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# Detection of *p*-nitroaniline released from degradation of 4,4´-dinitrocarbanilide in chicken breast during thermal processing

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## ABSTRACT

The diphenylurea 4,4´-dinitrocarbanilide (DNC) is the residue of concern left in edible tissues of broilers fed diets containing the anticoccidial nicarbazin. When chicken meat is submitted to thermal processing, *p*-nitroaniline (*p*-NA) is expected from DNC degradation. Then, this work aimed at evaluating whether thermal processing of DNC-containing chicken meat induces *p*-NA appearance. First, a hydrolysis assay was performed in aqueous solutions at 100 °C in different pH, confirming that DNC cleavage yields *p*-NA. Then, a novel LC-MS/MS method was used to detect traces of this aromatic amine in DNC-containing chicken breast fillets subjected to cooking methods. Our evidences showed *p*-NA occurrence in such chicken meat samples, which corroborated results from hydrolysis assay. The *p*-NA appearance in fillets was rather discrete during boiling treatment, but its concentration became pronounced over time for grilling, frying and roasting, achieving respectively 326.3, 640.0 and 456.9 *μ*g/kg. As far as we are concerned, no other research identified degradation products from DNC residue in heat-processed chicken fillets. Therefore, this study leads to further approaches to assess impacts on food safety.

**Keywords:** nicarbazin, poultry, chicken fillet, DNC, *p*-NA, heat treatment, cooking method, LC-MS/MS

## INTRODUCTION

Coccidiosis is a common disease in poultry farming caused by protozoa from the *Eimeria* genus. The control of this pathogen is achieved by regular use of in-feed anticoccidials. However, concerns regarding residue deposition of these additives in chicken meat are often raised.[1]

Nicarbazin (NCZ) is one of the most commonly used anticoccidials in poultry feed.[2] NCZ comprises an equimolar complex of 4,4´-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethyl-pyrimidine (HDP).[3] When this additive is administered to broilers, the unchanged DNC accumulates in muscle tissues and organs[4,5], therefore it represents the NCZ marker residue analyzed in monitoring programs.

Although surveillance plans identified non-compliant samples for NCZ residue (> 200 $\mu$g/kg)[2,6], studies have shown the disappearance of incurred DNC in chicken fillets along thermal processing.[7,8] However, details behind parent DNC decay in heat-processed chicken meat remain unclear, especially with respect to the proper identification of the degradation products. For instance, while chicken meat is submitted to heat treatment, *p*-nitroaniline (*p*-NA) can be expected from DNC breakdown.[7,8]

The *p*-NA is claimed to cause cancer[9,10] and methemoglobinemia.[11–13] Its occurrence in cooked chicken tissues is a matter of concern. Therefore, as a sequence of the research underway in our laboratory, we focused on revisiting the DNC-containing cooked chicken samples[7] to detect, by liquid chromatography tandem mass spectrometry (LC-MS/MS), possible *p*-NA traces

released during thermal processing. As a secondary approach, we verified the *p*-NA formation from the DNC cleavage in aqueous solution.

## EXPERIMENTAL SECTION

### Hydrolysis Assay

*Reagents.* 4,4´-dinitrocarbanilide (97%) and *p*-nitroaniline (> 99%) were acquired from Sigma-Aldrich Co. (St Louis, MO, USA). All other reagents were of analytical grade.

*Solutions.* Stock solutions of DNC (600 *μ*mol/L) and *p*-NA (725 *μ*mol/L) were prepared in dimethylformamide (DMF). The following buffer solutions were prepared in ultrapure water: 50 mmol/L phthalate pH 2, 50 mmol/L phthalate pH 4, 50 mmol/L phosphate pH 6, 50 mmol/L phosphate pH 8, and 50 mmol/L borate pH 10. All buffers contained 1 mol/L KCl.

*General procedure.* DNC hydrolysis was performed at different pH (2, 4, 6, 8 and 10) at 100 °C during pre-established times (0, 2, 4, 6, 8 and 10 h). For each pH value, eighteen screw-cap 15 mL test tubes containing a boiling bead were used. Each tube contained 50 *μ*L of the DNC solution at 600 *μ*mol/L, 250 *μ*L of DMF and 2.7 mL of the respective buffer solution (initial concentration of DNC in assay was 10 *μ*mol/L). After vortexing the reaction mixture, the tubes were placed in a sand-bath, and subsequently heated in an oven at 100 °C. Then, at each set time, three test tubes were withdrawn and immediately transferred to an ice-water bath (5–10 °C) for quenching the reaction.

*Determination of p-NA.* The amount of the aromatic amine released from DNC hydrolysis was determined on the basis of the Bratton-Marshall diazotization according to a procedure adapted from previous studies.[14,15] Thus, after cooling the reaction mixture, the following reagents were successively added into the tubes: 500 $\mu$L of 2 mol/L HCl, 50 $\mu$L of 2% (w/v) $NaNO_2$ (aq.), 50 $\mu$L of 10% (w/v) sulfamic acid (aq.), and 200 $\mu$L of 0.1% (w/v) *N*-(naphthyl)-ethylene diamine dihydrochloride monomethanolate (aq.). The volume was adjusted to 5 mL, and the absorbance was measured at 545 nm on a Varian Cary 50 UV-VIS spectrophotometer. Based on this same procedure, *p*-NA calibration curves within the range of 3–40 $\mu$mol/L were prepared in each buffer for quantification of the amine ($R^2 > 0.99$).

*Degradation rate constant*. The pseudo first-order rate constants ($k_1$') for DNC hydrolytic degradation were detailed in Supporting Information file.

**Chicken Breast Samples**

This study was a complementary part of our recent research involving the fate of NCZ residues in chicken meat. All details upon broiler production, breast sampling, thermal processing, general cooking procedures, and sample preparation were previously described.[7]

*DNC-containing chicken breast fillets (Thermal processing experiment)*. From that same set of chicken breast samples earlier prepared, a total of 180 portions from experiment 1 (see subsection *Thermal Processing* in Bacila et al.[7]) were selected for *p*-NA analysis. Briefly, at that particular experiment, thermal processing of the chicken breast fillets (portions with 50–60 g) was accomplished by conventional cooking methods over set times, as follows: boiling at 5, 10, 15,

20 and 25 min; grilling at 15, 30, 45, 60 and 75 min; frying at 5, 10, 15, 20 and 25 min; and roasting at 15, 30, 45, 60 and 75 min. A total of six replicates (i.e., 6 breast fillet portions) were prepared for each set time of a cooking method. DNC-containing raw breast fillets (6 portions per cooking method) represented the initial condition (zero-time) of thermal processing experiment.

*DNC-free chicken breast fillets (control samples)*. Four skinless breast fillets (*pectoralis major* muscle) were collected from 42-old-day broilers fed diets without NCZ. Each chicken breast was divided into 6 portions (50–60 g). One by one, the portions were sorted by chance and assigned to a cooking method. Four portions, representing the control samples of each cooking method, were subjected to heating until the core temperature achieved 70 °C (boiling for 15 min; grilling for 60 min; frying for 15 min; roasting for 45 min). The remaining four raw portions represented the control samples without heat treatment (identical to the blank samples). All breast portions were freeze-dried according to the previous procedure of sample preparation.[7] Heat-processed control samples were analyzed to confirm that DNC is the only precursor of *p*-NA throughout thermal processing. Raw portions were analyzed to declare breast fillets as free of *p*-NA as well.

*DNC-free chicken breast fillets (blank samples)*: Six skinless breast fillets (*pectoralis major* muscle) were collected from 42-old-day broilers fed diets without NCZ. Chicken breasts were divided into 6 portions (50–60 g). After subjecting to the freeze-drying procedure[7], these samples declared as *p*-NA free were used for method validation.

## *p*-NA Determination in Chicken Breast by LC-MS/MS

*Reagents.* LiChrosolv® acetonitrile (ACN) was supplied by Merck (Darmstadt, Hessen, Germany). *p*-nitroaniline (> 99%), aniline (> 99.5%), and benzoyl chloride (99%) were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). All other reagents were of analytical grade.

*Solutions*. *p*-NA stock standard solution at 1,000 *μ*g/mL was prepared in ACN. Working solutions of *p*-NA were daily prepared at 100 and 10 *μ*g/mL by diluting the stock solution with ACN. Aniline used as internal standard (IS) was dissolved in ACN to result in a IS stock solution of 1,000 *μ*g/mL. The IS working solution was daily prepared at 10 *μ*g/mL by diluting IS stock solution with ACN. The derivatizing reagent was prepared by diluting 200 *μ*L of benzoyl chloride in 10 mL of ACN. All solutions were protected from light and stored at -20 °C.

*Extraction of chicken meat samples.* The Supporting Information (Figure S1) brings the details of the method steps in flow chart scheme. In order to extract *p*-NA, about 2.5 g of ground freeze-dried sample (equivalent to 4–10 g in wet basis) was weighed into a 50 mL conical polypropylene tube and fortified with 50 *μ*L IS working solution at 10 *μ*g/mL. Then, 20 mL of 0.5 mol/L perchloric acid solution (aq.) was added, and the tube was vigorously shaken in a vortex mixer for 30 s. Further, the tube was shaken for 30 min on a “wrist action” shaker. Centrifugation was carried out at 3500*g* and 20 °C for 10 min. The supernatant was filtered (nylon hydrophilic membrane, 33 mm, 0.45 *μ*m), and collected into another 50 mL conical tube for use in the later steps of liquid-liquid extraction and derivatization. Blank samples fortified at 500 *μ*g/kg were used as quality control (QC) in every batch of analysis. A total of 10 QC fortified samples were analyzed.

*Salting-out assisted liquid-liquid extraction (SALLE) and derivatization.* First, 5 mL of the extract were transferred into a 15 mL conical polypropylene tube already containing 1.5 g NaCl and 0.5 g Na-citrate dihydrate. After adding 0.5 mL of 5 mol/L NaOH (aq.), the tube was vigorously shaken by hand for salt dissolution. ACN (2 mL) was added, and the tube was shaken again. Phase separation was achieved by centrifugation at 4370*g* for 10 min at 20 °C. An aliquot of the upper ACN phase (500 *µ*L) was transferred to a 2 mL microtube containing the derivatizing reagent (50 *µ*L) and ACN (450 *µ*L). The solution was incubated in an oven at 40 °C for at least 15 hours (overnight) and then centrifuged at 15,000*g* for 5 min at 20 °C. After the derivatization step, 700 *µ*L of the solution were transferred to a 2 mL vial for injection into the LC-MS/MS. Determinations were performed in duplicate.

*LC-MS/MS determination.* The analyses were performed using a LC system Surveyor Plus (Thermo, USA). Separations were carried out in a Kinetex C18 100 Å analytical column (100 mm x 4.6 mm, 5 *µ*m pore size, Phenomenex) combined with a C18 guard column (SecurityGuard™ Ultra, Phenomenex). Column temperature: 30 °C. Injection volume: 10 *µ*L. A combination of two mobile phases (A and B) was used with a constant flow rate at 1.0 mL/min. Mobile phase A: water with 0.1% formic acid (v/v). Mobile phase B: ACN with 0.1% formic acid (v/v). Separations were achieved with a gradient program described as follows: 95% A (0–0.5 min), 30% A (0.5–6 min, maintained until 10 min), and 100% B (10–12 min, held until 13.5 min), 95% A (13.5–15 min). The mobile phase ratio was changed linearly for each ramp. All compounds were eluted out of the column within 15 min. The autosampler was operated at 10 °C and rinsed with 50:50 water:ACN (1.2 mL) after each injection.

MS measurements were carried out on a triple-quadrupole mass spectrometer Quantum Access Max (Thermo, USA). Solutions of the benzoyl-derivatives from *p*-NA and aniline (at 10 mg/L in ACN) were directly infused through an integrated syringe pump at 10 *μ*L/min for tuning the MS spectrometer using electrospray ionization in positive mode. Under these conditions, the precursor ion and the respective product ions of each derivative were identified. In addition, the spray voltage (3.0 kV) and the collision energies (CE) were optimized. For *p*-NA derivative, the protonated molecular ion $[M+H]^+$ at *m/z* 242.9 was selected as the precursor ion, while the product ions at *m/z* 105.2 (CE 19 eV) and *m/z* 77.3 (CE 35 eV) were set for quantification and confirmation, respectively. The protonated molecular ion $[M+H]^+$ at *m/z* 198.0 was selected as the precursor ion for aniline derivative whose ion product at *m/z* 105.2 (CE 19 eV) was set for quantification and the other at *m/z* 77.3 (CE 35 eV) for confirmation. Retention time was also used for analyte confirmation. By infusing the same solution of derivatives into the MS spectrometer with mobile phase (50:50 water:ACN, both with 0.1% formic acid) at 1 mL/min, the source conditions were optimized, as follows: vaporized temperature at 348 °C; capillary temperature at 350 °C; sheath gas pressure at 50 psi; auxiliary gas pressure at 45 psi. Nitrogen was used as nebulizer gas and argon as collision gas at a pressure of 1.9 mTorr. The data were processed by using the Xcalibur™ 2.1 software.

*Validation.* Some performance criteria of the analytical method were evaluated by an in-house validation, as advised by the Commission Decision 2002/657/EC.[16] Specificity was assessed by checking for interferences around retention time of the benzoyl-derivatives (from *p*-NA and aniline) on chromatograms of blank samples (*n*=20) and heat-processed control samples

(*n*=4 for each cooking method) before and after spiking the analyte and the IS. To evaluate false-positive *p*-NA responses from remnant DNC breakdown during sample preparation, 100 *μ*L of DNC solution at 100 mg/mL were spiked on seven blank samples for each day of validation. The relative abundance of the confirmatory transition in relation to determinative transition was 20%. Linearity ($R^2$ > 0.98) was evaluated by preparing a matrix-matched calibration curve (using blank samples) containing the IS (aniline) at six levels of *p*-NA (100, 500, 1,000, 1,500, 2,000, and 2,500 *μ*g/kg) with three replicates each. Accuracy and precision were determined using blank samples fortified at four concentration levels: 200, 500, 1,000, and 2,000 *μ*g/kg. Six replicates of each level were analyzed on three different days. The accuracy was assessed through recovery (80–110%) for each level. Coefficients of variation (CV < 15%) were calculated to indicate precision in terms of intra- and inter-day repeatability. The overall matrix effect was evaluated using heat-processed control samples (of all cooking methods) spiked at 200, 500 and 1,000 *μ*g/kg (three replicates of each level for grilled samples; four replicates of each level for boiled, fried or roasted samples). The limits of detection (LOD) and quantification (LOQ) were determined according to International Conference on Harmonization guidelines[17], as follows: LOD = 3*σ*/S and LOQ = 10*σ*/S, where *σ/S* is the signal to noise ratio.

## RESULTS AND DISCUSSION

**Method for *p*-NA Determination.** First, *p*-NA was extracted from freeze-dried chicken meat samples with an acid aqueous solution at room temperature. After pH adjustment (pH > 8) for *p*-NA deprotonation, a salting-out assisted liquid-liquid extraction with ACN was carried out to isolate the mentioned analyte. Afterward,

the aromatic amine was derivatized with benzoyl chloride to produce the respective derivative, determined by LC-MS/MS according to fragmentation pathway (Figure 1).

**Validation.** Some analytical parameters of the novel method were evaluated. The specificity was verified since no interference (*m/z* 242.9 and *m/z* 105.2) in blank samples was evidenced around *p*-NA derivative retention time by checking LC-MS/MS chromatograms. When these samples were spiked with DNC and submitted to preparation, no amide signal was observed, indicating the absence of DNC breakdown along the analytical procedure. This procedure avoids false-positive responses for *p*-NA resulting from the remnant DNC in thermally-processed samples. The method was linear within the range of 100–2,500 *μ*g/kg in chicken breast ($R^2$ > 0.99) and showed acceptable accuracy (recovery) and precision (repeatability), according to data in Table 1. Overall recovery complied with EU guidance within the recommended limits for fortified levels evaluated.[16] Coefficients of variation indicated that precision did not exceed the limit of 15% for inter-day repeatability. Including all heat-processed control samples, the overall matrix effect was neglected based on following recoveries for the respective spiking levels: 95–119% (CV < 17%) at 200 *μ*g/kg; 94–103% (CV < 5%) at 500 *μ*g/kg; 92–105% (CV < 8%) at 1,000 *μ*g/kg. The LOD and LOQ for monitoring the target compound in meat were 10 *μ*g/kg and 30 *μ*g/kg, respectively. In summary, all minimum requirements were achieved, proving the suitability of this method. For analytical quality assurance, analysis of QCs together with real sample batches resulted in a mean recovery of 103% (CV = 6.9%), ensuring a reliable data set.

**Findings.** Attempts to find *p*-NA in heat-processed chicken meat were not conducted without first demonstrating its release from DNC hydrolysis. The stability of this diphenylurea was assessed in buffered aqueous solutions (pH 2–10) with *p*-NA monitoring as the azo-coupling derivative.[14,15,18] Surprisingly, the aromatic amine was not detected when fixing the hydrolysis temperature at 70, 80 or 90 °C. In contrast, DNC was susceptible to cleavage at 100 °C, yielding free *p*-NA in both acidic and alkaline media. This hydrolytic decomposition proceeded slowly at 100 °C, with continuous reduction of the reaction rate at increasing pH, as indicated by the respective $k_1'$ values: 0.066 $h^{-1}$ at pH 2; 0.061 $h^{-1}$ at pH 4; 0.057 $h^{-1}$ at pH 6; 0.050 $h^{-1}$ at pH 8; and 0.029 $h^{-1}$ at pH 10 (Table S1 in Supporting Information presents linear regression parameters). Our results are aligned, in part, with previous reports. For analytical purposes, Nose et al.[19] achieved quantitative *p*-NA formation only when performing the DNC hydrolysis at 150 °C. Tarbin et al.[8] provided further evidence on DNC depletion in aqueous solution at 100 °C, mentioning that *p*-NA can be formed. As reported by Audu and Heyn[14], hydrolysis of DNC-like *N*,*N'*-diphenylureas, giving rise the corresponding amines, takes place at very slow rates indeed.

Laudien and Mitzner proposed mechanisms of acid and basic catalysis for phenylureas hydrolysis *via* nucleophilic attack on carbonyl carbon.[20,21] These mechanisms are appropriate to clarify the course of hydrolytic breakdown investigated herein. Based on a structure-reactivity relation established by the same authors, the high resistance of DNC to hydrolysis makes sense if the electron-withdrawing effect promoted by nitro groups is considered. Such substituents markedly reduce the electron density on the N atoms adjacent to carbonyl, what hinders the protonation step involved in acid catalysis, and

adversely affect the reaction velocity. Furthermore, electron-attracting influence of nitro groups increases the acidity of proton in NH-aryl moiety. This means that DNC deprotonation is favored in alkaline solutions, leading to formation of its unreactive conjugate pair in the media. Actually, the predominance of this competitive equilibrium inhibits basic hydrolysis to the point of making it slower than acidic medium reaction.

The fact that DNC degrades to *p*-NA in aqueous solutions did not exclude other alternatives for residue disappearance in chicken meat subjected to cooking methods. The suggested pathway towards *p*-NA would be no more than speculation unless this aromatic amine was identified in actual heat-processed fillets previously prepared.[7] Thus, we sought for the detection of *p*-NA traces in such chicken samples by means of LC-MS/MS to define part of the DNC fate.

According to chromatograms shown in Figure S2 (see Supporting Information), the target amine was not detected (< 10 µg/kg) in DNC-containing raw fillets (samples that represented zero-time in thermal processing experiment). The absence of *p*-NA was also verified in DNC-free breast fillets (control samples) either *pre*- or *post*-heating by the cooking methods (Figure S3 in Supporting Information). Both remarks show that *p*-NA formation in chicken meat originates only from DNC degradation. Therefore, *p*-NA occurrence in breast muscle cannot be related to endogenous deposition resultant from its own impurity in NCZ consumed by broilers.[9,22] In addition, the release of this amine is not associated to decomposition of another naturally-occurring precursor in chicken meat matrix.

Meanwhile, typical LC-MS/MS profiles (Figure 2) revealed unequivocally the occurrence of *p*-NA (retention time at 9.5 min) in cooked chicken fillets. Similar chromatographic profiles were observed for the samples heated either by boiling, grilling, frying, or roasting. In essence, this finding explains into an acceptable reason why DNC disappears in chicken meat along heat treatment. As far as we are concerned, no other research has reported this information before.

Table 2 shows the *p*-NA concentration in DNC-containing chicken meat during thermal processing by different cooking methods. For this set of samples, some data are missing because relatively low concentrations have been observed in these assays (values between LOD and LOQ of the analytical method); nevertheless, this analytical limitation does not diminish the research merit. The appearance of *p*-NA in chicken fillets became pronounced in the course of time for grilling, frying, and roasting. Otherwise, the lowest concentrations were observed during boiling.

At first glance, such reticent accumulation of the aromatic amine caused some surprise when crosschecking the earlier remarks[7], in which more than 50% of the initial DNC had already been degraded in the minimum cooking time of all the applied methods (5 min for boiling, 15 min for grilling, 5 min for frying, and 15 for roasting). In response to this decomposition, a sudden increase in free *p*-NA content within the same period was expected, as outlined by the qualitative scheme in Figure 3. However, this behavior for *p*-NA concentration was not confirmed, what suggests a less simplistic reading about the fate of the amine during chicken meat heating.

The *p*-NA accumulation in thermally-processed breast fillets remains unclear, but probably it depends on factors beyond degradation itself. Apparently, the amine levels left in cooked meat were more like a consequence of DNC cleavage subtracted by losses in concomitant events. One of them, that for now cannot be ignored, refers to the moisture transport over cooking.

For well-stated reasons, interstitial water in the meat is naturally expelled by heating influence.[23,24] This fact gives a reasonable chance for *p*-NA leaching through the juices exuded from the fillets. However, besides the exudation reaching a limit at some point, the water transport does not occur only in liquid form. A significant moisture loss occurs directly by evaporation[24,25], a key-process for modeling mass transport under grilling[26,27], deep-fat frying[28–30] and roasting[31–33] conditions. Then, as water escaped from the meat in vapor phase, the *p*-NA migration out of the portion was disrupted, leading to impregnation of its traces in grilled, fried and roasted fillets. By prolonging the exposure time, the increase in amine concentration may have been a result of interaction between these drying and retention effects. When a limited screening was accomplished by LC-MS/MS, amine traces were found in boiling water. Although the quantification was not done, boiling probably provided *p*-NA extraction by hot water resulting in the low concentrations in chicken meat, differently from the other cooking methods. Further causes that explain this discrete behavior in boiling still need elucidation.

Besides the issues reported in this study, a relevant feature of DNC such as its thermal profile could not escape our notice, given its implication on outcomes. As recently verified by means of thermal analysis techniques, DNC is a thermo-labile compound whose decomposition occurs at 252 °C and results in *p*-NA as well.[34] Instead of introducing another perspective, this evidence

indicated that DNC degradation proceeds by hydrolytic breakdown. Thermally induced decomposition was not considered because the temperature of chicken fillets (in the core and the boundaries) did not exceed 200 °C in any of the cooking procedures.[7]

Our evidences prove for the first time the *p*-NA release from incurred DNC in chicken breast fillets submitted to thermal processing. Thus, the heating effect on both the DNC content and possible relevant degradation products in chicken meat should be considered. The findings are a pioneering milestone in anticoccidial-deriving degradation products. Based on our results, we suggest further research not only to identify the factors of each cooking method that determine the net *p*-NA accumulation, but also to verify whether the levels found are indeed a matter of concern regarding food safety.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Additional information on rate constant (Table S1), *p*-NA extraction procedure (Figure S1); chromatograms of breast fillets containing DNC (Figure S2) and free from DNC (Figure S3) (PDF).

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## Notes

The authors declare no competing financial interest.

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**Table 1. Accuracy and Precision for *p*-NA Determination in Chicken Breast Meat**

| fortified level (*μ*g/kg) | day | recovery (%)[a] | intra-day repeatability (CV, %) [a] | recovery (%)[b] | inter-day repeatability (CV, %)[b] |
|---|---|---|---|---|---|
| 200 | 1 | 103.8 | 11.0 | 96.6 | 9.7 |
| | 2 | 97.1 | 8.9 | | |
| | 3 | 92.7 | 14.8 | | |
| 500 | 1 | 104.2 | 7.5 | 101.2 | 6.6 |
| | 2 | 100.9 | 7.5 | | |
| | 3 | 98.7 | 4.2 | | |
| 1,000 | 1 | 108.7 | 8.2 | 99.1 | 8.9 |
| | 2 | 93.4 | 1.9 | | |
| | 3 | 97.7 | 7.5 | | |
| 2,000 | 1 | 113.6 | 10.9 | 102.2 | 14.6 |
| | 2 | 89.4 | 1.8 | | |
| | 3 | 102.9 | 14.8 | | |

[a]($n$ = 6); [b]($n$ = 18)

Intra- and inter-day repeatability means precision.

Recovery means accuracy.

**Table 2. Concentration of *p*-NA in DNC-Containing Chicken Meat Over Cooking Times**

| cooking method | cooking time (min) | *p*-NA (*μ*g/kg)[a] |
|---|---|---|
| boiling | 0 [b] | < LOD |
| | 5 | LOD–LOQ |
| | 10 | LOD–LOQ |
| | 15 | LOD–LOQ |
| | 20 | 38.4 ± 3.7 |
| | 25 | 51.9 ± 4.9 |
| grilling | 0 [b] | < LOD |
| | 15 | LOD–LOQ |
| | 30 | 60.6 ± 9.0 |
| | 45 | 141.2 ± 31.5 |
| | 60 | 210.4 ± 43.1 |
| | 75 | 326.3 ± 47.6 |
| frying | 0 [b] | < LOD |
| | 5 | 36.9 ± 8.4 |
| | 10 | 82.5 ± 13.6 |
| | 15 | 78.0 ± 6.5 |
| | 20 | 200.6 ± 52.7 |
| | 25 | 640.0 ± 143.3 |
| roasting | 0 [b] | < LOD |
| | 15 | < LOD |
| | 30 | LOD–LOQ |
| | 45 | 79.8 ± 18.5 |
| | 60 | 191.7 ± 48.0 |
| | 75 | 456.9 ± 50.7 |

[a] Average value of 6 replicates for each cooking time. Data were corrected, considering weight loss during freeze-drying process[7].

[b] The zero-time represents the DNC-containing raw chicken fillets.

LOD: 10 *μ*g/kg; LOQ: 30 *μ*g/kg; LOD–LOQ: among LOD and LOQ values

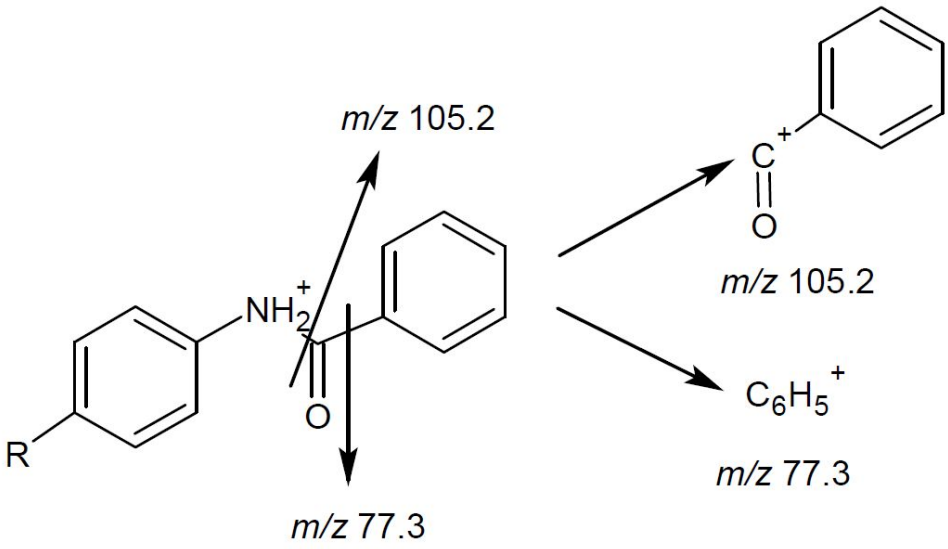
**Figure Captions**

**Figure 1.** Fragmentation pathway of benzoyl derivatives from *p*-NA and aniline (IS).

**Figure 2.** LC-MS/MS chromatograms showing *p*-NA traces in DNC-containing chicken breast fillets submitted to thermal processing by different cooking methods.

**Figure 3.** Simplified qualitative scheme to illustrate the behavior of DNC and *p*-NA concentrations in chicken breast fillets submitted to different cooking methods. Straight line represents the DNC and *p*-NA profiles found experimentally. Dotted line indicates the expected *p*-NA profile. The DNC profile has been adapted from our data previously reported.[7]

**Figure 1.**




*p*-NA derivative *m/z* 242.9 (R = $NO_2$)

Aniline derivative *m/z* 198.0 (R = H)

**Figure 2.**

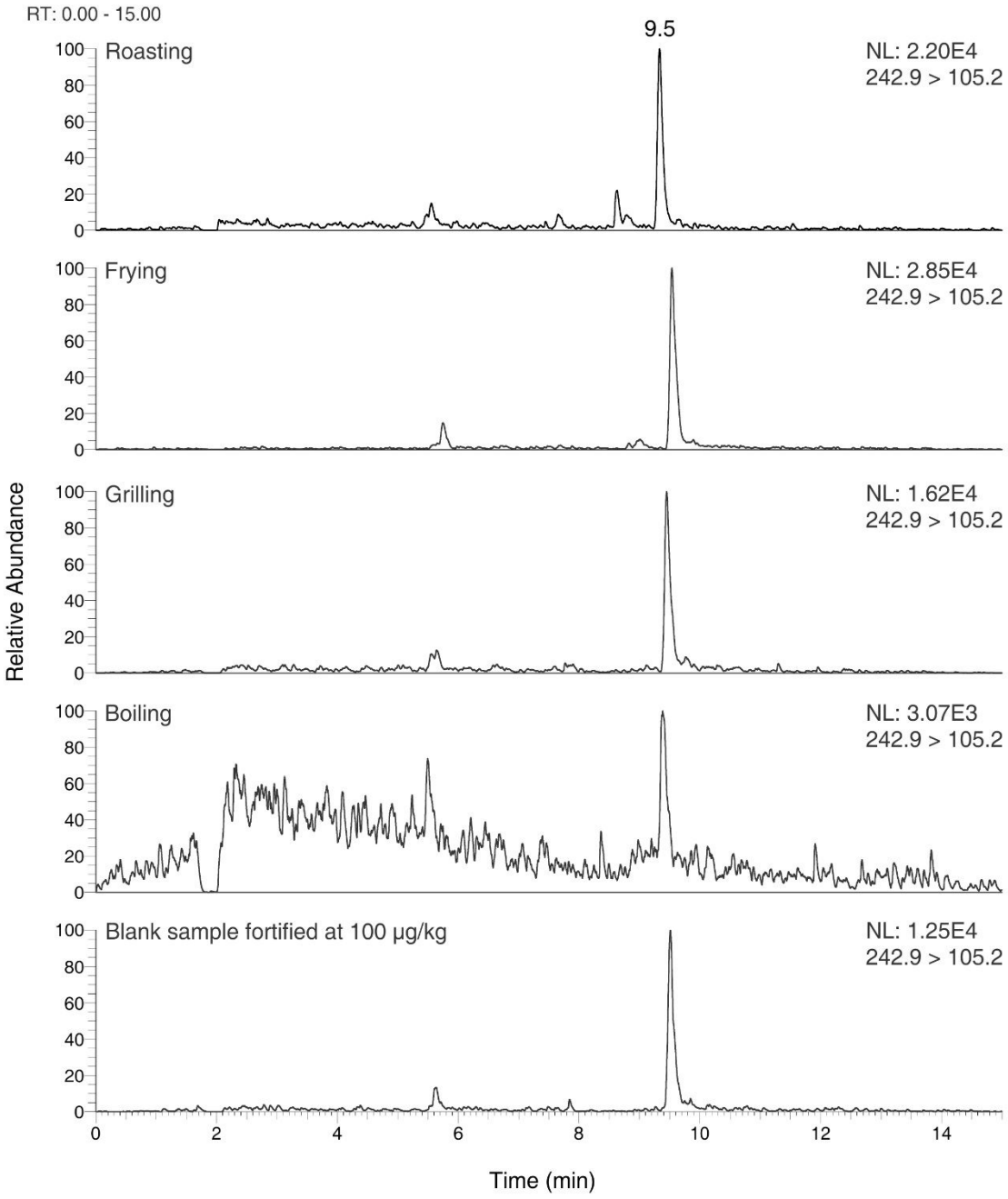
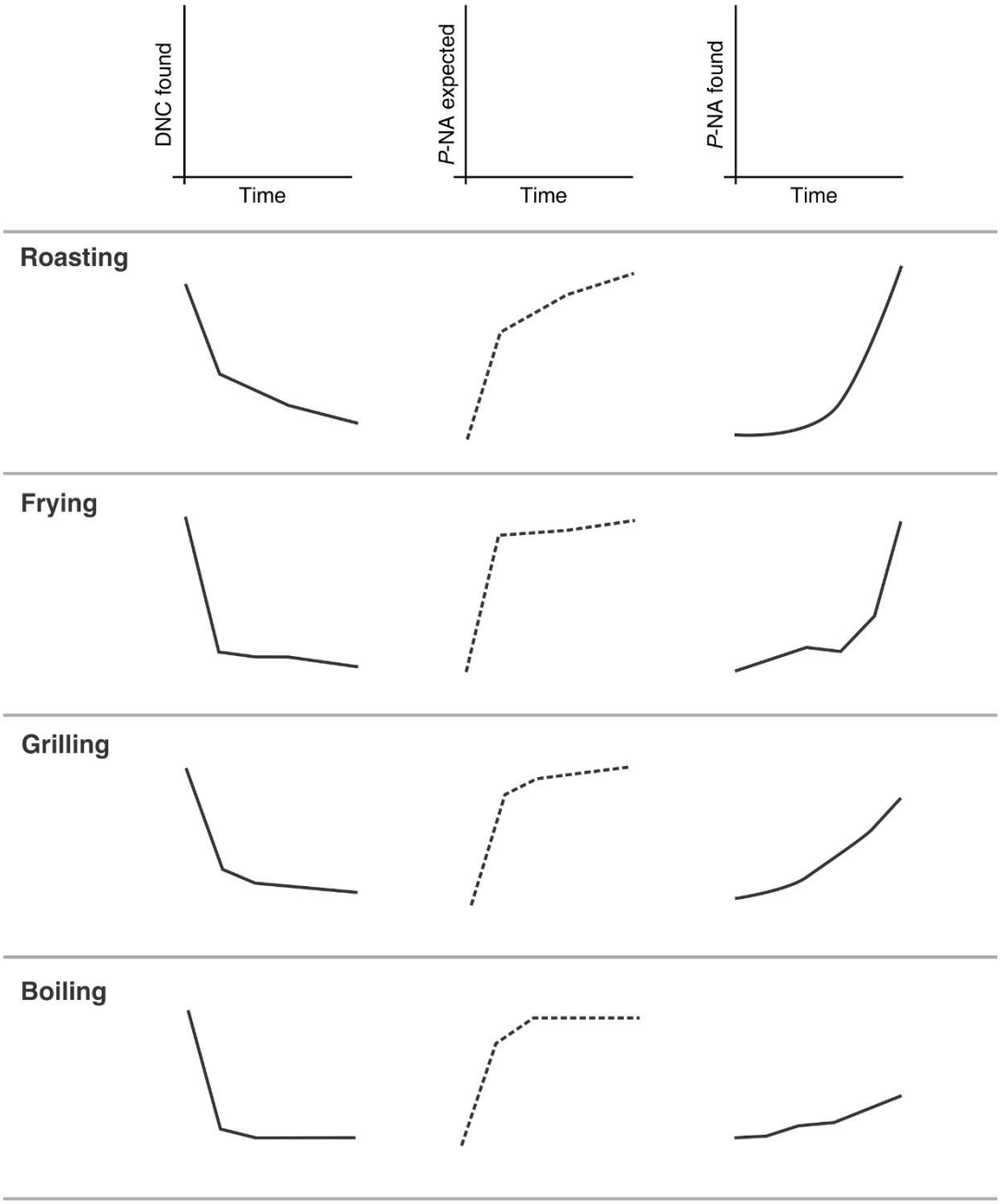

RT: 0.00 - 15.00
9.5
Roasting
NL: 2.20E4
242.9 > 105.2
Frying
NL: 2.85E4
242.9 > 105.2
Grilling
NL: 1.62E4
242.9 > 105.2
Boiling
NL: 3.07E3
242.9 > 105.2
Blank sample fortified at 100 µg/kg
NL: 1.25E4
242.9 > 105.2
Relative Abundance
Time (min)

**Figure 3.**


DNC found
Time
P-NA expected
Time
P-NA found
Time
Roasting
Frying
Grilling
Boiling