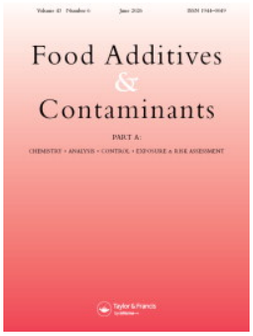

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Comparison of *N*-nitrosamine formation mechanisms in vacuum and modified atmosphere packaged sausages using mathematical modelling

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ABSTRACT

This study investigated the comparative effects of vacuum and modified atmosphere packaging (MAP; 70% CO_2 + 30% N_2) on the formation dynamics of seven volatile N-nitrosamines (N-nitrosodimethylamine (NDMA), N-nitrosomethylethylamine (NMEA), N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP), and N-nitrosodibutylamine (NDBA)) in sausages during 12 weeks of refrigerated storage (4 °C). Weekly Gas Chromatography-Mass Spectrometry (GC-MS) analysis combined with two-way variance analysis (ANOVA) revealed that storage duration significantly increased all target nitrosamine concentrations ($p < .005$). Beyond standard statistical evaluation, a kinetic modelling approach was employed to elucidate the distinct reaction mechanisms driven by packaging atmospheres. The results demonstrated that the packaging type fundamentally dictated the formation kinetics. Notably, NDMA followed an "Acidity Controlled Delayed Growth" model under MAP, exhibiting a critical lag phase of 2.1 weeks (t_{lag}) before accumulation commenced, whereas it remained below detection limits in vacuum packages. Furthermore, NPYR exhibited "Exponential Acceleration" kinetics in MAP, contrasting with the "Rapid Equilibrium" behaviour observed for NDBA and NDEA under vacuum conditions. These models achieved high determination coefficients ($R^2 > 0.90$), mathematically demonstrating that packaging atmospheres shift underlying reaction pathways—from rapid saturation in vacuum to acid-catalyzed exponential growth in MAP. These findings underscore the necessity of evaluating nitrosamine dynamics within specific packaging contexts to ensure the chemical safety of processed meat products.

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Volatile N-nitrosamines; processed meat; process contaminants; GC-MS; kinetic modelling

Introduction

Nitrate and nitrite, crucial additives in processed meat products, play significant roles in shaping the diverse chemical characteristics within these products. Processing methods such as curing and smoking, in particular, contribute to the formation of N-nitroso compounds (NAs) (Domingo and Nadal 2017). Nitrite serves critical functions in processed meats, including colour development, stabilisation, and notably, as an important antimicrobial agent against *C. botulinum* (Perea-Sanz et al. 2020). However, the use of nitrite inevitably leads to the formation of N-nitrosamines (NAs), which are recognised as chemicals posing risks to consumer health (Chaudhary et al. 2025). These compounds are known to emerge particularly when cured, salted, or emulsified meat products are fried or grilled (Yurchenko and Mölder 2007; Özbay 2022b).

The formation of NAs is a highly complex process, not solely confined to processing methods. As noted by Rywotycki (2003), NAs can be present even in untreated and nitrite-free meats; factors such as animal species, dietary patterns, and seasonal variations can influence NA levels in raw meats (Rywotycki 2003). In processed meat products, NA formation is influenced by a multitude of complex chemical reactions occurring during processes including cooking, storage, packaging, and production (Özbay & Sireli, 2022). Numerous processed meat products, such as sucuk, smoked or grilled meat, sausages, salami, and kebabs, have been the subject of studies concerning NA formation (Ozel et al. 2010; Sannino

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and Bolzoni 2013; Herrmann et al. 2015; Sallan et al. 2020; Özbay 2022a). Owing to the wide variability in products, compositions, and processing techniques, significant research has been conducted on diverse meat products and production methods across the world.

From a health risk perspective, specific N-nitrosamine (NA) derivatives, including N-nitro sodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitroso-di-*n*-propylamine (NDPA), N-nitrosodibutylamine (NDBA), N-nitrosomethyle thylamine (NMEA), nitrosopyrrolidine (NPYR), and nitrosopiperidine (NPIP), are of particular concern due to their carcinogenic effects. The U.S. Environmental Protection Agency (EPA) has drawn attention to the health effects of these derivatives, emphasising that foetuses, infants, and children represent more sensitive groups regarding their mutagenic effects (EPA 2016). Consequently, monitoring NA levels in foods and assessing compliance with limits set by organisations such as the World Health Organisation (WHO) (e.g. 10 µg/kg) is important for public health (Cintya et al. 2019; Abdullah et al. 2022).

Beyond production and cooking processes, it is recognised that the health risks associated with NAs persist during storage, highlighting the critical importance of this phase (Li et al. 2024). Storage duration and conditions significantly influence NA formation. For instance, in a study examining traditional blood and liver sausages, it was reported that total NA levels, which were undetectable or very low on the day of purchase, increased substantially after one day of storage at room temperature (to 4.0 µg/kg for blood sausage and 6.7 µg/kg for liver sausage) (Domańska-Blicharz et al. 2004). Similarly, sausages from Belgian markets were found to contain high levels of NPIP at the end of their shelf life (specifically 12.3 µg/kg in 'Pepper Salami') (De Mey et al. 2014). During the maturation process, increases in the amounts of NAs such as NDMA, NDEA, and NPYR have also been reported, with this increase linked to chemical and microbiological reactions occurring during the process (Xiao et al. 2018).

Packaging methods and atmospheric conditions are also critical factors affecting NA formation. Ahn et al. (2002) stored sausages irradiated at different doses under both aerobic and vacuum conditions. While NDMA was not detected after 4 weeks in vacuum-packaged sausages irradiated at 20 kGy, 4.7 µg/kg of NDMA was formed in sausages stored under aerobic conditions under otherwise similar treatment. In the same study, non-irradiated sausages stored aerobically contained 24.9 µg/kg of NPYR, whereas the NPYR level decreased to 3.3 µg/kg in sausages irradiated at 5 kGy and stored under identical conditions. These findings demonstrate that both the packaging atmosphere (vacuum vs. aerobic) and protective treatments (irradiation) significantly influence NA formation (Ahn et al. 2002).

The existing literature establishes that storage duration and packaging conditions exert significant effects on NA levels in processed meat products. However, the complex and often poorly understood mechanisms of NA formation are frequently highlighted. In this context, understanding how the packaging atmosphere modulates the complex interactions governing the formation pathways of specific NAs in processed meat products is of considerable importance.

Therefore, this study aims to compare the effects of vacuum and Modified Atmosphere Packaging (MAP; 70% CO_2 + 30% N_2) conditions on the formation profiles and levels of various volatile N-nitrosamines (NDMA, NMEA, NDEA, NDPA, NPYR, NPIP, NDBA) in sausages during a 12-week shelf life under refrigerated storage. Accordingly, the primary objective of this research is to investigate the mathematical model and statistically significant differences in the formation patterns and quantities of specific N-nitrosamines during storage under vacuum versus MAP packaging. Concurrently, discussing the potential underlying reasons for these observed differences constitutes another key objective of the study.

Materials and methods

Chemicals and standards

All reagents utilised in the analytical procedures were of analytical grade purity or higher. A certified standard mixture of nitrosamines (EPA 521 Nitrosamine Mix, 2000 ppm in dichloromethane; from Sigma-Aldrich, USA). This standard

contained N-nitrosodimethylamine (NDMA), N-nitrosodibutylamine (NDBA), N-nitrosodi-*n*-propylamine (NDPA), N-nitrosomethylethylamine (NMEA), N-nitrosodiethylamine (NDEA), nitrosopyrrolidine (NPYR), and nitrosopiperidine (NPIP). Considering the established potential carcinogenicity of N-nitrosamines, requisite personal protective equipment (PPE), specifically masks and gloves, was employed throughout the study duration. All chemical waste generated during the experimental process was disposed of in an environmentally sound manner, managed under the supervision of a certified waste disposal company. Working standard solutions for calibration were prepared at various concentrations *via* serial dilution of the stock standard using dichloromethane (for gas chromatography; Merck, Germany) and subsequently stored at −18 °C until required for analysis.

Samples

Experimental sausage samples for this investigation were sourced from the Ankara Meat and Milk Institution, Sincan Meat Plant facility, where they were produced under controlled conditions. The experimental sausage batter (mix) was formulated with the following ingredients: fatty beef (77.8%), ice (15.5%), starch (3.9%), fine salt (1.2%), nitrite-containing salt (0.4%), polyphosphates (0.31%), red paprika (0.31%), black pepper (0.19%), white pepper (0.12%), coriander (0.09%), smoke essence (0.06%), ginger (0.06%), food-grade colourant (0.013%), and ascorbic acid (500 ppm). The experimental sausages were of the scalded-emulsified type, produced following standard commercial protocols. The process involved the preparation of a fine meat emulsion (batter), followed by sequential thermal treatments including drying (55 °C, 24 min), smoking (60 °C, 16 min), and multiple scalding/cooking stages (70–75 °C) until a final core temperature of 72–75 °C was reached. Subsequent to standard production, the obtained samples were divided and assigned to two distinct packaging treatments: vacuum packaging and Modified Atmosphere Packaging (MAP) utilising a gas mixture of 70% CO_2 and 30% N_2. Transportation to the laboratory was conducted under refrigerated conditions (4 °C) using an in-vehicle refrigeration unit. Sampling occurred on a weekly basis, commencing from the day of production (week 0) and continuing throughout the designated 12-week shelf-life period. This regime yielded a total of 26 experimental packages (13 MAP and 13 vacuum-packaged units). The experimental design involved two packaging factors and thirteen time points, with independent analyses for each condition conducted in triplicate. This resulted in a total dataset comprising 78 analytical results for each individual nitrosamine analyte. Throughout the 12-week study, sausage packages assigned for each specific week were kept under refrigeration at 4 °C until the day of extraction. Once the extraction procedure was completed, the resulting organic extracts were stored in a freezer at −18 °C to ensure chemical stability until the GC-MS analysis was performed

Methods

Determination of N-nitrosamines

The extraction of N-nitrosamines (NAs) was performed according to the methodology previously described by Özbay and Şireli (Özbay & Sireli, 2021). Sausage samples were subjected to comminution and homogenisation using a Philips blender (HR1316/00, Istanbul, Turkey). A precise aliquot of 20 g from each homogenised sample was weighed using a KERN precision balance (ABJ 220–4NM, Ballingen, Germany) and subsequently transferred into individual beakers.

To each prepared sample, 40 mL of dichloromethane (DClM) (Merck, Dichloromethane for gas chromatography, Darmstadt, Germany) was added. The resulting mixtures were subjected to ultrasonic treatment for 15 min in a VWR ultrasonic water bath (USC-TH, Leicestershire, England). Following sonication, the samples were filtered through S & H Labware filter paper (Ø125 mm, Ankara, Turkey), and the filtrate was collected. The collected filtrate was then subjected to a secondary extraction with an additional 40 mL of DClM in the ultrasonic water bath for another 15 min. The combined filtrate from these steps was subsequently concentrated using a rotary evaporator (Heidolph, Hei-VAP Advantage, Schwabach, Germany) operated at 30 °C to

remove the DClM solvent. The resultant residue (extract) was reconstituted in 1 mL of methanol (Merck, Methanol for chromatography, Darmstadt, Germany), filtered through a 0.45 µm polyethersulfone (PES) syringe filter (Sartorius Stedim, Gottingen, Germany), and transferred into glass autosampler vials.

Prior to analysis, the sample extracts within the vials were homogenised using a vortex mixer (VELP Scientifica, Velata, Italy). The vials were then sealed, wrapped with parafilm, and stored under refrigeration until gas chromatography-mass spectrometry (GC-MS) analysis. Volatile N-nitrosamine analysis was carried out using an Agilent Technologies 7890 A Gas Chromatograph coupled to an Agilent Technologies 5975 C Mass Selective Detector (MSD) (Santa Clara, CA, USA). Chromatographic separation was achieved using a DB624 capillary column (30 m length, 0.25 mm internal diameter, 1.40 µm film thickness; Agilent, Santa Clara, CA, USA).

For quantification, a calibration curve was constructed using seven standard solutions meticulously prepared from the certified nitrosamine mixture (EPA 521 Nitrosamine Mix, 2000 ppm; Sigma-Aldrich, USA), covering a concentration range of 0.5 to 75 µg/L. The linearity of the response for the various NAs was confirmed, with correlation coefficients (R^2) determined to be between 0.9623 and 0.9895.

Method detection limits (LOD) and quantification limits (LOQ) were established. The minimum detectable concentration for each analyte was identified, and ten replicate measurements were performed at this concentration. The LOD was calculated as three times the standard deviation (SD) of these measurements, while the LOQ was calculated as ten times the SD. The calculated LOD values for the target volatile N-nitrosamines (VNAs) ranged from 0.06 to 0.17 µg/L.

Method accuracy was evaluated through a recovery experiment. A sample matrix previously determined to have low endogenous VNA content was selected and fortified with standard solutions at three concentration levels (2, 4, and 6 ng/g, equivalent to ppb). The observed recoveries ranged from 87.7% to 95.8%. Method precision was assessed by determining reproducibility (% Relative Standard Deviation, %RSD). Ten replicate analyses were performed on a standard solution at a concentration of 50 ng/mL (ppb). The overall validation of the developed GC-MS method was performed in adherence to the principles outlined in the Eurachem method validation guidelines (Magnusson and Örnemark 2014).

Statistical analysis

Statistical evaluation of the experimental data was performed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA). A two-way Analysis of Variance (ANOVA) was employed to determine the main effects of packaging type (Vacuum vs. MAP) and storage time (0–12 weeks), as well as the significance of their interaction, on the measured concentrations of each of the seven target volatile nitrosamines. The experimental design involved two packaging factors and thirteen time points, with analyses for each condition conducted in triplicate. This resulted in a total dataset comprising 78 analytical results (13 time points × 2 packaging types × 3 replicates) for each individual nitrosamine analyte. Statistical significance was accepted at the $p < 005$ level.

Mathematical modeling methodology

In this study, a dynamic modelling approach was developed to simulate the formation dynamics of seven different N-nitrosamine (NA) species in sausages, utilising the interpolation of experimental data (Weeks 0–12) and kinetic assumptions based on literature. The modelling process consisted of two main stages: (1) Curve fitting analysis of experimental data, and (2) Predictive modelling of formation kinetics. This approach relies on the theoretical assumption - supported by established work - that the distinct physicochemical conditions (such as potential pH shifts and oxidation levels) and microbial dynamics induced by packaging type (Vacuum and MAP) specifically modulate NA formation rates. While these parameters were not directly measured in this study, they served as the mechanistic basis for selecting the specific kinetic models applied to the nitrosamine data.

The time-dependent concentration changes (C(t)) of the analysed NA species were defined

using the following kinetic functions, utilising the least squares method to minimise error (Labuza 1984; Van Boekel 2008; Atkins et al. 2014).

Formation kinetic models

Acidity controlled delayed growth model (NDMA)

It was hypothesised that NDMA formation is triggered only after the pH drops below a specific threshold. This behaviour, observed under MAP conditions, was modelled using a piecewise linear function with a lag phase:

$$CNDMA(t) = \begin{cases} \sim 0, & t \le tlag \\ kNDMA.(t - \text{tlag}), & t > tlag \end{cases}$$

Here t_{lag}, represents the critical time required for acidification to initiate the reaction, and k_{NDMA} represents the formation rate constant. Under vacuum conditions, concentrations were considered negligible as the theoretical t_{lag} exceeded the storage period.

Exponential acceleration model (NPYR)

The surge in NPYR, particularly observed in MAP packaging, exhibited kinetics accelerated by the catalytic effect of acidity. This behaviour was expressed by an exponential function consistent with first-order autocatalytic reaction kinetics:

$$CNPYR(t) = Cbase + \propto .e^{\beta.t}$$

Logarithmic saturation model (NPIP)

It was assumed that NPIP formation depends on specific precursors (e.g. piperidine from black pepper) present in limited quantities, and the reaction rate decreases to reach an equilibrium point as these precursors are depleted:

$$CNPIP(t) = C0 + A.\ln(1 + k.t)$$

Rapid equilibrium model (NDBA and NDEA)

The rapid initial increase followed by stabilisation observed in vacuum packaging was modelled as a fast equilibrium reaction occurring under non-oxidative conditions:

$$C(t) = Cmax.(1 - e^{-k.t})$$

Cumulative accumulation model (NMEA)

The steady increase in NMEA concentration was associated with the continuous release of precursors into the medium due to microbial proteolysis and decarboxylation activities throughout storage, defined by zero-order kinetics (linear increase):

$$CNMEA(t) = C0 + kacc.t$$

Late-stage critical surge model (NDPA)

A quadratic acceleration model was used to describe the sudden concentration spike observed in the late stages of storage (especially after Week 7 in Vacuum):

$$CNDPA(t) = \begin{cases} Cbase, & t < tcrit \\ Cbase + \gamma.(t - tcrit)^2, & t \ge tcrit \end{cases}$$

Result and discussion

The present investigation evaluated the temporal concentration dynamics of seven volatile N-nitrosamines (NAs) in sausages subjected to either vacuum or Modified Atmosphere Packaging (MAP; 70% CO_2, 30% N_2) during refrigerated storage (4 °C) over a 12-week period. The analytical findings depicting these changes are illustrated in Figures 1 and 2, while the outcomes of the corresponding statistical analyses are summarised in Table 1.

General effects of storage time and packaging type

It is established that nitrosamines (NAs) can be found in processed meat products (Özbay & Sireli, 2021; Özbay 2022a; Sallan et al. 2023; Deveci and Tek 2024). The results of the statistical analysis (Table 1) indicate that storage time ('Week') exerted a highly significant ($p < .005$) increasing effect on the levels of all examined NAs. The graphs presented in Figures 1 and 2 corroborate this general upward trend. The increase in NA concentrations with prolonged

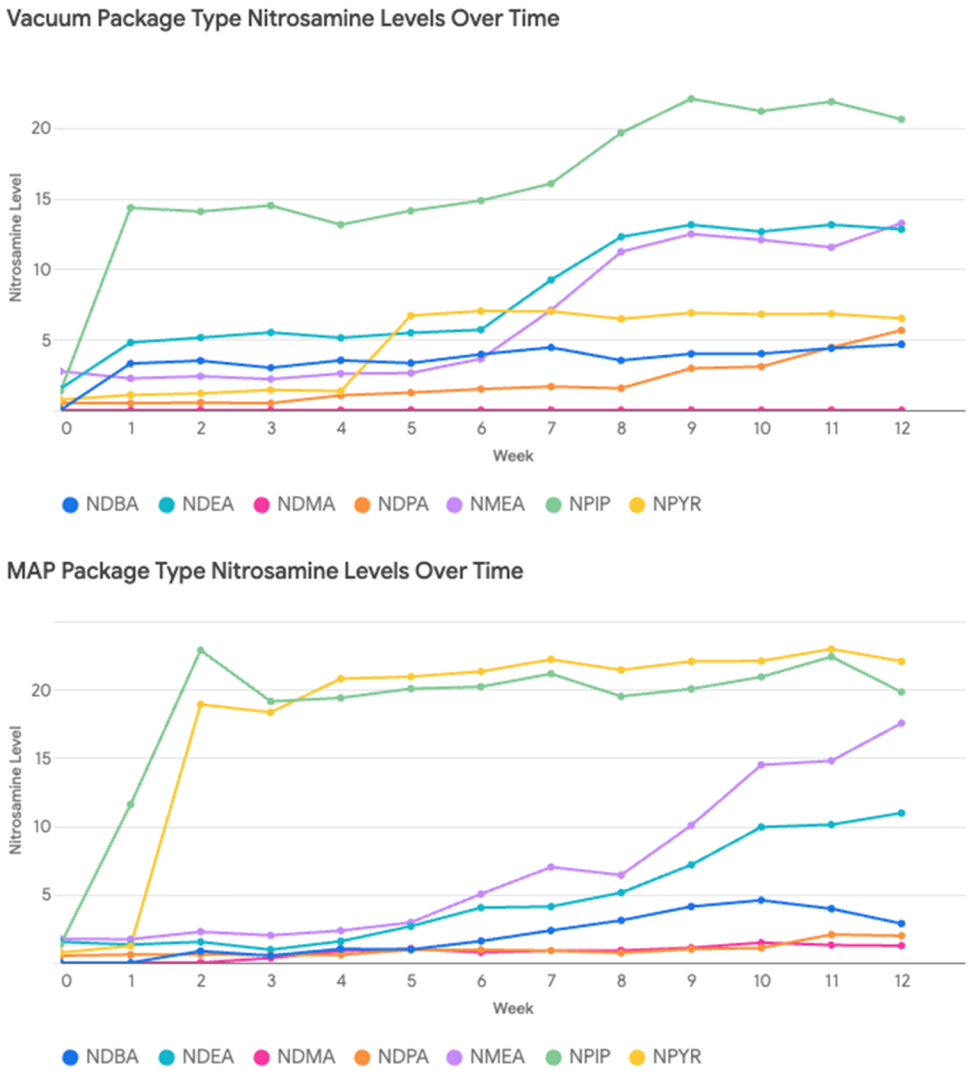

Figure 1. Temporal profiles of individual volatile N-nitrosamines (μg/kg) in sausages stored under vacuum and modified atmosphere packaging (MAP) over 12 weeks.

storage time is a well-documented finding in the literature (Domańska- Blicharz et al. 2004; Scheeren et al. 2015; Li et al. 2024). This increase is primarily attributed to ongoing chemical and microbiological reactions throughout the storage period (Xiao et al. 2018). Specifically, the formation or accumulation of amine precursors resulting from microbial activity (Domańska and Różańska 2003; Domańska- Blicharz et al. 2004) and the oxidation of the meat's own components (lipids, proteins) (De Mey et al. 2017), followed by their reaction with nitrosating agents, contribute to this rise. NA formation has even been observed in presumably sterile canned products depending on time and temperature (Jurak et al. 2013), highlighting the critical role of the storage process itself in NA formation dynamics (Li et al. 2024). It has also been reported that N-nitrosamine levels increase with the amount of added sodium nitrite and may be further enhanced during processes such as roasting or *in vitro* digestion (Niklas et al. 2023).

Packaging type ('Package') was also observed to have a statistically significant effect on NA formation (Table 1, $p < .005$, except for NMEA and NPIP). Examination of Figures 1 and 2 clearly reveals that the NA profiles in vacuum and MAP packaging exhibit distinct differences. This finding indicates that the packaging atmosphere affects NA formation by modifying the chemical and microbiological environment within the product (Özbay & Sireli, 2022). For instance, a study by Ahn et al. (2002) found no NDMA detection in vacuum-packaged irradiated

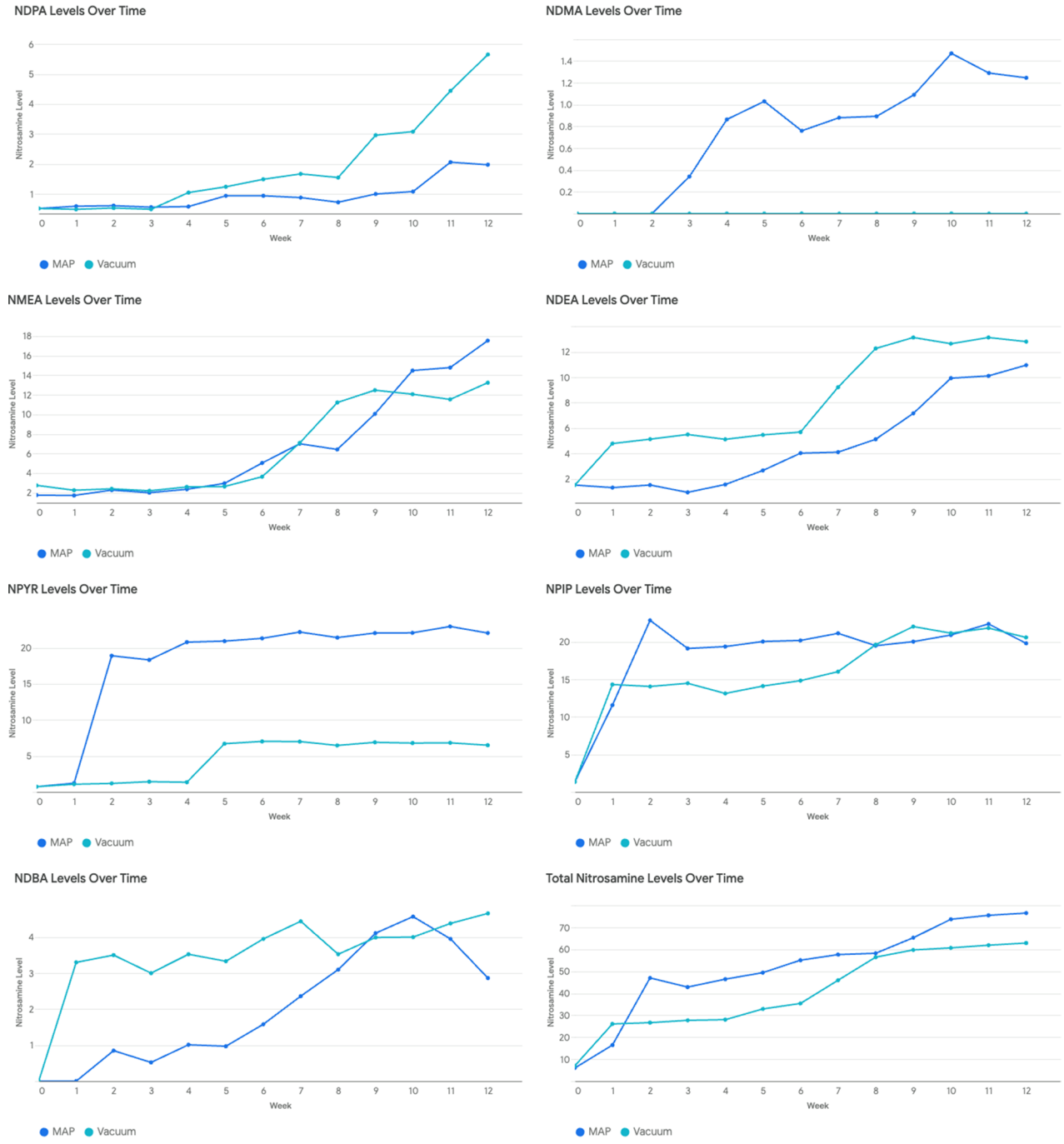

Figure 2. Comparative levels of specific and total volatile N-nitrosamines (µg/kg), highlighting differences between MAP and Vacuum packaging atmospheres.

sausages, whereas NDMA formation occurred in those stored under aerobic conditions. Similarly, it is expected that the MAP atmosphere containing high CO_2 (Rousset and Renerre 1991; Brown et al. 2018) and vacuum conditions promote the development of different microbial flora (Rousset and Renerre 1991; Yang et al. 2009) and lead to different chemical reaction pathways.

The interaction between storage time and packaging type ('Week * Package') was also found to be statistically significant for NDMA, NMEA, NDEA, NPYR, and NDBA (Table 1, $p < .005$). This implies that the packaging type differentially influences the rate of NA formation throughout the storage period. Statistical analysis indicated no significant interaction for NPIP (p=.792), but

Table 1. Statistical analysis results.

	NDMA	NMEA	NDEA	NDPA	NPYR	NPIP	NDBA	Total NAs
Week	.000	.000	.000	.000	.000	.000	.000	.000
Package	.000	.443	.000	.016	.000	.209	.000	.000
Week * Package	.000	.000	.000	.000	.000	.792	.019	.000

p-values < 0.005 are considered statistically significant.

a significant interaction was found for NDBA (p=.019). These interactions are also visually evident in the different slopes and intersections depicted in Figures 1 and 2.

Changes in individual nitrosamines and associated mechanisms

NPIP (nitrosopiperidine)

Our findings show that NPIP was among the NAs reaching the highest concentrations during storage in both vacuum and MAP-packaged sausages (Figure s 1 and 2). This aligns with numerous studies in the literature (De Mey et al. 2014; Scheeren et al. 2015). Piperidine, the primary precursor for NPIP, is known to originate frequently from black pepper used in meat product formulations (Domańska and Kowalski 2003; Yurchenko and Mölder 2007). Piperidine can react directly with the nitrosating agent to form NPIP. Furthermore, it has been suggested that cadaverine, formed from the bacterial decarboxylation of amino acids (e.g. lysine), can convert to piperidine through deamination and cyclisation, thereby contributing to NPIP formation (Sallan et al. 2023). In our study, the high initial NPIP level in vacuum packaging followed by an increase after week 7, compared to the more continuous increase in MAP packaging, suggests that formation kinetics might differ under varying conditions. Domańska and Kowalskı (2002) reported that even short-term storage at room temperature significantly increased NPIP levels (Domańska and Kowalskı 2002). Kaban et al. (2022) noted an association between NPIP formation, water activity (aw), and residual nitrite (Kaban et al. 2022).

NPYR (nitrosopyrrolidine)

NPYR was another NA reaching notable levels, particularly in MAP packaging (Figures 1 and 2). Precursors for NPYR include compounds such as proline, putrescine, and spermidine (De Mey et al. 2017). It is known that the proline content naturally present in meat can increase during storage (Gray and Collins 1977) and biogenic amines like putrescine/spermidine can be formed through microbial activity. Red pepper has also been reported as a potential source of NPYR precursors (Yurchenko and Mölder 2007). A decrease in pH has been reported to promote NPYR formation (Sallan et al. 2020; Kaban et al. 2022). This could explain the potentially higher NPYR levels in MAP packaging, which has the potential to lower pH due to dissolved CO_2 (Dermiki et al. 2008).

NDMA (nitrosodimethylamine)

One of the most striking differences in this study was observed for NDMA. While remaining below the limit of detection in vacuum packaging, NDMA was formed and increased over time in MAP packaging (Figure 2). This finding partially aligns with Ahn et al. (2002), who reported no NDMA formation under vacuum, but its formation under MAP conditions underscores the importance of the packaging atmosphere. Kaban et al. (2022) reported an association between NDMA formation and pH. In the same study, an increase in NDMA was observed during initial storage, followed by a significant decrease during extended storage, which was attributed to potential degradation by microbial activity, chemical transformation, or volatility. The continuous increase observed in MAP packaging in the present study might stem from differences in the product matrix or microbial flora. Increased NDMA formation during maturation has also been reported elsewhere (Xiao et al. 2018).

NDBA (nitrosodibutylamine)

NDBA levels remained relatively low in both packaging types and showed only a limited increase over time (Figure 2). This observation is fully consistent with the findings of Domańska and Kowalskı (2002), who reported low levels of NDBA in stored processed meat products with only a minor increase during storage.

NDPA (nitrosodi-n-propylamine)

In our study, NDPA levels remained lower compared to NPIP and NPYR and exhibited a slow increase over time (Figure 2). This finding contrasts with reports by Scheeren et al. (2015), who found high amounts of NDPA in some processed meats, and Ferysiuk and Wojciak (2020), who reported a decrease in NDPA during storage of canned pork (Ferysiuk and Wojciak 2020). These discrepancies may arise from differences in the product type studied, formulation, and analytical methodologies employed.

NMEA (nitrosomethylethylamine) & NDEA (nitrosodiethylamine)

These two NAs also demonstrated a gradual increase over time in both packaging types (Figures 1 and 2). These increases can be explained within the general framework of NA formation mechanisms, involving the generation and subsequent nitrosation of precursor amines during storage (Xiao et al. 2018).

Potential mechanisms during storage

Nitrosamine formation fundamentally occurs through the reaction of a nitrosating agent (typically HNO_2 or N_2O_3 derived from nitrite) with secondary or tertiary amines (Honikel 2008; Voice et al. 2015). The observed NA increases and profiles in this study are associated with complex mechanisms occurring during storage:

Presence and formation of precursor amines

Only a small fraction of the nitrite added to meat participates in NA formation, while the majority undergoes other transformations (Herrmann 2014). Amines required for NA formation can be naturally present in meat (peptides, free amino acids - Herrmann 2014) or can be formed or increase in quantity during storage through microbial activity (proteolysis - (Kieliszek et al. 2021), biogenic amine formation *via* amino acid decarboxylation - (De Mey et al. 2017; Sallan et al. 2023) or chemical degradation (lipid/protein oxidation - (De Mey et al. 2017); rancidity - (Combet et al. 2010). Furthermore, spices serve as significant sources of specific amine precursors (e.g. piperidine, pyrrolidine precursors) (Domańska and Kowalskı 2002; Yurchenko and Mölder 2007).

Chemical environment (pH, aw)

The pH of the medium significantly influences the rate of nitrosation reactions. Generally, acidic pH (optimum around pH 3.5) accelerates the reaction (Honikel 2008; De Mey et al. 2014). Lactic acid produced by starter cultures or indigenous microflora activity can lower the pH, potentially promoting NA formation (especially NPYR) (Sallan et al. 2020). Carbonic acid formed from high CO_2 levels in MAP can also reduce pH (Dermiki et al. 2008). A decrease in pH also facilitates the conversion of nitrite to nitric oxide (Sallan et al. 2023). Water activity (aw) has also been associated with the formation of certain NAs (e.g. NPIP) (Kaban et al. 2022).

Microbial activity

Microorganisms can increase NA levels by reducing nitrate to nitrite (Domańska and Różańska 2003; Domańska- Blicharz et al. 2004) or by directly producing NAs (Ayanaba and Alexander 1973). Concurrently, they contribute indirectly by generating precursor amines (as mentioned above). Conversely, certain microorganisms (especially Lactic Acid Bacteria - LAB) may also play a role in reducing NA formation by degrading nitrite or suppressing the growth of biogenic amine-producing bacteria (Perea-Sanz et al. 2020; Sallan et al. 2023). The packaging type dictates the developing microbial flora (LAB, Enterobacteriaceae, Pseudomonas, etc.), thereby influencing these effects (Rousset and Renerre 1991; Dermiki et al. 2008; Yang et al. 2009; Brown et al. 2018).

In summary, the general increase in NAs observed during storage likely initiates with the reaction of initial nitrite and amine precursors (from meat, spices). However, the continuation and potential acceleration of this increase are sustained by the ongoing enzymatic activity (proteolysis, decarboxylation) of the active microbial flora (especially LAB), which continuously generates new amine precursors (free amino acids, biogenic amines) that react with available nitrosating agents.

Furthermore, the distinct NA profiles between vacuum and MAP packaging primarily arise from

the different dominant microbial populations selected by the respective gas atmospheres and the differential effects of these atmospheres on the product's chemical environment (particularly pH and redox potential). The lower pH potentially induced by high CO_2 in MAP may favour faster nitrosation reactions under acidic conditions (e.g. NDMA, NPYR formation), whereas the strict anaerobic conditions under vacuum might limit the formation of certain precursors or specific microbial metabolisms, leading to a different NA profile (e.g. NDMA being < LOD).

Considering precursor availability, the generally higher levels of NPIP and NPYR compared to other NAs are likely directly related to the abundance of specific raw materials used in the formulation (e.g. black pepper containing piperidine - NPIP source) and/or specific precursors naturally present in the meat or formed during storage (e.g. proline, putrescine - NPYR source), as well as the susceptibility of these precursors to nitrosation. The presence of these dominant precursors plays a more decisive role in shaping the overall NA profile compared to other amines.

The study results suggest potential competition among common resources for the formation of NAs. The primary shared resource for the formation of all N-nitrosamines is the nitrosating agent derived from nitrite (HNO_2, N_2O_3, etc.). The total amount of nitrite is initially fixed and decreases over time through various reactions (binding to myoglobin, oxidation, NA formation, etc. (Herrmann 2014). If the total amount of different amine precursors exceeds the available nitrosating agent, or if the formation/regeneration rate of the nitrosating agent is slow, competition among amines for this agent will commence.

Concurrently, different amines react with the nitrosating agent at different rates. This rate depends on the amine's structure, ambient pH, temperature, and other factors. Amines that react faster (or are more abundant) consume the available nitrosating agent more quickly, leading to faster formation of their respective NAs. This situation may leave less nitrosating agent available for amines that react more slowly. The observation in this study that NPIP and NPYR generally form faster and reach higher levels (Figures 1 and 2) suggests that piperidine and pyrrolidine (or their precursors) are either more abundant (e.g. from the spice mix) or react faster with the nitrosating agent under these conditions. This rapid formation might indirectly limit the formation rate of slower-forming NAs like NDBA and NDPA, as they consume the common nitrosating agent resource more rapidly.

This implies that even if the nitrosating agent is not completely depleted or precursors are continuously regenerated over the 12-week period, potential still exists for the formation of all NAs. However, the clear dominance of NPIP and NPYR could be indicative of the aforementioned kinetic competition or precursor abundance. The slower increase of other NAs might be interpreted as them being "outcompeted" by reactions producing these dominant NAs.

In conclusion, kinetic competition, particularly for the common and potentially limited nitrosating agent, can be considered. This competition manifests not necessarily as a direct suppression where the increase of one NA causes a decrease in another, but rather as an influence on the relative formation rates of different NAs. Faster-forming or more abundant precursor NAs (like NPIP and NPYR in this study) utilise the nitrosating agent more efficiently, reaching higher levels, while potentially limiting the rate of increase for slower-forming NAs (like NDBA, NDPA). The data do not show complete suppression of one NA by another within this timeframe but suggest an interaction affecting their relative formation quantities and rates.

Mathematical modelling of N-nitrosamine formation kinetics

To elucidate the mechanisms driving the distinct NA profiles observed under Vacuum and MAP conditions, the experimental data were fitted to the kinetic models described in the methodology. The calculated kinetic parameters and R^2 are presented in Table 2. The high determination coefficients ($R^2 > 0.90$ for most models) indicate that the proposed mechanistic models successfully describe the NA formation behaviours.

Table 2. Calculated kinetic parameters and determination coefficients (R^2) for N-nitrosamine formation in sausages under Vacuum and MAP conditions.

Analyte	Model Applied	Packaging	Model Parameters (Calculated)	R^2
NDMA	Delayed Growth	MAP	t_{lag} = 2.1 weeks; k_{NDMA} = 0.14 µg/week	0.94
		Vacuum	No significant formation ($k \sim 0$)	–
NPYR	Exponential Acceleration	MAP	C_{base} = 1.2;α = 0.85; β = 0.42 $week^{-1}$	0.92
	Linear	Vacuum	k = 0.51 µg/week (Low rate)	0.89
NPIP	Logarithmic Saturation	MAP	C_0 = 1.5; A = 7.8; k = 0.9	0.96
	Logarithmic Saturation	Vacuum	C_0 = 1.8; A = 8.2; k = 0.7	0.95
NMEA	Linear Accumulation	MAP	k_{acc} = 1.45 µg/week	0.98
	Linear Accumulation	Vacuum	k_{acc} = 0.98 µg/week	0.97
NDPA	Quadratic Surge	Vacuum	tcrit = 7.0 weeks; γ 0.18	0.95
	Linear	MAP	k = 0.12 µg/week (Slow increase)	0.88
NDBA	Rapid Equilibrium	Vacuum	C_{max} = 3.5; k = 1.8 $week^{-1}$ (Fast saturation)	0.93
	Linear Accumulation	MAP	k = 0.35 µg/week	0.91
NDEA	Rapid Equilibrium	Vacuum	C_{max} = 5.2; k = 1.5 $week^{-1}$	0.94
	Linear Accumulation	MAP	k = 0.88 µg/week	0.96

Conclusions

This study confirms that N-nitrosamine (NA) formation during the refrigerated storage of sausages is a dynamic process, significantly influenced by both storage duration and packaging type. The application of kinetic modelling in this study provided a quantitative characterisation of these dynamic behaviours, mathematically describing the differing formation pathways with high determination coefficients. The distinct reaction orders observed—specifically the 'Rapid Equilibrium' model ($R^2 > 0.93$) describing NDBA and NDEA saturation in vacuum versus the 'Exponential Acceleration' of NPYR in MAP—mathematically demonstrate that the packaging atmosphere fundamentally alters reaction pathways, not just final concentrations

The observed variations are a reflection of the complex interplay between the sources and formation rates of precursor amines, the chemical characteristics of the product matrix (particularly pH), and shifts in the composition and activity of the microbial flora. Considering the inherent diversity among different meat products, formulations, and processing/storage conditions (Rywotycki 2003; Ozel et al. 2010; Sannino and Bolzoni 2013; Özbay & Sireli, 2022; Özbay 2022a), it underscores the importance of assessing the risks and underlying mechanisms of NA formation individually for each specific product type.

The findings of this study have important implications for the meat processing industry and food safety regulators. The observation that vacuum packaging significantly suppresses the formation of certain carcinogenic nitrosamines, such as NDMA, underscores the value of choosing appropriate packaging strategies to minimise chemical hazards during storage. The identification of a critical lag phase for NDMA formation in MAP provides a concrete scientific basis for adjusting 'Best Before' dates based on chemical safety thresholds rather than just sensory spoilage. This is further supported by the kinetic finding that NDMA formation in MAP exhibits a 'Delayed Growth' mechanism with a critical lag phase, a risk factor absent under vacuum conditions. This insight can guide manufacturers in selecting packaging methods that extend shelf life while also reducing potential health risks. Furthermore, the findings suggest that for products intended for long-term refrigerated storage (beyond 8 weeks), vacuum packaging may offer a superior safety profile by suppressing specific carcinogenic pathways that are otherwise triggered in modified atmospheres. Additionally, the study highlights the need for dynamic risk assessments that consider storage conditions and packaging type, rather than solely focusing on initial processing parameters.

These kinetic models can be integrated into HACCP (Hazard Analysis and Critical Control Points) programs as predictive tools for chemical risk management, allowing manufacturers to optimise packaging selection and nitrate/nitrite levels based on the intended shelf-life and storage conditions. Regulatory authorities may also consider packaging-specific guidelines when setting limits for nitrosamine levels in processed meats.

Although this study provides a detailed evaluation of VNA formation under two packaging conditions during refrigerated storage, several limitations should be acknowledged. First, the study focused solely on seven VNAs, excluding other possible nitrosamine species that may form under different storage or formulation scenarios. Second, no direct microbiological analyses were

conducted to confirm the proposed effects of packaging on microbial ecology, which were inferred based on the literature. Third, while the proposed kinetic models successfully fitted the experimental data, they are semi-empirical and require further validation across different temperatures to determine activation energies. Fourth, the results are specific to a particular sausage formulation and may not be directly generalisable to other meat products with different ingredients or processing conditions. Lastly, environmental factors such as temperature fluctuations and light exposure, which may also influence NA formation, were not varied and controlled under ideal laboratory conditions. It is important to note that the developed kinetic models aim to describe the formation of chemical contaminants (nitrosamines) rather than serving as general indicators of product spoilage. While this study focuses on chemical safety, it is acknowledged that nitrosamine kinetics are intrinsically linked to the product's evolving microflora and physicochemical state (pH and gas composition). The absence of real-time monitoring for these parameters is a limitation; however, the models were specifically selected to reflect the mechanistic shifts (e.g. acidification-driven lag phases) expected under different packaging atmospheres

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Author contributions

CRediT: **Sena Özbay**: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing.

Ethical approval

This research does not need ethical approval.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Data will be made available on request.

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