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A reference isotope dilution headspace GC/MS method for the determination of nitrite and nitrate in meat samples

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Summary

A novel method for the determination of nitrite and nitrate in meat products is presented. The samples were ground and extracted in hot water with the presence of $^{15}NO_2^-$ and $^{15}NO_3^-$ internal standards. The solution was buffered with sodium bicarbonate and reacted with triethyloxonium tetrafluoroborate to convert nitrite and nitrate into $EtNO_2$ and $EtONO_2$. Such derivatives could be detected by headspace GC/MS in positive chemical ionization mode with 0.05 µg g^{-1} NO_2^- and 1.0 µg g^{-1} NO_3^- LOD. The method was used for NO_2^- and NO_3^- quantitation in the 0.5-300 and 2.5-300 µg g^{-1} ranges. The method was applied for the analysis of fifteen meat products. Despite minimal sample preparation, the headspace sampling ensured a clean chromatography for over 135 analyses (throughput 10 samples/hour). The proposed method offers selective GC/MS detection combined with high-precision isotope dilution calibration, it is suitable for metrological applications, and can support regulations on meat safety (European Commission, 2011).

Keywords

Meat Products; Nitrates/Nitrites/Nitrosamines; Isotope Dilution; Headspace Analysis; GC/MS

Running Head

GC/MS method for nitrite and nitrate in meat

Introduction

During the last decade, the scientific community has largely debated on the role of nitrate (and nitrite) in the human diet and, to date, a clear picture about benefits and risks associated with these nitrogen compounds is still a matter of discussion and controversy (Katan, 2009; Bedale *et al.*, 2016). The benefits advocating a nitrate-rich diet are rooted in the role of this nutrient to sustain the nitrate-nitrite-NO pathway with demonstrated improvements to the cardiovascular function (Hord *et al.*, 2009; Weitzberg & Lundberg, 2013). The risks associated with nitrate and nitrite are linked to the disposition of these anions to generate *N*-nitrosamine during cooking and along the digestive system, increasing the probability of developing certain types of cancer (Sinha *et al.*, 2009; Cross *et al.*, 2010; Ferrucci *et al.*, 2012, Catsburg *et al.*, 2014).

Although the highest contribution to dietary nitrate is given by leafy vegetables, a certain degree of scrutiny has been dedicated to meat products, where nitrite and nitrate have been historically employed for curing purposes (Honikel, 2008; Badale *et al.*, 2016). Such additives have been introduced in meat products under the form of sodium or potassium salts, or by means of vegetable powders and beetroot juice which are a rich source of nitrate (Sebranek *et al.*, 2012; Ko *et al.*, 2017; Riel *et al.*, 2017; Sucu & Turp, 2018).

Due to the potential adverse effects of NO_2^- and NO_3^- when associated with meat, regulations have been established to limit their amount in food and cured meat products (European Commission, 2011; Merino *et al.*, 2016) and analytical testing for residual nitrite and nitrate is required to demonstrate regulatory compliance.

Due to the complex meat matrix, the determination of nitrite and nitrate in these materials is still a challenging task (Merino *et al.*, 2017). Official methods employ photometry detection in combination with Griess diazotization reaction (ISO 3091:1975; ISO 2918:1975). This derivatization is only applicable for nitrite, whereas nitrate requires a critical reduction step to nitrite usually performed on Cd/Cu. Although this photometric method is low-cost and allows

high-throughput, it requires careful sample clean-up for removal of proteins, fat, and other matrix components than could interfere with the assay. Other photometric (Altunay *et al.*, 2017; Ensafi *et al.*, 2004) and direct electrochemical methods (Campos *et al.*, 2010; Somer *et al.*, 2016) share similar limitations due to matrix effects and preferential response toward nitrite only. To date, most popular methods for simultaneous determination of nitrite and nitrate are based on liquid separation. Della Betta *et al.*, 2016 proposed an elegant capillary zone electrophoresis (CZE) method with UV detection which could provide results within 30 s. Although CZE is a well suited technique for inorganic anions, CZE instruments are not widely diffused in testing laboratories and current methods for nitrite and nitrate mostly employ ion chromatography and C_{18} reverse phase HPLC with conductivity (Iammarino & Di Taranto, 2012; Lopez-Moreno *et al.*, 2016; D'Amore *et al.*, 2019), UV (Siu & Henshall, 1998; Hsu *et al.*, 2009; Chetty *et al.*, 2019), or mass spectrometry detection (Saccani *et al.*, 2006; Siddiqui *et al.*, 2015) although direct LC/MS methods for nitrite and nitrate are more commonly applied to simpler sample matrices (Khan *et al.*, 2013; Khan *et al.*, 2016;). Despite such methodologies have reached a broad diffusion in food testing laboratories, liquid chromatography is typically lengthy (20-30 min per sample) and require a certain level of maintenance due to the complexity of the meat matrix.

In this study, we propose a simple gas chromatography mass spectrometry (GC/MS) method for the determination of nitrite and nitrate in meat. The procedure is based on the conversion of nitrite and nitrate into volatile $EtNO_2$ and $EtONO_2$ by means of simple, fast, single-step triethyloxonium tetrafluoroborate aqueous chemistry at room temperature (D'Ulivo *et al.*, 2009; Pagliano *et al.*, 2018). Such derivatives can be sampled from the headspace with remarkable matrix simplification, detected within 6 min of GC/MS analysis, and quantified by high-precision isotope dilution (ID). ID is regarded as a primary method of analysis (Richter, 1997) with an essential role for metrological applications including characterization of Certified Reference Materials (Pagliano *et al.*, 2019). The strength of isotope dilution derives from the use of $^{15}NO_2^-$ and $^{15}NO_3^-$ isotopically enriched internal standards which allows compensation for sample

losses, incomplete derivatization and irreproducibility of headspace sampling and injection. The novel procedure was validated and compared with independent procedures for nitrite and nitrate in meat.

Material and methods

Reagents, samples and standards

Nitrite and nitrate in cured meat samples were quantified against primary standards obtained from Sigma-Aldrich (P/N 74246, 1000 mg L^{-1} nitrate in water; P/N 67276, 1000 mg L^{-1} nitrite in water at pH 11). Working solutions were prepared by gravimetric dilution. $K^{15}NO_3$ (P/N NLM-765-1, 99%+ ^{15}N) and $Na^{15}NO_2$ (P/N NLM-658-1, 99%+ ^{15}N) isotopically enriched internal standards were supplied from Cambridge Isotope Laboratories. Triethyloxonium tetrafluoroborate (P/N 90520), sodium bicarbonate (P/N 31437), sodium tetraborate decahydrate (P/N B3545, borax), acetonitrile (P/N 34851), sodium carbonate solution (P/N 56169, 0.1 M), and sodium bicarbonate solution (P/N 36486, 0.1 M) were obtained from Sigma-Aldrich. A solution of triethyloxonium tetrafluoroborate was prepared in a PFA vial by adding 5 g of Et_3OBF_4 to 5 mL of pre-cooled (−20 ± 1 °C) acetonitrile. This solution was manipulated in a fume hood for the shortest period of time, and it was stable for one month when stored at −20 °C. The Et_3OBF_4 solution was used for nitrite and nitrate derivatization and leftovers were hydrolyzed in water before disposal. Sample preparation and dilutions were obtained gravimetrically with a Mettler-Toledo MS204S balance and ultrapure water was generated with a Thermo Scientific GenPure UV xCAD plus system (18.2 MΩ cm^{-1} at 25 °C). Fifteen cured meat samples were acquired locally and employed for method development. The samples were chosen to cover a wide spectrum of matrices including sausages, salami, pepperoni, ham, bacon, chicken liver pâté, prosciutto, capocollo, and Bologna sausage (Table S1). The samples were kept at 4 ± 1 °C until the day of analysis.

Nitrite and nitrate extraction and derivatization

A 50 g aliquot of sample was homogenized using a blade food processor. According to common practice, nitrite and nitrate are extracted from the meat matrix in hot water with (ISO 2918:1975; ISO 3091:1975; Della Betta *et al.*, 2016) or without (D'Amore *et al.*, 2019) borax. A 2 L volume of 0.5% aqueous borax was prepared and spiked with 10 mg L^{-1} $^{15}NO_2^-$ and $^{15}NO_3^-$ isotopically enriched internal standards. Such solution is called here the "internal standard" and was used throughout all measurements. 20.0 g of internal standard was used to extract 4.0 g of the homogenized meat sample. Extraction was performed in a 40 mL glass vial placed in a hot block at 70 ± 1 °C (20 min, shaken repeatedly). The extracted sample was cooled down and then 1 mL extract was transferred into a 10 mL headspace vial for CTC autosampler along with 60 mg $NaHCO_3$ and 50 µL Et_3OBF_4 solution in acetonitrile. The vial was shaken and, after 30 min, the nitrite and nitrate derivatives were analyzed by static headspace GC/MS.

GC/MS detection

Nitrite and nitrate were chemically converted into $EtNO_2$ and $EtONO_2$, both suitable for sensitive detection by GC/MS in positive chemical ionization (PCI) mode. A 5973 Hewlett-Packard GC/MS system with a CTC CombiPAL autosampler was used. The autosampler program allowed for incubation of the vial at 60 °C for 2 min following the sampling of 1.5 mL of static headspace and injection in the GC/MS. The syringe was held at 70 °C and flushed by N_2 for 6 min after every injection to avoid carry over. The injection was performed in 7:1 split mode in a narrow 1 mm ID split liner at 120 °C. Separation was obtained in constant flow mode (1 mL min^{-1} He) on a 30 m x 0.250 mm ID x 1.40 µm film DB-624 column (6% cyanopropyl dimethylpolysiloxane) with the following oven program: 50 °C for 1.5 min, 20 °C min^{-1} up to 140 °C (6 min of run time). The transfer line was set at 220 °C and detection was obtained in PCI mode with CH_4 as reagent gas at 1 mL min^{-1} (250 °C MS source, 150 °C MS quadrupole). The MS was autotuned with the standard routine and acquisition was obtained in Single Ion

Monitoring (SIM) mode with a gain factor of 1. After a solvent delay of 3.7 min, the nitrate ethyl-derivative ($EtONO_2$) was detected at 4.2 min by monitoring *m*/*z* 92 ($EtO^{14}NO_2H^+$) and 93 ($EtO^{15}NO_2H^+$). The nitrite ethyl-derivative ($EtNO_2$) eluted at 4.8 min and was detected at *m*/*z* 76 ($Et^{14}NO_2H^+$) and 77 ($Et^{15}NO_2H^+$). Each mass was acquired with a dwell time of 50 ms.

Isotope dilution calibration

Isotope dilution (ID) calibration was used for quantitation. Fundamentals and calculation examples of ID method are reported elsewhere (Pagliano *et al.*, 2015; Campanella *et al.*, 2017). Briefly, the calibration solutions were prepared in the range 0.5-300 and 2.5-300 μg g^{-1} NO_2^- and NO_3^-, including one reagent blank. The blends for ID calibration were prepared by mixing 4.0 g of the calibration solution with 20.0 g of internal standard, which must be from the same batch used for meat extraction. Derivatization of the calibration blends was obtained similarly to the sample: a 1 mL volume of the calibration blend was transferred into a 10 mL headspace vial for CTC autosampler with 60 mg $NaHCO_3$ and 50 μL Et_3OBF_4 solution. Both calibration and sample blends were measured by GC/MS within the same sequence. The isotope ratio (r_{AB}) was obtained by dividing the chromatographic peak area of the ^{14}N signal over the area of the ^{15}N signal. For example, in the case of nitrate, the peak area at *m*/*z* 92 was divided by the one at *m*/*z* 93. As reported in the supplementary information, the r_{AB} (along with the gravimetric preparation data) was used for quantitation.

Ion chromatography control method

The performance of the proposed method was compared against an ion chromatography control method. Nitrite and nitrate extraction was obtained as reported by D'Amore *et al.*, 2019. Briefly, 4.0 g of homogenized sample was transfer in a plastic bottle with 80 mL of water. The container was placed in a bain-Marie at 80 ± 1 °C for 5 min. After cooling, the extract was filtered at 0.2 μm (Fisher Scientific, Basix, nylon) and analyzed by ion chromatography with both UV and

conductivity detection. A single pump Thermo Scientific ICS-5000$^+$ SP-5 was used for the measurements. Separation was obtained within a 30 min isocratic run (4.5 mM Na_2CO_3, 0.8 mM $NaHCO_3$, 1.0 mL min^{-1} at 30 °C) on a Dionex AS23 column (ID 4 mm; length 250 mm) with a Dionex AG23 guard column (ID 4 mm; length 50 mm). 25 µL sample was injected in push full mode and suppressed conductivity was obtained with a Dionex self-regenerating suppressor (AERS 500, 4 mm) operating with a 25 mA applied current. Conductivity was read at 35 °C (20 Hz collection rate), whereas UV detection was performed at 210 nm with a Dionex ICS-5 variable wavelength detector (VWD).

Results and Discussion

Derivatization and GC/MS detection

The proposed method is based on a simple derivatization for conversion of nitrite and nitrate into volatile molecules. As we reported previously (Pagliano *et al.*, 2011; Pagliano *et al.*, 2014), in an aqueous medium at room temperature, Et_3OBF_4 converts nitrite into EtONO (t_{boil} 17 °C) and nitrate in $EtONO_2$ (t_{boil} 86.9 °C). In this study, for the first time, we noticed that the *O*-ethylated derivative is not the only product generated by interaction of nitrite with Et_3OBF_4. As reported in Fig. S1, the nitrite *N*-ethylated derivative ($EtNO_2$, t_{boil} 114.4 °C) was also identified. With respect to EtONO, which is a gas under laboratory conditions, the $EtNO_2$ has better chromatographic retention and elutes free of interferences. For this reason, the GC/MS signal of $EtNO_2$ and $EtONO_2$ were used as proxy for nitrite and nitrate determination.

As both compounds are volatile, they can be detected at the headspace level separated from the complex meat matrix. This is a key feature of the method which results in significant procedural advantages. Since the matrix was not injected into the GC/MS, no sample cleanup/deproteinization/filtration/centrifugation was required. Therefore, the sample preparation was very simple and fast: the sample was extracted in water with the ^{15}N internal

standards and 1 mL of the resulting solution was treated with 60 mg $NaHCO_3$ and 50 µL Et_3OBF_4 solution. The chromatography was very clean (Fig. 1), the compounds could be eluted within 6 min with no deterioration of the analytical column. Notably, after 135 consecutive analyses of various meat samples, we could not observe appreciable drifts of the baseline or retention times (Fig. S2). With respect to the classical photometry and ion chromatography methods, which need direct analysis of the meat aqueous extract, headspace GC/MS provides methodological advantages for sample preparation and allows a throughput of 10 samples per hour, about five times faster with respect to ion chromatography.

When analyzing nitrite and nitrate, attention should be paid to the potential interconversion occurring at the sample preparation stage. It is known that under an acidic pH, the nitrite can convert to nitrate (Pagliano *et al.*, 2014). For this reason, extraction and derivatization were carried out at pH>7 using borax and sodium bicarbonate to buffer the medium. Within the conditions used for extraction, we verified that no nitrite/nitrate interconversion occurred. For this purpose, a 4.0 g sample of cured meat was spiked with 2 mg $^{15}NO_2^-$ following extraction in 0.5% borax at 70 ± 1 °C for 20 min. The extract was analyzed for $^{15}NO_3^-$. Less than 0.1% conversion of $^{15}NO_2^-$ to $^{15}NO_3^-$ was observed. The same experiment was also performed in the other direction and less than 0.5% conversion of $^{15}NO_3^-$ to $^{15}NO_2^-$ was detected. This result shows that the risks of analyte interconversion within the extraction/derivatization are negligible.

Extraction volume

This study was meant to explore GC/MS as a diagnostic tool for nitrite and nitrate analysis in meat samples, therefore the analyte extraction was simply adapted from consolidated methods (ISO 2918:1975; ISO 3091:1975; Della Betta *et al.*, 2016; D'Amore *et al.*, 2019). Since the headspace GC/MS procedure can efficiently handle complex matrices, for extraction we reduced the typical 1:10 sample:water ratio to 1:5. To demonstrate that the method was robust toward this variation, we monitored nitrite and nitrate response at different water:sample ratios

including 1:2.5, 1:5, 1:7.5, and 1:10. This ruggedness test was performed with four meat samples (smoked ham, baked ham, Bologna sausage, and smoked sausage). As presented in Fig S3 (Tables S2-S3), the method was not perturbed by variations in the extraction volume.

Analytical response

Fig. 2 shows the calibration plot obtained in the 0.5-300 and 2.5-300 μg g^{-1} region for nitrite and nitrate. Although an isotope dilution plot is formally described by a rational function (Pagliano *et al.*, 2015), the deviations from linearity working with the ^{14}N:^{15}N isotope ratio are usually modest. Calibration data in Fig. 2 was collected over twenty days of measurements. Under the same MS tuning settings, the calibration plot was very reproducible from day-to-day. Tables S4-S5 report the raw calibration data and show that the inter-day RSD (*n* = 6) for the response of the standards was better than 1% for NO_2^- above 1.0 μg g^{-1} and better than 3% for NO_3^- above 5.0 μg g^{-1}. The sensitivity of the method was also adequate for the analysis of meat samples: as reported in Fig. 1, 0.089 μg g^{-1} NO_2^- could be detected with a signal-to-noise ratio of 63 whereas 0.56 μg g^{-1} NO_2^- was measured with an inter-day RSD of 2.4% (*n* = 4, Table S4). 0.05 μg g^{-1} NO_2^- was established as the LOD and 0.5 μg g^{-1} NO_2^- as the LOQ. For nitrate, 1.18 μg g^{-1} NO_3^- was measured with a signal-to-noise ratio of 10.6. LOD and LOQ were established at 1.0 and 2.5 μg g^{-1} NO_3^-, respectively. At LOQ, the nitrate inter-day RSD was 6.8% (*n* = 4, Table S5). LOD and LOQ were evaluated experimentally by analyzing standards close to detection limit. The LODs were established at concentrations detectable with S/N ratio >9 with a reagent blank contributing less than 35% to the analytical signal. The LOQs were established at concentrations measurable with an inter-day reproducibility better than 10% (S/N ratio >18) and with a reagent blank contributing less than 20% to the analytical signal. The criterion adopted for the estimation of LODs and LOQs was more conservative than required by common validation guidelines.

Analysis of cured meat samples

The method was tested for the analysis of fifteen cured meat products (Tables 1-2). For each sample, three aliquots were prepared for GC/MS analysis and one aliquot for ion chromatography. The three extracts prepared for GC/MS were analyzed in triplicate with the proposed PCI GC/MS method and also with an electron ionization (EI) GC/MS method previously proposed for seawater analysis (Pagliano *et al.*, 2011). The aliquot for ion chromatography was analyzed in duplicate with both conductivity and UV detection. Nitrite results are presented in Table 1 where a good agreement within the three analytical procedures was obtained. Notably, the samples with the highest nitrite content (10-30 μg g^{-1} NO_2^-) were the ones cured with the cultured celery extract (Table S1). For the analysis of nitrate (Table 2) the PCI and EI GC/MS method were in a good agreement, whereas the ion chromatography method produced higher nitrate estimate by 10-20 μg g^{-1} NO_3^-. This bias can be explained considering the selectivity issues associated with ion chromatography. It is known that, on a carbonate/bicarbonate column, separation of nitrate from matrix components – like the glucose-6-phosphate – is weak and may give rise to positive interference (Saccani *et al.*, 2006).

The results obtained by PCI GC/MS were in line with recent surveys published on the topic (Sullivan *et al.*, 2012; Nuñez De González *et al.*, 2012; Iacumin *et al.*, 2019).

Spike recovery test

To further validate the performance of PCI GC/MS, we perform a three-level spike recovery test on three meat products. Three sample aliquots were spiked with a known amount of nitrite and nitrate (10, 40, and 110 μg/g NO_2^- and NO_3^-), whereas the fourth was analyzed as-is. As shown in Table 3, quantitative spike recovery were obtained for both NO_2^- and NO_3^-.

Sample equilibration

In this study, our main focus was to test the PCI GC/MS detection and we assumed that standard extraction procedures for nitrite and nitrate are fit for analytical purpose. Most of the published method in this field, including official methods, are based on a hot-water extraction of the sample for 15-20 min with or without borax (ISO 2918:1975; ISO 3091:1975; Della Betta *et al.*, 2016; D'Amore *et al.*, 2019). With the aid of the ^{15}N internal standards, we tested the efficiency of this approach.

After extraction, we expected that incurred NO_x^- (x = 2, 3) would be fully homogenized (equilibrated) with $^{15}NO_x^-$ internal standards in such a way that the $^{14}NO_x^-/^{15}NO_x^-$ ratio would not change in time. To test this assumption, we monitored by PCI GC/MS the $^{14}NO_x^-/^{15}NO_x^-$ ratio at different extraction time. 4.0 g sample was mixed with 20.0 g internal standard solution following extraction at 70 ± 1 °C for 10 – 15 – 20 – 30 – 60 – 120 min. According to standard procedures, complete extraction should be obtained within 20 min at 70 ± 1 °C. On the contrary, results in Fig 3 and Table S6 show that nitrite and nitrate response kept growing for over 120 min. The differences in extraction efficiency at 20 min and 120 min were between 5-30% depending on analyte and matrix type. This effect is remarkable and it may be due to strong analyte-matrix bounding which could require more aggressive extraction conditions. More investigations will be needed to understand the root cause for this effect and establish a more robust extraction protocol.

Effect of freeze-dry

The stability of nitrite and nitrate was tested after freeze-dry. All meat products were homogenized in a blade blender and frozen at −80 °C. The samples were transferred in a Thermo Scientific 5L ModulyoD freeze-drier for 5 days. The material was then crushed in a mortar and kept at −20 ± 1 °C until analysis. Mass loss for all sample is reported in Table S7. Only 1 g freeze-dried sample was used for extraction/analysis. The results, normalized to the wet basis, are listed in Table 4. Consistent with the previous observation, the freeze-dry process

had also made nitrite and nitrate more available toward extraction and the recovery was 5-30% higher with respect to results in Tables 1-2, with the exception of one material where the nitrite recovered in the freeze-dried sample was almost double. These results proved the stability of nitrite and nitrate toward the freeze-dry process which may be of interest for applications in the production of Reference Materials for quality control.

Conclusion

Nitrite and nitrate salts are historical additives employed for curing meat products (Honikel, 2008; Badale *et al.*, 2016) and their determination in meat samples is required to demonstrate regulatory compliance (European Commission, 2011). In this study, we have validated a novel analytical approach suitable for the determination of residual nitrite and nitrate in cured meat matrices. After analyte extraction, a simple aqueous chemistry was employed to convert NO_2^- and NO_3^- into $EtNO_2$ and $EtONO_2$ which were readily separated from the complex matrix under the form of a vapour. Headspace GC/MS allowed sensitive and specific detection of the derivatives within 6 min of chromatography. Furthermore, the use of MS detection allowed the use of primary, high-precision isotope dilution quantitation with $^{15}NO_2^-$ and $^{15}NO_3^-$ internal standards. This detection method was validated by spike recovery tests, ruggedness tests, and by comparison with established methodologies. With respect to methods based on liquid chromatography, PCI GC/MS has notable procedural advantages, requires minimal instrumental maintenance, and allows the performance characteristics required for metrological applications.

The proposed method was tailored on existing protocols for the extraction of nitrite and nitrate from the meat matrix which are based on a simple hot water extraction of the ground sample for up to 20 min. Most notably, the ruggedness experiments aimed to test the efficiency of the extraction had shown that a 20 min hot water extraction with borax may not be sufficient to quantitatively remove nitrite and nitrate from the matrix and an underestimation of 5-30% was

observed. Analysis of freeze-dried samples have confirmed this tendency. More research on grounding/extraction protocols may be required.

Data Availability Statement

Research data are not shared.

Ethical Guidelines

Ethics approval was not required for this research.

Conflict of Interest

We have no conflict of interest to declare.

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Display Items

Figure 1 PCI GC/MS chromatograms for nitrite (*m*/*z* 76) and nitrate (*m*/*z* 92). a. Nitrite signal at LOD. b. Nitrate signal at LOD. c. Nitrite signal observed in meat samples. d. Nitrate signal observed in meat samples

Figure 2 Isotope dilution PCI GC/MS calibration plot for nitrite and nitrate. The small deviation from linear response can be accounted by using a rational function for fitting (Pagliano *et al.*, 2015)

Figure 3 Increase of the analytical response in function of the extraction time. 4.0 g ground smoked ham was mixed with 20.0 g of internal standard solution following incubation at 70 ± 1 °C. The isotope ratio was measured at different time

Table 1 Determination of nitrite (μg g^{-1}) in processed meat products without prior desiccation

Table 2 Determination of nitrate (μg g^{-1}) in processed meat products without prior desiccation

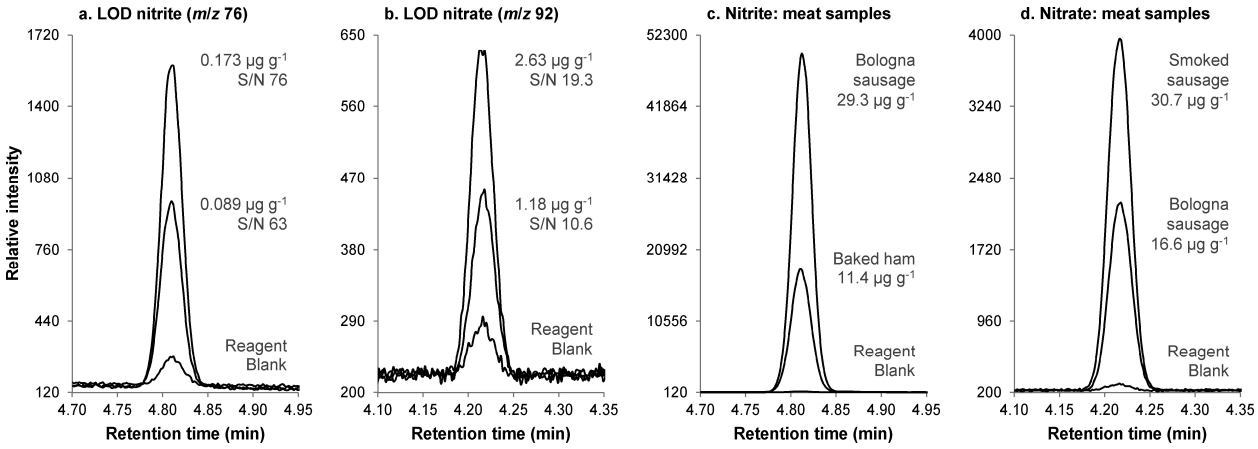
Table 3 Results from a nitrite and nitrate three-level spike recovery test on three meat products

Table 4 Determination of nitrite and nitrate on the freeze-dried meat samples.

Supplementary Files

Supplementary_data.pdf: supplementary data for method development and validation

Supplementary_calculation.xlsx: supplementary information for isotope dilution calculation



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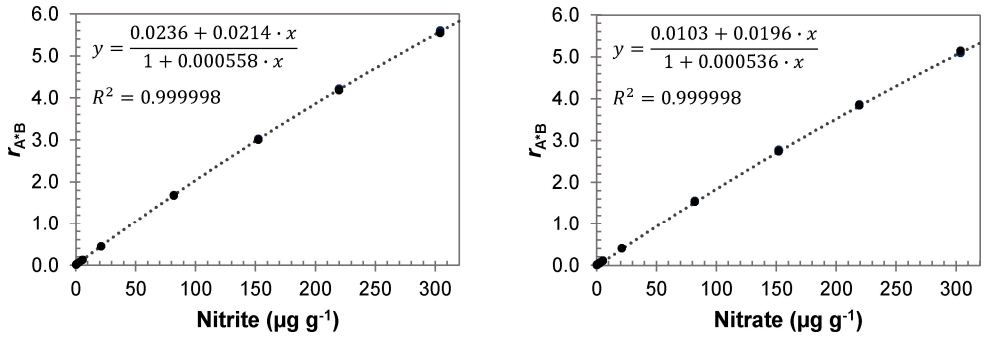

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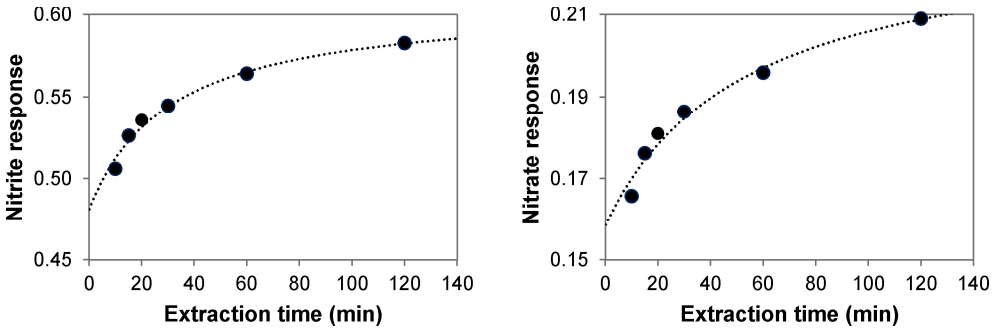

Figure 3 Increase of the analytical response in function of the extraction time. 4.0 g ground smoked ham was mixed with 20.0 g of internal standard solution following incubation at 70 ± 1 °C. The isotope ratio was measured at different time

Table 1 Determination of nitrite (μg g^{-1}) in processed meat products without prior desiccation

Sample matrix	**PCI GC/MS**[a]	**EI GC/MS**[b]	**Ion chromatography**[c]
	proposed method	Pagliano *et al.*, 2011	Conductivity, UV
Sausage	0.80 (0.07)	<LOD	N/D
Smoked sausage A	3.65 (0.05)	3.3 (0.1)	4.2 (0.7)
Smoked sausage B	3.35 (0.10)	3.7 (0.1)	4.8 (1.6)
Smoked salami	0.90 (0.03)	<LOD	N/D
Salami	1.04 (0.03)	<LOD	N/D
Chicken liver	4.29 (0.11)	3.7 (0.3)	N/D
Pepperoni A	1.91 (0.14)	1.7 (0.4)	N/D
Pepperoni B	2.61 (0.06)	<LOD	N/D
Smoked ham	24.58 (0.07)	24.1 (0.5)	23.2 (2.0)
Baked ham	11.43 (0.13)	10.9 (0.4)	9.7 (0.3)
Smoked bacon	1.33 (0.05)	<LOD	2.4 (0.9)
Bacon	0.27 (0.05)	<LOD	N/D
Prosciutto	<LOD	<LOD	N/D
Capocollo	1.59 (0.05)	1.7 (0.1)	N/D
Bologna sausage	29.30 (0.05)	29.7 (1.1)	28.5 (2.6)

N/D = not detected

[a] Proposed isotope dilution PCI GC/MS method. The results are the average from three independent sample preparations (standard deviation reported in parenthesis)

[b] Isotope dilution EI GC/MS method from Pagliano *et al.*, 2011. The results are the average from three independent sample preparations (standard deviation reported in parenthesis)

[c] Ion chromatography control method. One extracted sample was measured by conductivity and UV (210 nm) detection. The average is reported along with the absolute difference between the two detection approaches (in parenthesis)

Table 2 Determination of nitrate (μg g^{-1}) in processed meat products without prior desiccation

Sample matrix	**PCI GC/MS**[(a)]	**EI GC/MS**[(b)]	**Ion chromatography**[(c)]
	proposed method	Pagliano *et al.*, 2011	Conductivity, UV
Sausage	5.3 (0.1)	5.5 (0.1)	N/D
Smoked sausage A	30.7 (0.3)	31.0 (0.4)	50.8 (0.0)
Smoked sausage B	17.4 (0.2)	17.6 (0.1)	35.0 (1.7)
Smoked salami	17.2 (0.1)	17.2 (0.1)	36.9 (0.0)
Salami	<LOD	<LOD	18.2 (1.2)
Chicken liver	29.8 (0.1)	29.7 (0.1)	99.1 (0.7)
Pepperoni A	40.6 (2.4)	41.1 (2.2)	54.2 (0.9)
Pepperoni B	53.8 (0.4)	53.9 (0.6)	64.3 (1.5)
Smoked ham	8.5 (0.1)	8.7 (0.1)	29.0 (3.3)
Baked ham	8.6 (0.1)	8.6 (0.1)	24.1 (0.0)
Smoked bacon	10.9 (0.1)	10.7 (0.1)	30.4 (0.0)
Bacon	2.3 (0.2)	2.6 (0.2)	22.9 (2.2)
Prosciutto	<LOD	<LOD	N/D
Capocollo	2.5 (0.1)	2.9 (0.1)	16.3 (1.6)
Bologna sausage	16.6 (0.2)	17.0 (0.1)	34.5 (4.6)

N/D = not detected

[(a)] Proposed isotope dilution PCI GC/MS method. The results are the average from three independent sample preparations (standard deviation reported in parenthesis)

[(b)] Isotope dilution EI GC/MS method from Pagliano *et al.*, 2011. The results are the average from three independent sample preparations (standard deviation reported in parenthesis)

[(c)] Ion chromatography control method. One extracted sample was measured by conductivity and UV (210 nm) detection. The average is reported along with the absolute difference between the two detection approaches (in parenthesis)

Table 3 Results from a nitrite and nitrate three-level spike recovery test on three meat products

Sample matrix	Nitrite (μg g^{-1})		Nitrate (μg g^{-1})	
	Added	Recovered	Added	Recovered
Bacon	10.5	10.4 (99.1%)	10.5	10.5 (100.6%)
Bacon	41.1	41.0 (99.6%)	41.2	41.5 (100.5%)
Bacon	126.1	125.8 (98.2%)	126.0	126.3 (100.2%)
Pepperoni	10.5	10.3 (98.2%)	10.5	9.5 (90.2%)
Pepperoni	40.9	40.6 (99.3%)	41.0	38.0 (92.6%)
Pepperoni	107.9	108.3 (100.3%)	107.9	106.6 (98.8%)
Smoked sausage	10.5	10.5 (100.1%)	10.5	10.7 (102.0%)
Smoked sausage	40.2	40.0 (99.4%)	40.4	40.4 (100.0%)
Smoked sausage	111.7	111.3 (99.7%)	111.7	111.4 (99.8%)

Table 4 Determination of nitrite and nitrate on the freeze-dried meat samples.

Sample matrix	Nitrite (μg g^{-1})[a]		Nitrate (μg g^{-1})[a]	
	NO_2^- (μg g^{-1})	Recovery (%)	NO_3^- (μg g^{-1})	Recovery (%)
Bologna sausage	31.12	106%	19.2	116%
Smoked ham	25.82	105%	9.7	114%
Baked ham	11.50	101%	9.7	113%
Pepperoni	4.91	188%	66.9	124%
Smoked sausage	4.11	113%	35.9	117%

[a] Values are reported on wet-basis and recovery was calculated with reference to Tables 1-2