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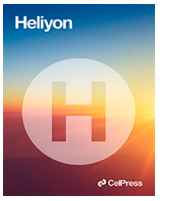

Research article

Volatile N-nitrosamines in Spanish commercial meat products and in fermented sausages prepared with different ingoing amounts of nitrate and nitrite

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ABSTRACT

Spanish commercial dry fermented sausages and dry hams, manufactured with and without nitrate and/or nitrite have been screened for volatile N-nitrosamine (VNA) content. VNAs have been also quantified in experimental fermented sausages prepared with known ingoing amounts of curing salts. Solid phase microextraction followed by tandem quadrupole gas chromatography/mass spectrometry (GC-QQQ-MS) analysis allowed the identification and quantification of 8 VNAs, 5 of which were detected in the samples. The highest concentration of VNAs found in the commercial products was 5.4 μg/kg. The most frequently detected VNAs were N-nitrosodiphenylamine and N-nitrosopyrrolidine. Principal component analysis and cluster analysis did not show correlation between the content of VNAs and the use of nitrate/nitrite in the formula. In the experimental sausages N-nitrosodiphenylamine and N-nitrosopyrrolidine were only detected (0.55 μg/kg total concentration) when 150 mg/kg of both nitrate and nitrite were added to the formula without any antioxidant. The levels of VNAs detected in this study are similar to those reported in the literature in different fermented meat products and dry hams.

1. Introduction

Sodium and potassium nitrate and nitrite are curing additives traditionally used in meat products. In the case of uncooked cured meats, these additives develop an important role as antimicrobial agents, among other functions. In particular, they inhibit pathogenic bacteria such as *Clostridium botulinum* and *Listeria monocytogenes* [1,2]. However, the use of these additives has been controversial for decades due to their involvement in the formation of N-nitroso compounds. In the year 2018, the International Agency for Research on Cancer (IARC) published a monograph evaluating the carcinogenic risks associated with the consumption of processed meats, and recommended limiting their intake due to their association to colorectal cancer [3]. This carcinogenic effect has been related, among other compounds, to the presence of N-nitrosamines and/or nitrite in meat products [4].

N-nitrosamines include volatile (VNAs) and non-volatile (NVNAs) amines [5]. VNAs are more potent carcinogens than NVNAs, although their amounts are usually much lower. The VNAs most commonly found in meat are N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP) [6]. NDMA and NDEA are classified as

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Group 2A carcinogens (probably carcinogenic) to humans, whereas other VNAs that are also present in meat products are classified as Group 2B (possibly carcinogenic) [7]. NDEA appears to be the most potent carcinogen among VNAs, followed by NDMA, NPYR and NPIP [8–10]. Only a few countries have established maximum levels of N-nitrosamines in meat products, either for total content or for individual N-nitrosamines, such as 3 μg/kg for processed meats in China [11], 10 μg/kg for bacon in the USA [12], 10–15 μg/kg in general for meat products in Canada [13], and 30 μg/kg for cecina in Chile [14]. At the moment, in the European Union there is no available legislation regulating the presence of N-nitrosamines in food or drinking water [15].

Nitrosation reactions are influenced by the composition and manufacturing process of the product, through factors such as pH, temperature, ingredients and the course of ripening. When added to meat, nitrite is present in a highly pH-dependent equilibrium with nitrous acid. Under strongly acidic conditions, the concentration of nitrous acid increases, and turns into highly nitrosating species, such as nitric oxide [16]. Optimum pH for N-nitrosamine formation is 2.5–3.5, which favors the production of nitrous acid and nitric oxide, while amines are still in their non-protonated form, thus being more reactive [13]. Compounds such as ascorbate and erythorbate are well known nitrosation inhibitors, as they readily bind nitric oxide, and therefore, they are used to reduce N-nitrosamine formation in meat products [17,18]. Nitrosation is significantly accelerated at temperatures above 130 °C, as it occurs in fried bacon and grilled or fried sausages [17,19]. On the other hand, Iammarino et al. [20] showed in a study using different cooking techniques on bacon and fresh sausages that heat can also influence the oxidation state of nitrite and therefore the potential for nitrosamine formation.

The concern regarding the use of nitrate and nitrite due to the risk of N-nitrosamine formation has led regulations to become

Table 1
Type of curing salt and antioxidants included in the formula of the commercial products as indicated on the label.

Product/brand	KNO_3	$NaNO_2$	Antioxidants
Salami 1	+	+	Sodium ascorbate
Salami 2	+	+	Sodium erythorbate, Rosemary extract
Salami 3	+	+	Sodium erythorbate, Rosemary extract
Salami 4	–	+	Sodium ascorbate
Salami 5	+	+	Sodium ascorbate
Salami 6	+	+	Sodium ascorbate
Chorizo 1	+	+	Sodium ascorbate, Sodium citrate
Chorizo 2	+	+	–
Chorizo 3	+	+	Sodium ascorbate
Chorizo 4	+	–	Sodium ascorbate
Chorizo 5	+	+	Sodium ascorbate
Chorizo 6	–	+	Sodium ascorbate
Chorizo 7	–	–	–
Chorizo 8	–	–	–
Chorizo 9	–	–	–
Chorizo 10	–	–	–
Chorizo 11	–	–	–
Chorizo 12	–	–	–
Salchichón 1	+	+	Sodium erythorbate
Salchichón 2	+	+	Sodium ascorbate
Salchichón 3	+	+	Sodium erythorbate
Salchichón 4	+	+	Sodium ascorbate
Salchichón 5	+	+	Sodium ascorbate
Salchichón 6	+	+	Sodium erythorbate/Vegetable extracts
Salchichón 7	–	–	–
Salchichón 8	–	–	–
Salchichón 9	–	–	–
Salchichón 10	–	–	–
Salchichón 11	–	–	–
Fuet 1	+	–	Sodium ascorbate
Fuet 2	+	–	Sodium ascorbate, Sodium citrate
Fuet 3	+	+	Sodium erythorbate
Fuet 4	+	+	Sodium erythorbate
Fuet 5	+	–	Sodium lactate, Rosemary extract
Fuet 6	–	+	Sodium ascorbate
Dry ham 1	+	+	Sodium ascorbate
Dry ham 2	+	+	Sodium ascorbate
Dry ham 3	+	+	Sodium ascorbate
Dry ham 4	+	+	Sodium erythorbate
Dry ham 5	+	+	–
Dry ham 6	+	+	Sodium ascorbate
Dry ham 7	–	–	–
Dry ham 8	–	–	–
Dry ham 9	–	–	–
Dry ham 10	–	–	–
Dry ham 11	–	–	–
Dry ham 12	–	–	–

progressively more restrictive. In the European Union, as a result of a scientific opinion by EFSA [21], regulations changed the term "indicative ingoing amount" by "maximum ingoing amount" and established limits for addition. EU Regulation 1333/2008 on food additives and EU Regulation 1129/2011 establishing the additive list limited the addition of nitrate and nitrite to dry fermented products to 150 mg/kg each (or 250 mg/kg of nitrate in long-ripening products when no nitrite is added). This background led to a re-evaluation of the use of nitrate and nitrite as food additives. In 2017, EFSA published two scientific opinions, concluding that the maximum ingoing amounts currently authorized in meat are safe for consumers, but recommended further studies on the levels of nitroso compounds formed in different meat products with known ingoing amounts of nitrate/nitrite [22,23]. Although in the re-evaluations of nitrate and nitrite, the formation of N-nitrosamines in the body from curing salts added at the approved levels to meat products is considered of low concern for human health, meat and meat products are considered the main food category contributing to N-nitrosamine exposure from foods [15]. New EU Regulation 2023/2108 on the use of nitrate and nitrite has established a reduction of the maximum ingoing amounts of these additives by October 2025, specifically 80 mg/kg of nitrite expressed as NO_2 ion and 90 mg/kg of nitrate expressed as NO_3 ion.

Spain is a traditional producer of dry fermented sausages and dry hams. Of the total national production, more than 70,000 tons of sausages and 48,000 tons of hams are exported to European countries, the USA and China, among others [24]. The aim of this study was to assess the concentration of VNAs in the most popular types of fermented sausages and dry hams in the Spanish market. In addition, since there is limited literature correlating VNA formation with known ingoing amounts of nitrate and nitrite, we have addressed this issue in a dry fermented sausage.

2. Materials and methods

2.1. Samples

2.1.1. Commercial products

A selection of different meat products (n = 47) among the most commonly consumed in Spain were purchased in different retail markets. Different brands of each type of product were selected: salami (6), fuet (6), salchichón (11), chorizo (12) and dry ham (12). Two units of each brand and product were purchased. All salami and fuet samples contained curing salts in their formula, while salchichón, chorizo and dry ham were available with and without nitrate/nitrite. The formulation of the products is shown in Table 1. The most usual formula was a mixture of potassium nitrate, sodium nitrite and sodium ascorbate.

2.1.2. Experimental sausages

Spanish salchichón was manufactured according to the traditional ingredients and fermentation-ripening parameters of Mediterranean products [25–27]. Nine batches were prepared with different concentrations of potassium nitrate and sodium nitrite (0, 75, 100, 125 and 150 mg/kg each), and with (500 mg/kg) and without sodium ascorbate. The levels of nitrate and nitrite were set according to the current maximum ingoing amounts allowed by EU Regulation 1129/2011 (150 mg/kg each) and progressive reductions to investigate the influence of these ingredients in the formation of VNAs. Two complete and independent sausage making experiments were performed on two different days. For each sausage preparation, two sausages were manufactured per batch and analyzed for the content of VNAs (n = 4).

The mixtures were prepared by grinding lean pork (70 %) and pork fat (30 %) in a mincer (Garhe MR-9, Amorebieta, Spain) equipped with an adjustable plate set at a hole diameter of 6 mm. After mixing, 2.5 % NaCl, 3 % lactose, 0.5 % dextrose, 0.25 % ground black pepper, 1 % water, and the selected concentrations of nitrate/nitrite, and ascorbate were added to the batter. The mixtures were also added with starter cultures: *Staphylococcus carnosus* and *Staphylococcus xylosus* (10^6 cfu/g) and *Lactobacillus fermentum* (10^7 cfu/g). Cultures were prepared from frozen stocks which were transferred to Tryptone Soy Broth (Pronadisa, Madrid, Spain) and pH 5.7 Man-Rogosa-Sharpe broth (Pronadisa) for staphylococci and *L. fermentum*, respectively. Tubes were incubated at 37 °C for 24 h. Then, grown cultures were subcultured under the same conditions until the stationary growth phase was reached. For inoculum preparation, subcultures were centrifuged at 10,000×*g* at 4 °C, 10 min; supernatants were discarded and the pellets were resuspended in sterile saline solution (0.85 % NaCl) to reach the target concentration.

The different batters were stuffed into 70 mm diameter collagen casings, obtaining pieces of approximately 200 g. The sausages were ripened in a climatic chamber Binder KMF 115 (Binder GmbH, Tuttlinger, Germany) with the following temperature and relative humidity (RH) program: 24 h at 19 °C and 87 % RH, 24 h at 15 °C and 83 % RH, and 28 days at 12 °C and 80 % RH. Samples were taken at the end of ripening.

For the physico-chemical characterization of the experimental sausages, pH was measured using a Hanna HI98161 pH-meter with a penetration electrode FC2023 (Hanna Instruments, Woonsocket, USA). Water activity was measured in sausage slices (3 mm thick) using a dew point hygrometer AquaLab 4 TE (Meter Group, Pullman, USA) at 25 °C. Moisture content was determined by dehydration at 100 °C until constant weight. All parameters were measured by triplicate.

2.2. Reagents and materials for nitrosamine analysis

A standard mix solution of 9 VNAs in methanol (EPA 8270/Appendix IX Nitrosamines Mix), at a concentration of 2000 μg/mL each, was purchased from Sigma Aldrich (St. Louis, MO, USA). This solution contained N-nitrosodibutylamine (NDBA), N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), N-nitrosodiphenylamine (NDPhA), N-nitrosodipropylamine (NDPA), N-nitrosomethylethylamine (NMEA), N-nitrosomorpholine (NMOR), N-nitrosopiperidine (NPIP) and N-nitrosopyrrolydine (NPYR). N-

nitrosopyrrolidine-d8 (NPYR-d8), at a concentration of 10 μg/mL in acetonitrile, was used as internal standard (Analytical Standard Solution, Saint Jean d'Illac, France).

A Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber (50/30 mm thickness, 2 cm length) was used for solid phase microextraction (SPME) (Supelco, Bellefonte, PA, USA). Before analysis the fiber was preconditioned in the injection port of the gas chromatograph at 270 °C for 3 h, according to the manufacturer's instructions.

2.3. Nitrosamine extraction

VNAs were extracted and analyzed according to Sun et al. [28] with some modifications. One hundred grams of each product were ground in a mincer, and 2 g aliquots were transferred to a headspace glass vial (15 mL), to which 7 mL of distilled water and 100 μL of NPYR-d8 (1 mg/L) were added. The vials were sealed with a PTFE/silicone septum (Supelco) and placed on a CombiPAL autosampler (Agilent Technologies Santa Clara, CA, USA) for shaking at 400 rpm, and incubated at 65 °C for 10 min. Afterwards, the SPME fiber was exposed to the headspace for 30 min while maintaining the sample at 65 °C.

2.4. GC-MS analysis

The analytes adsorbed by the fiber were desorbed at 250 °C for 10 min in the injection port of an Agilent 8890 gas chromatograph fitted to an Agilent 7000C triple quadrupole mass spectrometer (GC-QQQ-MS). The column used was a HP-5MS UI (5 % phenylmethylpolysiloxane) (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent). The oven temperature was programmed at 35 °C for 3 min, then increased to 200 °C at a rate of 7 °C/min, and then to 300 °C at a rate of 50 °C/min, followed by an isothermal period of 2 min. The carrier gas was helium at a flow rate of 1 mL/min. The mass spectrometer operated in electron impact mode with an electron energy of 70 eV. Detection was performed in Multiple Reaction Monitoring (MRM) mode. The transitions selected for each VNA and the optimal collision energies are listed in Table 2. Compounds were identified by comparing their mass spectra with those of authentic standards and those contained in the NIST20 (National Institute of Standards and Technology, Gaithersburg, MD) Mass Spectral Library, and also by comparison of their retention times with those obtained for the standards. The area of each peak was integrated using Mass Hunter workstation software (Agilent).

Calibration curves of VNA standards were prepared for the quantification of VNAs in the samples with concentration ranging from 0.025 to 10 μg/L. The method did not allow the quantification of NDMA. Other authors, using DVB/CAR/PDMS fiber for SPME, observed the poorest sensitivity for NDMA extraction [29]. On the other hand, Sun et al. [28] also obtained the highest limit of quantification for this VNA. The low sensitivity for NDMA could be due to its lower molecular mass, which could result in poorer mass spectral properties [30].

For the rest of VNAs, the limit of detection (LOD) and the limit of quantification (LOQ) were determined by a signal-to-noise ratio of 3:1 and 10:1 provided by Mass Hunter software, respectively (Table 2). Recovery and relative standard deviation (RSD%) were calculated using spiked samples of raw pork processed as described in 2.3. Samples (n = 3) were spiked with the standard mix solution of VNAs at two levels, 0.5 and 5 μg/kg. Recoveries ranged from 73 to 125 % and 71–102 % at the levels of 0.5 and 5 μg/kg, respectively. The RSD% values were below 15 %, except for NMOR at the lowest spike level and NPYR at the highest (Table 2).

Table 2
GC-QQQ-MS parameters of volatile N-nitrosamine analysis.

Compound	Retention time (min)	Precursor ion (*m/z*)	Collision energy (V)	Product ion (*m/z*)	LOD (μg/kg)	LOQ (μg/kg)	Spike level[a] 0.5 μg/kg		Spike level[a] 5 μg/kg	
							Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
NMEA	7.0	88	4 3	71 72	0.12	0.40	91	5.9	86	0.8
NDEA	8.8	102	4 12	85 44	0.23	0.76	100	9.6	83	2.5
NPYR	12.8	100	7 10	70 43	0.12	0.40	95	4.5	102	16.7
NMOR	12.9	116	2 15	86 56	1.16	3.83	125	29.7	84	10.4
NDPA	13.0	130 101	10 10	43 70	0.23	0.76	84	6.9	92	5.4
NPIP	13.7	114	7 25	84 55	0.52	1.72	114	5.8	79	5.7
NDBA	17.1	158	10 10	141 99	0.06	0.20	73	9.0	71	10.7
NDPhA	23.4	199 170	10 10	169 168	0.09	0.30	106	6.7	96	8.2

[a] Calculated in raw pork (n = 3).

2.5. Data processing

Data from commercial products underwent preprocessing prior to further analysis. This preprocessing was performed using the R programming language [31]. Autoscaling was applied to the data, standardizing each variable to have a mean of 0 and a standard deviation of 1. Additionally, all values that fell below the LOD were replaced with half the value of the LOD.

Following preprocessing, cluster analysis was conducted by the Gap Statistic method, which is widely accepted for determining the optimal number of clusters in a dataset [32]. The optimal number of clusters was determined using the 'factoextra' package in R [33].

A Principal Component Analysis (PCA) was performed subsequent to the cluster analysis. This data elaboration was also executed using the 'factoextra' package in R. The PCA generated a comprehensive view of the underlying structure in the dataset, allowing for the identification of patterns and associations. As a part of this analysis, confidence ellipses were constructed in the PCA bi-plot, each encompassing 50 % of the samples within the respective clusters.

Table 3
Volatile N-nitrosamine content (μg/kg) in different Spanish meat products[a].

Product/brand	NO_3 and/or NO_2	NDEA	NPYR	NPIP	NDBA	NDPhA	Sum of NAs
Salami 1	Cured	ND	ND	ND	ND	4.40 ± 0.27	4.40 ± 0.27
Salami 2	Cured	ND	ND	ND	ND	1.30 ± 0.11	1.30 ± 0.11
Salami 3	Cured	ND	4.40 ± 0.16	ND	ND	ND	4.40 ± 0.16
Salami 4	Cured	ND	ND	ND	ND	ND	ND
Salami 5	Cured	0.29 ± 0.10	ND	ND	ND	ND	0.29 ± 0.10
Salami 6	Cured	ND	ND	ND	ND	ND	ND
Chorizo 1	Cured	ND	ND	ND	ND	1.80 ± 0.17	1.80 ± 0.17
Chorizo 2	Cured	ND	1.50 ± 0.10	ND	ND	1.01 ± 0.17	2.51 ± 0.13
Chorizo 3	Cured	ND	0.56 ± 0.11	ND	0.30 ± 0.11	0.93 ± 0.20	1.79 ± 0.14
Chorizo 4	Cured	ND	ND	ND	ND	0.87 ± 0.11	0.87 ± 0.11
Chorizo 5	Cured	ND	ND	ND	ND	ND	ND
Chorizo 6	Cured	ND	ND	ND	ND	ND	ND
Chorizo 7	Uncured	ND	ND	ND	ND	ND	ND
Chorizo 8	Uncured	ND	ND	ND	ND	1.76 ± 0.0.7	1.76 ± 0.07
Chorizo 9	Uncured	ND	ND	ND	ND	ND	ND
Chorizo 10	Uncured	ND	ND	ND	ND	ND	ND
Chorizo 11	Uncured	ND	ND	ND	ND	ND	ND
Chorizo 12	Uncured	ND	ND	ND	ND	ND	ND
Salchichón 1	Cured	ND	ND	1.42 ± 0.16	ND	0.98 ± 0.08	2.40 ± 0.12
Salchichón 2	Cured	ND	1.27 ± 0.06	ND	ND	ND	1.27 ± 0.06
Salchichón 3	Cured	ND	ND	ND	ND	0.93 ± 0.08	0.93 ± 0.08
Salchichón 4	Cured	ND	0.50 ± 0.14	ND	0.41 ± 0.08	0.93 ± 0.10	1.84 ± 0.11
Salchichón 5	Cured	ND	ND	ND	ND	ND	ND
Salchichón 6	Cured	ND	0.30 ± 0.07	ND	ND	ND	0.30 ± 0.07
Salchichón 7	Uncured	ND	ND	ND	ND	1.76 ± 0.13	1.76 ± 0.13
Salchichón 8	Uncured	ND	ND	ND	1.22 ± 0.14	1.01 ± 0.17	2.23 ± 0.16
Salchichón 9	Uncured	ND	0.58 ± 0.08	ND	ND	ND	0.58 ± 0.08
Salchichón 10	Uncured	ND	0.31 ± 0.07	ND	ND	ND	0.31 ± 0.07
Salchichón 11	Uncured	ND	ND	ND	ND	ND	ND
Fuet 1	Cured	ND	ND	ND	ND	5.41 ± 0.40	5.41 ± 0.40
Fuet 2	Cured	ND	1.91 ± 0.16	ND	ND	0.98 ± 0.14	2.89 ± 0.15
Fuet 3	Cured	ND	4.42 ± 0.24	ND	ND	ND	4.42 ± 0.24
Fuet 4	Cured	ND	ND	ND	ND	ND	ND
Fuet 5	Cured	ND	1.10 ± 0.10	ND	0.94 ± 0.18	1.01 ± 0.08	3.05 ± 0.12
Fuet 6	Cured	ND	ND	ND	ND	ND	ND
Dry ham 1	Cured	ND	0.87 ± 0.07	ND	ND	2.20 ± 0.13	3.07 ± 0.10
Dry ham 2	Cured	ND	0.44 ± 0.08	1.33 ± 0.08	ND	0.90 ± 0.18	2.67 ± 0.12
Dry ham 3	Cured	ND	3.18 ± 0.25	ND	ND	ND	3.18 ± 0.25
Dry ham 4	Cured	ND	ND	0.90 ± 0.14	ND	ND	0.90 ± 0.14
Dry ham 5	Cured	0.30 ± 0.07	1.48 ± 0.13	ND	ND	ND	1.78 ± 0.10
Dry ham 6	Cured	ND	ND	ND	ND	ND	ND
Dry ham 7	Uncured	ND	2.72 ± 0.17	ND	ND	1.21 ± 0.10	3.93 ± 0.13
Dry ham 8	Uncured	ND	ND	1.33 ± 0.13	ND	0.90 ± 0.14	2.23 ± 0.13
Dry ham 9	Uncured	ND	ND	ND	ND	ND	ND
Dry ham 10	Uncured	ND	ND	ND	ND	3.31 ± 0.16	3.31 ± 0.16
Dry ham 11	Uncured	0.87 ± 0.06	ND	ND	ND	ND	0.87 ± 0.06
Dry ham 12	Uncured	ND	ND	ND	ND	ND	ND

[a] Each value is the mean of two different product units ± the standard deviation.

3. Results and discussion

3.1. Volatile N-nitrosamine content in commercial meat products

Different methods can be used for the analysis of VNAs, some of which are complex, time-consuming and generate high amounts of solvent residues. Recently, SPME extraction is gaining interest, since it combines simplicity, high sensitivity and cleanliness. The DVB/CAR/PDMS fiber permitted the quantification of eight VNAs (Table 2), and five VNAs were detected in the samples: NDEA, NPYR, NPIP, NDBA and NDPhA (Table 3).

3.1.1. VNAs in fermented sausages

Table 3 shows the VNAs detected in the sausages samples. NPYR and NDPhA were the most frequently detected VNAs, in 31 and 43 % of the samples, respectively, while NDBA was detected in 4 samples, and NDEA and NPIP only in one of them. The highest levels corresponded to NDPhA, with a maximum concentration of 5.4 μg/kg, and NPYR with 4.4 μg/kg, whereas the maximum levels for the other VNAs were lower. Thus, NDEA, which is considered the most potent carcinogen among VNAs, was only detected in one salami sample at a level of 0.29 μg/kg. As it will be further discussed, this VNA was not frequently detected in a significant percentage of fermented sausages analyzed by other authors, although, if present, concentrations up to 4.1 μg/kg have been reported [34]. On the other hand, NPIP and NDBA were detected at maximum levels of 1.4 μg/kg and 1.2 μg/kg, respectively.

The total concentration of detected VNAs per sample ranged from 0.29 to 5.4 μg/kg, the latter corresponding to a fuet sample in which only NDPhA was found. No VNAs were detected in 13 sausages, 7 cured and 6 uncured, corresponding to 37 % of the commercial samples analyzed in this study. All the sausages analyzed were raw and ripened, and are typically consumed without any heat treatment. Chorizo typically contains garlic and paprika, while salami, fuet, and salchichón are seasoned with black pepper. Cured and uncured varieties of chorizo and salchichón were investigated, while salami and fuet were not available in their uncured versions. Chorizo, salchichón and salami are typically high-acid fermented products (pH usually equal or below 5.0), while fuet has a smaller diameter (<30–40 mm) and higher pH (5.3–6.2) [26].

Various studies have been recently conducted in several countries on the content of VNAs in different meat products. Some of these studies have been carried out in Denmark, which national provisions regarding the use of nitrite in meat products are more stringent than those of EU Regulation 1333/2008, setting a maximum ingoing amount of 100 mg/kg of nitrite in salami instead of 150 mg/kg [35]. Herrmann et al. [36] in salami, chorizo and pepperoni available in the Danish market detected NPYR in 42 % of the samples (mean value of 2.1 μg/kg), NDEA in 25 % (mean value of 0.3 μg/kg) and NPIP in 21 % (mean value of 0.1 μg/kg). These concentrations are similar to the data reported in our study for NPYR and NDEA, and higher for NPIP. The presence of NPYR and NPIP in meat products is attributed to spices like black pepper and paprika, which contain precursor alkaloids such as pyrrolidine and piperidine [6]. In our study, NPYR was more frequently detected in products manufactured with black pepper (salami, salchichón and fuet) than in chorizo, which is seasoned with paprika. NPIP was only detected in a cured salchichón. In meat products from the Danish market, Niklas et al. [5] found a higher content of black pepper derived VNAs in salami. NPIP levels ranged from 0.3 to 2.9 μg/kg, and the concentration of NPYR ranged from not detected to 4.4 μg/kg.

Studies have also been carried out in Belgium. De Mey et al. [37] in different North and South European dry fermented sausages available in the Belgian market, did not detect VNAs in 53 % of the samples analyzed, while when they were detected, the total amount was lower than 5.5 μg/kg, except for one sample. This level is similar to that observed in our study for fermented sausages. The dominant VNA was NPIP, which was detected in 28 % of the samples, while NPYR was rarely detected (3 %), and NDEA was not detected at all. On the other hand, in the above mentioned study, Herrmann et al. [36] also analyzed fermented sausages commercialized in Belgium, and NPYR and NPIP were detected in 78 % of the samples, at mean concentrations of 2.7 and 0.3 μg/kg, respectively. After comparing Danish and Belgian products, Hermann et al. [36] highlighted the lack of clear differences in their nitrosamine content, despite the more restrictive legislation in Denmark regarding the maximum permitted levels of nitrate and nitrite.

Yurchenko and Mölder [38] quantified VNAs in salamis purchased in the Estonian market, accounting for a total mean content of 3.9 μg/kg. NPYR showed the highest levels (0.93 μg/kg), while NPIP was detected at the lowest concentration (0.64 μg/kg). These authors also indicated a relationship between the presence of both VNAs and the use of spices in meat processing.

Fermented sausages from the Turkish market have also been screened for VNA content by different authors. In a study on sucuk, salami and other fermented sausages, Ozel et al. [39] found a total concentration ranging from 0.63 to 4.7 μg/kg. As for individual VNAs, NDEA, NPYR and NPIP ranged from not detected to a maximum of 1.7, 1.4 and 2.7 μg/kg, respectively. NDBA was not detected in 56 % of the samples and the concentration was in most cases below 0.56 μg/kg. However, Ozbay and Sireli [34] detected NDBA in all the samples of different salami varieties at levels of 0.3–4.6 μg/kg, as well as NPIP, at 0.25–7.8 μg/kg. NDEA and NPYR ranged from not detected to approximately 4.1 and 5.7 μg/kg, respectively. In a recent study on sucuk, Kizilkaya et al. [19] reported a maximum total VNA content of 1.5 μg/kg, with NPIP levels below 0.95 μg/kg, whereas NDEA, NPYR and NDBA were not detected.

Finally, in China, where the maximum ingoing amount of nitrite is established in 150 mg/kg, Wang et al. [11] found NDBA, NPYR and NDPhA in more than 85 % short-time fermented sausage samples. NDPhA levels reached a maximum of 12.3 μg/kg, higher than the concentration observed in our study. Almost 30 % of the samples analyzed by these authors showed a total VNA concentration higher than 10 μg/kg.

The different results reported in the literature, both qualitative and quantitative, may be partly explained by factors intrinsic to the products, such as formulation and processing. However, as recommended by the EFSA Panel on Contaminants in the Food Chain, there is a need to standardize a sensitive analytical method for the quantification of N-nitrosamines in foods in view of the implementation of official controls [15].

3.1.2. VNAs in dry hams

Cured and uncured dry hams were also analyzed in our study. No VNAs were detected in 25 % of the products. NPYR and NDPhA were detected in 5 out of 12 samples (42 %), whereas NDEA and NPIP were detected in 2 and 3 products, respectively (Table 3). NDBA was not detected in any ham sample. NPYR was detected at a maximum level of 3.2 μg/kg, NDPhA at 3.3 μg/kg, NPIP at 1.3 μg/kg, and NDEA at 0.87 μg/kg. VNAs were not detected in 3 hams, corresponding to one cured and two uncured. The maximum total content detected in hams was 3.9 μg/kg in an uncured product.

There are fewer studies in the literature dealing with the study of VNAs in dry cured hams. Herrmann et al. [36] analyzed dry and cooked ham samples commercialized in Denmark. Their results did not differentiate among both types of ham, but NPYR was the most frequently detected VNA (7 out of 8 samples) at a mean level of 1.2 μg/kg. Also in cooked and dry hams from the Belgian market, NPYR was found in 71 % of the samples at a mean concentration of 1.5 μg/kg. Again, when comparing Danish and Belgian products, Hermann et al. [36] did not observe clear differences in their VNA content, despite the more restrictive legislation in Denmark.

3.1.3. Principal Component Analysis

The PCA applied to the dataset extracted two principal components (PC) that accounted for 46.3 % of the total variance (PC1: 24 %, PC2: 22.3 %). The first principal component (PC1) had significant contributions from variables NPYR and NDPhA, while the second

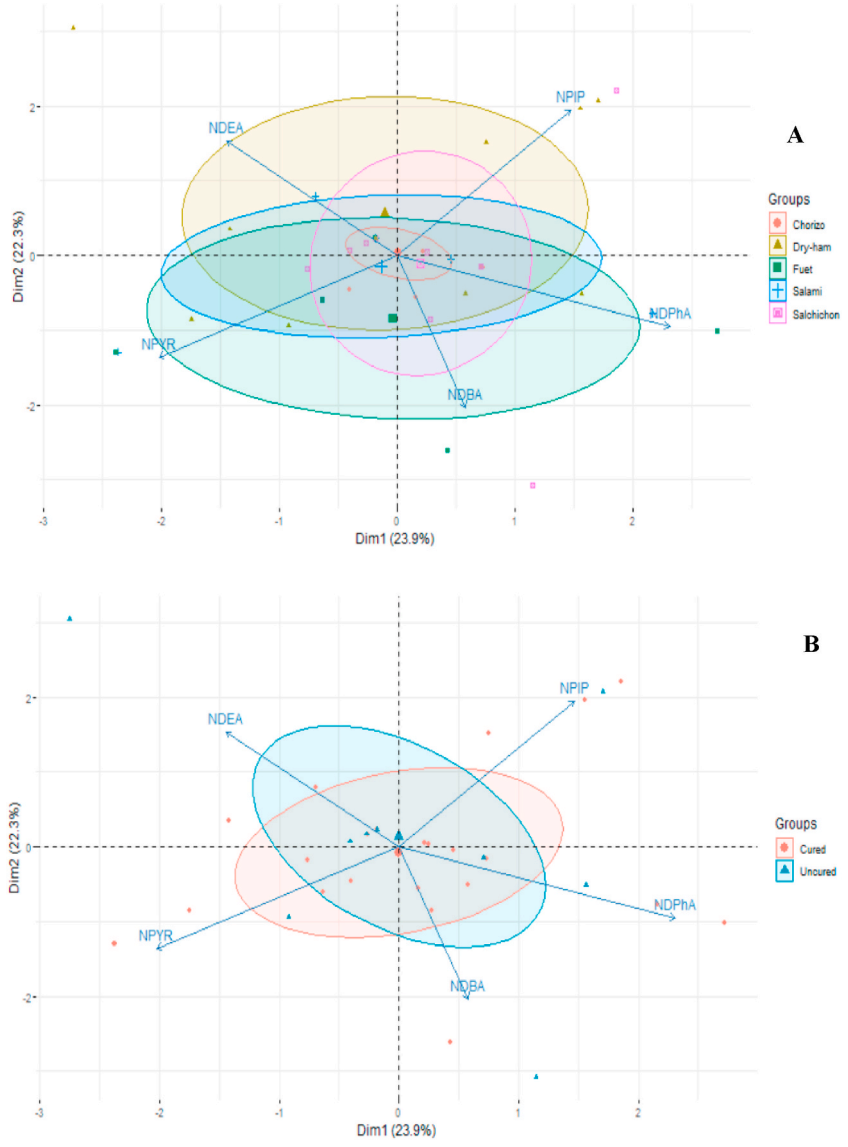

Fig. 1. Principal component analysis of meat samples using the variables NDEA, NPYR, NPIP, NDBA and NDPhA. Each point in the plot represents an individual sample. Different colors are used to differentiate the type of product (A) or the presence/absence of curing salts (B). Ellipses encompass 50 % of the samples within each cluster.

principal component (PC2) was largely influenced by NDBA and NPIP [Fig. 1(A and B)]. The analysis revealed two distinct clusters, with one corresponding to salami 1, fuet 1 and uncured dry ham 10. All three products shared a commonality of high NDPhA levels compared to other samples in the dataset.

No clear correlations were observed when trying to group samples based on the presence/absence of nitrate/nitrite in the formula or on the product type. Similarly, despite the variance explained by the PCA, the differentiation between product types based on the variables could not be clearly established. The detection of VNAs in uncured meat products could be explained by the natural presence of nitrate and nitrite in the ingredients. In this way, Iacumin et al. [40] reported threshold values of 42, 28 and 24 mg/kg for naturally present nitrate, and 14, 10 and 6 mg/kg for nitrite in pork meat, pork fat and salt, respectively. On the other hand, in cured products, added nitrite rapidly binds to different meat components, mainly proteins, including myoglobin, and also lipids [41], which would limit the formation of nitrosamines, together with the presence of ascorbate or erythorbate in formula.

3.2. Physico-chemical parameters of the experimental sausages

The initial pH of the sausage batter was approximately 5.7–5.8 and decreased along ripening to 4.7–4.8. The a_w was reduced from initial values around 0.96 to approximately 0.86–0.87 in the final product. The moisture content at the end of ripening was 34–35 % (data not shown). No significant differences ($p > 0.05$) were found for any of these parameters among batches.

3.3. Volatile N-nitrosamine content in experimental sausages

Some authors have investigated the correlation between residual nitrate and/or nitrite and N-nitrosamine formation in cured meat products, showing no or limited correlation [5,19,36,37]. In our study we have investigated the reciprocal relationship between VNA concentration and the ingoing amounts of nitrate and nitrite.

Table 4 shows the VNA content in the experimental sausages manufactured with different concentrations of nitrate, nitrite and sodium ascorbate. Only the sausages with the maximum nitrate and nitrite concentration (150 mg/kg), and without ascorbate, showed detectable levels of some VNAs, that is 0.35 μg/kg of NPYR and 0.20 μg/kg of NDPhA. Therefore, no correlation could be established since we did not detect VNA presence in the majority of the batches. The protective effect of ascorbate was observed at the highest ingoing amounts of nitrate and nitrite. As mentioned, in sausages nitrite enters into a dynamic equilibrium, highly dependent on pH, with nitrous acid (HNO_2). Before fermentation takes place, at pH 5.5, nitrite is mainly found as nitrite anion (NO_2^-), but the pH decrease caused by lactic acid bacteria promotes the conversion of NO_2^- to HNO_2 [17]. Under acidic conditions, HNO_2 is unstable and easily decomposed to different nitrosating compounds. The presence of ascorbate favors the formation of nitric oxide (NO), while its absence promotes the formation of nitrosonium ion (NO^+), which is a more potent nitrosating agent [42].

There are not many studies in the literature relating VNA levels with the ingoing amounts of nitrate and nitrite. Sun et al. [43] studied the VNA profile in Harbin type sausages added with 90 mg/kg of nitrite and 1000 mg/kg ascorbate, and found 4 VNAs, NDEA, NPIP, NDPA and NDPhA, being the last one the most abundant by far, with levels up to 1600 μg/kg. These concentrations could be considered too high for a dry fermented sausage that was fermented and dried at ambient temperature (25 °C) during 9 days and not subjected to any thermal treatment.

Xiao et al. [44] studied the VNA profile of a dry fermented sausage prepared with 150 mg/kg of nitrite and without ascorbate or spices. The authors found NDMA, NDEA, and NPYR, which maximum content after 20 days of ripening were 1.2, 0.9 and 1.7 μg/kg, respectively, and accounting for a total VNA final content of 3.8 μg/kg. Despite the sausages prepared by Xiao et al. [44] did not contain spices, NPYR was the most abundant VNA. The formula also included wine, in which NDMA and NDBA have been detected at levels of 4.7–12.9 and 0.7–3.8 μg/L, respectively [45].

Finally, in a recent work, Hu et al. [46] detected 5 VNAs in fermented sausages manufactured with 150 mg/kg of nitrite and 500 mg/kg of ascorbate, at the end of a 2 week ripening period, with a total content ranging from 3.4 to 3.7 μg/kg, depending on the starter cultures used. NPYR was not detected and NDPhA levels reached 0.69 μg/kg, slightly higher than the concentrations detected in our study in the sausage batch in which VNAs were detected.

Table 4
Volatile N-nitrosamine content (mean ± standard deviation) in dry fermented sausages manufactured with different concentrations (mg/kg) of nitrate, nitrite and sodium ascorbate. The results are expressed as μg/kg.

Batch	KNO_3	$NaNO_2$	Ascorbate	NDEA	NPYR	NPIP	NDBA	NDPhA
1	150	150	500	–	–	–	–	–
2	150	150	0	–	0.35 ± 0.07	–	–	0.20 ± 0.04
3	125	125	500	–	–	–	–	–
4	125	125	0	–	–	–	–	–
5	100	100	500	–	–	–	–	–
6	100	100	0	–	–	–	–	–
7	75	75	500	–	–	–	–	–
8	75	75	0	–	–	–	–	–
9	0	0	0	–	–	–	–	–

4. Conclusion

The VNA levels detected in the commercial products analyzed in this study can be considered low and comparable to those reported in the literature in different fermented meats and dry hams. The highest content was 5.4 μg/kg in a sausage. The most frequently detected VNAs were NPYR and NDPhA. We did not observe a clear correlation between the presence/absence of nitrate/nitrite and the VNA content in the commercial products. No correlation could be established either with the ingoing amounts of nitrate/nitrite in experimental fermented sausages, since only the addition of 150 mg/kg of these additives in the absence of sodium ascorbate would lead to VNA formation at low levels.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Xavier F. Hospital: Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Manuela Fernández:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Paloma Morales:** Funding acquisition, Formal analysis. **Claudio Alba:** Formal analysis. **Ana I. Haza:** Funding acquisition, Formal analysis. **Eva Hierro:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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