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DETECTION OF *p*-NITROANILINE BY LC–MS/MS IN RAW AND COOKED BROILER BREAST

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ABSTRACT – Coccidiosis is a common disease in commercial broiler production. To control this disease, anticoccidials are commonly used. However, there are concerns about the deposition of residues on the tissues of broilers fed with anticoccidials in their diet. The Ministry of Agriculture monitors nicarbazin residues and has detected one of its molecules, dinitrocarbanilide (DNC). However, in thermal processing, *p*-nitroaniline (*p*-NA) can be formed, and if chicken meat is ingested excessively by people, *p*-NA is likely to cause liver damage in humans. Therefore, the objective of this research was to develop a method to detect *p*-NA by LC-MS/MS in thermally processed chicken breasts. The LOD and LOQ were 10 and 30 $\mu\text{g kg}^{-1}$ respectively. Up to 640 $\mu\text{g kg}^{-1}$ *p*-NA was detected in frying procedure. The results warn to monitor *p*-NA for the benefit of consumer health and maintenance of exports.

RESUMO – A coccidiose é uma doença comum na produção comercial de frangos de corte. Para controlar esta doença, são comumente usados anticoccidianos. No entanto, existem preocupações sobre a deposição de resíduos nos tecidos dos frangos alimentados com anticoccidianos na ração. O Ministério da Agricultura monitora resíduos de nicarbazina e tem detectado uma de suas moléculas, a dinitrocarbanilida (DNC). Porém, no processamento térmico, a *p*-nitroanilina (*p*-NA) pode se formar e, caso a carne de frango seja ingerida em excesso pelas pessoas, existe a probabilidade da *p*-NA ocasionar danos ao fígado de humanos. Portanto, o objetivo desta pesquisa foi desenvolver um método para detectar *p*-NA por LC-MS/MS em peito de frango submetido a processamentos térmicos. Os LOD e LOQ foram 10 e 30 $\mu\text{g kg}^{-1}$ respectivamente. Até 640 $\mu\text{g kg}^{-1}$ de *p*-NA foi detectada na fritura. Os resultados alertam para se monitorar a *p*-NA em benefício à saúde do consumidor e manutenção das exportações.

KEYWORDS: heat treatment; cooking method; dinitrocarbanilide (DNC); LC-MS/MS.

PALAVRAS-CHAVE: tratamento térmico; método de cozimento; dinitrocarbanilida (DNC); LC-MS/MS.

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1. INTRODUCTION

Brazil is the world's largest exporter of chicken meat, however most of its production remain in the domestic market (67%), with annual *per capita* consumption of 42 kg, higher than other types of meat (ABPA, 2019). In poultry production, the birds may be affected by coccidiosis, which is a common disease, causing damage to their intestines, decreasing daily weight gain or even provoking death of these animals, leading to great economic losses in meat producing countries.

In Brazil, losses due to subclinical coccidiosis reach \$ 19.1 million per year, being 62% at meat production and 38% during feed consumption. Considering that Brazil is the second largest producer and the first in chicken meat exports, the lack of coccidiosis control would jeopardize the country entire economy, which generates revenues of \$ 7.2 billion from chicken exports to about 140 countries (ABPA, 2019). Therefore, coccidiosis control is urged and is usually made by administering in-feed anticoccidials, being nicarbazin one of the most used. However, concerns regarding tissue deposition of its residues are being raised lately by the Ministry of Agriculture, Livestock and Food Supply (MAPA).

MAPA established a maximum concentration of 125 mg kg⁻¹ nicarbazin in feed, 10 days of withdrawal period and a maximum residue limit (MRL) of 200 µg kg⁻¹ nicarbazin as dinitrocarbanilide (DNC) in raw chicken meat (BRASIL, 2019). However, compliance with this legislation may not be sufficient to obtain a safe product for human consumption.

Studies (Bacila et al., 2018; Bacila et al., 2019a) have shown that nicarbazin can degrade to *p*-nitroaniline (*p*-NA), a substance with potential liver toxicity in humans (Health Council of the Netherlands, 2008; National Toxicology Program, 1993). Its control has been recommended and limited to be at the lowest achievable level by the European Union Commission since 2010 in chicken diet (EC, 2010). The MRL for *p*-NA has not yet been established by national and international regulatory agencies, probably because of scientific data absence and lack of analytical methodology for quantifying this molecule. To assist in that matter, this work aimed at developing a method by LC-MS/MS to detect *p*-NA in chicken breast and validate this method in chicken breast samples submitted to in-house cooking procedures.

2. MATERIAL AND METHODS

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 and the internal standard (IS) aniline stock solution, both at 1,000 µg mL⁻¹, were prepared in ACN. Working solutions were daily prepared at 10 µg mL⁻¹ by diluting the stock solution with ACN. The derivatizing reagent was prepared by diluting 200 µL of benzoyl chloride in 10 mL of ACN. All solutions were protected from light and stored at -20 °C.

Samples. In order to collect chicken breast samples, broilers were raised in Embrapa Suínos e Aves. Then the samples were freeze-dried until analysis. All details upon broiler production, breast sampling, thermal processing, general cooking procedures, and sample preparation were previously described (Bacila et al., 2018).



Thermal Processing. Conventional cooking methods over set times were performed: 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.

Extraction of *p*-NA. The amount of 2.5 g of ground freeze-dried chicken breast sample (equivalent to 4–10 g in wet basis) was weighed into a 50 mL polypropylene tube and fortified with 50 μ L IS (10 μ g mL⁻¹). Then, 20 mL of 0.5 mol L⁻¹ perchloric acid was added and shaken in a vortex (30 s). Further, the tube was shaken for 30 min on a “wrist action” shaker and centrifuged at 3500g for 10 min at 20 °C. The supernatant was filtered (nylon hydrophilic membrane, 33 mm, 0.45 μ m), and 5 mL of this filtrate was transferred into a 15 mL polypropylene tube already containing 1.5 g of NaCl and 0.5 g of Na-citrate dihydrate. After 0.5 mL of 5 mol L⁻¹ NaOH (aq.) was added, 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 centrifuging at 4370g for 10 min at 20 °C. An aliquot of the upper ACN phase (500 μ L) was transferred to a 2 mL tube containing the derivatizing reagent (50 μ L) and ACN (450 μ L). The solution was allowed to warm in an oven at 40 °C for at least 15 h (overnight) and then centrifuged at 15000g for 5 min at 20 °C. After the derivatization step, 700 μ L of the solution was transferred to a 2 mL vial for injection into the LC-MS/MS. Determinations were performed in duplicate.

Determination of *p*-NA by LC-MS/MS. The analyses were performed using a LC System Surveyor Plus (Thermo) coupled with a triple-quadrupole mass spectrometer Quantum Access Max (Thermo). Separations were carried out in a Kinetex C18 100 Å analytical column (100 \times 4.6 mm, 5 μ m pore size) combined with a C18 guard column. A combination of two mobile phases was used with a flow rate at 1.0 mL min⁻¹. Mobile phase A consisted of water with 0.1% formic acid (v/v). Mobile phase B consisted of ACN with 0.1% formic acid (v/v). The gradient program was: 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). Column temperature was 30 °C and injection volume was 10 μ L.

p-NA and aniline analysis were done using positive electrospray ionization and the optimized MS conditions were: spray voltage of 3.0 kV, 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. For *p*-NA, 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.

3. RESULTS AND DISCUSSION

Some analytical parameters of the novel method were evaluated (Table 1). 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. The method was linear within the range of 100–2500 μ g kg⁻¹ in chicken breast ($R^2 > 0.99$) and showed acceptable accuracy (recovery) and precision (repeatability).

Table 1 - Accuracy and precision for *p*-NA determination in chicken breast

Fortified level ($\mu\text{g kg}^{-1}$)	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.

Source: Bacila et al. (2019a).

Overall recovery complied with EU guidance within the recommended limits for fortified levels evaluated (EC, 2002). Coefficients of variation indicated that precision was within the limit of 15% for inter-day repeatability. Including all heat-processed control samples, the overall matrix effect was neglected based on recoveries for the respective spiking levels: 95–119% (CV < 17%) at 200 $\mu\text{g kg}^{-1}$; 94–103% (CV < 5%) at 500 $\mu\text{g kg}^{-1}$; 92–105% (CV < 8%) at 1,000 $\mu\text{g kg}^{-1}$. The LOD and LOQ for monitoring the target compound in chicken breast were 10 and 30 $\mu\text{g kg}^{-1}$, respectively. In summary, all minimum requirements were achieved, proving the method suitability. 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.

The continued use of nicarbazin depends on ensuring the safety of any residues or degradation products in chicken meat considering the effects during thermal processing. Through thermogravimetric analysis coupled to mass spectrometry it was observed that the decomposition of DNC occurs above 250 °C with *p*-NA formation (Bacila et al., 2019b).

The novel LC-MS/MS method developed herein was applied to detect *p*-NA traces considering different in-house cooking procedures over cooking times (Table 2). The *p*-NA detection in fillets was discrete during boiling treatment, but became pronounced over time for grilling, frying and roasting, achieving respectively 326.3, 640.0 and 456.9 $\mu\text{g kg}^{-1}$. As far as we are concerned, no other research identified degradation products such as *p*-NA from DNC residue in heat-processed chicken fillets. Therefore, this study leads to further approaches to assess impacts on food safety.

Table 2 - Concentration of *p*-NA in DNC-containing chicken meat over cooking times ^a

Cooking method	Cooking time (min)	<i>p</i> -NA ($\mu\text{g kg}^{-1}$) ^b
Boiling	0 ^c	< LOD
	5	LOD–LOQ
	10	LOD–LOQ
	15	LOD–LOQ
	20	38.4 ± 3.7
	25	51.9 ± 4.9
Grilling	0 ^c	< 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 ^c	< 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 ^c	< LOD
	15	< LOD
	30	LOD–LOQ
	45	79.8 ± 18.5
	60	191.7 ± 48.0
	75	456.9 ± 50.7

^a LOD: 10 $\mu\text{g kg}^{-1}$; LOQ: 30 $\mu\text{g kg}^{-1}$; LOD–LOQ: among LOD and LOQ values

^b Average value of 6 replicates for each cooking time. Data were corrected, considering weight loss during freeze-drying process (Bacila et al., 2018).

^c The zero-time represents the DNC-containing raw chicken fillets.

Source: Bacila et al. (2019a).

Our evidences showed that although *p*-NA could not be detected in the beginning (around 15 min) of some cooking procedures, its concentration increased over time and was present in chicken breast samples up to 640 $\mu\text{g kg}^{-1}$ at 25 min during frying. Is not possible to sign if the values encountered herein are subject of risk to consumers, because no regulatory agency in the world has regulated about the degradation products, only about DNC (MRL = 200 $\mu\text{g kg}^{-1}$). If we assume this limit and compared it to our *p*-NA results: grilling above 60 min, frying above 20 min and roasting slightly more than 60 min, should pose an alert to the public. However, as this study is the only one in the literature dealing with *p*-NA, more studies are needed to estimate risk.

These findings serve as a warning for the monitoring and control of this degradation product, providing subsidy as a start point to the safety of chicken breast consumption to domestic and foreign markets. Also, this methodology can be used in the quality control of agro-industries and inspection laboratories, thus helping in negotiating markets for chicken products that are of great relevance to the country's economy.



4. CONCLUSION

The method developed herein was able to properly quantify *p*-NA in chicken breast samples. It was observed that this amine concentration increased over time. Although it could not be detected in the beginning of some cooking procedures, it was observed that over time *p*-NA concentration was more pronounced, achieving the maximum amount ($640 \mu\text{g kg}^{-1}$) at 25 min during frying. As there is no legislation of *p*-NA concentration in chicken breast, the findings are a pioneering milestone in anticoccidial-deriving degradation products. Based on our results, we suggest further research to identify the factors of each cooking method that determine the net *p*-NA accumulation, and also to verify whether the levels found are indeed a matter of concern regarding food safety.

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