering that 11% of all the analyzed samples (n = 54) and 27% of the samples with NIT, NAT, and SOR (n = 22) have yielded quantifiable values of DNMP, these data demonstrate the importance of monitoring this mutagen. On the other hand, risk analysis is essential to set the concentrations that may lead to an increase in the incidence of cancer by the consumption of this compound [33].

Chromatograms of both spiked and real samples and were provided as Supplementary material (Figs. S1 and S2).

4. Conclusion

The lack of commercial standards may not discourage the development of new analytical methods intended to contribute to food safety. Once the importance of the DNMP analyte to science was clearly demonstrated, the viability of the commercial production of an analytical standard is only a matter of time. We succeeded to synthesize the DNMP standard and thus method development was enabled. Since all validation parameters yielded satisfactory results and quantifiable samples were found, the developed method proved to be a reliable, selective, and sensitive tool for DNMP measurements in meat products. We found that 11% of the samples assessed for method applicability presented quantifiable results of DNMP, confirming the usefulness of this measurement tool, which can contribute to future studies on this mutagen.

Declarations of interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2018.03.035.

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