

Effects of Boiling on Nitrofuran AOZ Residues in Commercial Eggs

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Summary

The aim of this study was to determine the effects of boiling on nitrofuran 3-amino-2-oxazolidinone (AOZ) residues in eggs. The use of furazolidone in food-producing animals is banned within the EU and Turkey. The nitrofuran AOZ residues in raw and boiled eggs were analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in a chromatographic run of 20 min. The method validation was done according to the criteria laid down in Commission Decision No. 2002/657 EC. Linearity was proved between 0 to 1.5 µg/kg, decision limit (CC α) was 0.70 µg/kg, detection capability (CC β) was 0.77 µg/kg, recovery values ranged between 88-97.9% and repeatability (CV) was 3-4.3%. The detected average nitrofuran AOZ residue level in 13 uncooked eggs by LC-MS/MS was 0.86±0.017 µg/kg which was increased to 2.42±0.037 µg/kg after boiling. In this study, it was surprisingly found that protein-bound side-chain metabolite, nitrofuran AOZ levels in eggs were significantly increased after boiling. This finding runs counter to the claim that heat process in general should decrease various antibiotic levels in food. The observed increase (P<0.001) in nitrofuran AOZ levels in boiled eggs relative to uncooked eggs may be due to enhanced efficiency of extraction in boiled samples. Therefore boiled eggs should be used for analysis of nitrofuran AOZ levels in order to obtain more reliable and more predictive results.

Keywords: Nitrofuran AOZ, Residue, Egg, LC-MS/MS, Validation, Heat process

Ticari Yumurtalarda Nitrofuran AOZ Kalıntıları Üzerine Kaynamanın Etkisi

Özet

Bu çalışmanın amacı, yumurtalarda nitrofuran 3-amino-2-okzazolidinon (AOZ) kalıntıları üzerine kaynamanın etkisini belirlemektir. Furazolidonun gıda değeri taşıyan hayvanlarda kullanımı Avrupa Birliği'nde ve Türkiye'de yasaklanmıştır. Çiğ ve haşlanmış yumurtalarda nitrofuran AOZ kalıntıları sıvı kromatografi-tandem kütle spektrometrisi (LC-MS/MS) kullanılarak 20 dakikalık süre içerisinde analiz edilmiştir. Uygulanan yöntemin validasyonu Avrupa Birliği Komisyon Kararı No 2002/657 EC 'de belirtilen kriterlere göre yapıldı. Doğrusallık 0-1.5 µg/kg, tayin limiti (CC α) 0.70 µg/kg, tayin kapasitesi (CC β) 0.77 µg/kg, geri kazanım değerleri %88-97.9, tekrarlanabilirlik (CV) %3-4.3 arasında gerçekleşti. LC-MS/MS ile 13 adet çiğ yumurtada 0.86±0.017 µg/kg miktarında tespit edilen ortalama değer haşlama sonucu bakılan aynı yumurta örneklerinde istatistik olarak önemli derecede artış göstererek 2.42±0.037 µg/kg ortalama değerlere yükselmiştir. Şaşırtıcı bir şekilde bu çalışmada, kaynamadan sonra yumurta içinde protein-bağlı yan-zincir metaboliti nitrofuran AOZ düzeylerinin arttığı tespit edildi. Bu bulgular genel olarak ısı işlemin gıdalarda çeşitli antibiyotik düzeylerini azaltması gerektiği iddiası ile ters düşmektedir. Pişmemiş yumurtalara göre haşlanmış yumurtalarda nitrofuran AOZ düzeylerinde gözlenen artış (P<0.001) haşlanmış örneklerde ekstraksiyon etkinliğinin artmasına bağlı olabilir. Bu nedenle nitrofuran AOZ seviyelerinin analizinde daha güvenilir ve anlamlı sonuçlar elde etmek amacıyla haşlanmış yumurtalar kullanılmalıdır.

Anahtar sözcükler: Nitrofuran AOZ, Kalıntı, Yumurta, LC-MS/MS, Validasyon, Isıl işlem

INTRODUCTION

Veterinary drugs are used in layer flocks by addition to feed or drinking water in order to prevent and treat

diseases, promote egg productions, assist in converting stress due to environmental changes, beak trimming,



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vaccination and other management practices. Improperly and illegal administration of these drugs can cause residue problems in eggs and these residues can cause severe harmful effects on consumers. In addition, trace amounts of antibiotic compounds in eggs favor the development of antibiotic-resistant bacteria [1-3].

Nitrofurans have been widely used in the treatment of gastrointestinal infections such as fowl cholera, coccidiosis and blackheads in laying hens. Nitrofurans, including furazolidone, in veterinary practice are banned in many countries, including Turkey, because of their mutagenic, carcinogenic and genatotoxic effects [4-7].

Parent compound furazolidone is rapidly and extensively metabolized *in vivo* (*in vivo* half life is less than a few hours) [8,9]. The side chain metabolites (3-amino-2-oxazolidinone, AOZ) can bind to proteins to form bound residues. These residues are extensively formed in liver, kidney, muscle tissues and also eggs [10-12]. They have a long residence time and their extraction from the tissues is very difficult [12-14].

Short half-life of nitrofurans has made screening for parent drugs difficult in food products, but the development of assays that can detect highly stable nitrofurans metabolites have been used to demonstrate the persistence of tissue-bound residues in egg and the other animal origin foods [9-11,15,16].

Several liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been developed for determination of nitrofurans metabolites in different tissues [17-21] and eggs [18]. The detection of AOZ in poultry eggs, was carried out by Mccracken *et al.* [11] using LC-MS.

Several studies have generally described drug residues in uncooked tissues and the other various of food product [18,20,22]. However, the effects of cooking on nitrofurans 3-amino-2-oxazolidinone (AOZ) in eggs have not been studied. It is important to determine if residues of nitrofurans AOZ are reduced or not by boiling procedure. Since egg is usually cooked before consumption, more findings about the effects of boiling on residues of nitrofurans AOZ are needed to determine the risks to the consumer from dietary exposure to these substances. We carried out this study to examine whether boiling has an effect on residues of nitrofurans AOZ in eggs.

MATERIAL and METHODS

Reagents and Water

2-NP-AOZ-D4 internal standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals such as dimethyl sulfoxide (DMSO), hydrochloric acid (HCl), 2-nitrobenzaldehyde, sodium hydroxide (NaOH), dipotassium hydrogen phosphate (K_2HPO_4), ethyl acetate,

methanol (MeOH) and acetic acid were HPLC grade and were purchased from Merck (KGaA, Darmstadt, Germany). The water used was purified with a Milli-Q water purification system from Millipore (Millex GV, 0.45 μ m).

Standard Solutions

The standard stock solution and internal standard were stored at -20°C in the dark and warmed up to room temperature before use. The working standard solution was stored at $+4^\circ\text{C}$ in the dark. DMSO was stored at room temperature and the other chemicals were stored at room temperature (22 to 25°C).

Samples

Samples of eggs were collected from different layer flocks as part of the "National Program of Residue Control", between 1 Jan 2010 - 31 December 2010. Nitrofurans AOZ was found positive from one poultry farm's layer flocks by LC-MS/MS. In this study we used nitrofurans AOZ positive 13 egg samples from these layer flocks.

Sample Preparation

The extraction was performed as described previously [23,24] with some minor modifications. Before boiling process, each egg (liquid) was scrambled, split and 2 ± 0.03 g of that was put into 50 ml centrifuge tubes. The remaining amount of each egg (yolk and albumen) were immersed in 100°C water for 10 min, removed and cooled immediately in a chilled water bath. We used the same extraction method for 10 min boiled (solid) eggs. 100 μ l 2-NP-AOZ-D4 internal standard and 5 ml 0.1 M HCl were added. They were vortexed for 60 sec and homogenized. Then 300 μ l 50 mM 2-nitrobenzaldehyde, which was dissolved in dimethylsulfoxide (DMSO), was added and mixed for 1 min by multireax vortex. They were incubated in the shaker incubator at 37°C for 16 h at 50 rpm. After incubation, samples were cooled to the room temperature. 700 μ l neutralization solution was added [1 M NaOH/0.1 M $K_2HPO_4 + 3H_2O$ (2:1, v/v)] [25]. They were vortexed for 1 min. 5 ml ethyl acetate was added onto it and they were mixed in the shaker incubator at room temperature for 10 min at 300 rpm, then to prevent soap forming between lipid and ethyl acetate, 3 ml n-hexane was added. Then they were centrifuged at 15°C for 10 min at 4000 x g. 6 ml of upper phase were taken and transferred into 15 ml glass tube, and dried under nitrogen flow in 5.0 psi pressure at 42°C . After the drying operation, 1 mL n-hexane was added into the tube and vortexed for 1 min. 750 μ l MeOH/deionized water (1:4, v/v) was added. They were mixed with the multireax vortex for 5 min. They were centrifuged at 15°C for 10 min at 2500 x g and the sample was taken from the lower phase by the help of an injector. Then this liquid part was filtered onto a 0.45 mm filter and 50 μ l filtrate is transferred to a microvial capped and adapted to the LC autosampler [23,24].

LC-MS/MS Analysis

This study was carried out by Tandem Gold Triple Quadrupole LC-MS/MS (ZIVAK, Kocaeli, Turkey) system. LC separation was achieved by using a Synergi™ Max-RP (4 µm, 150 x 2 mm) column. The mass spectrometer operated in selective reaction monitoring mode (SRM) with positive electro-spray interface (ESI). The mobile phase A and B used were 0.2% acetic acid and methanol, respectively. The column was thermostated at 40°C. The flow rate was 0.2 ml/min, with an injection volume of 50 µl. The linear gradient was: 0-3 min 80% A, 3-7min 50% A, 7-9 min 30% A, 9-9:50 min 25% A, 9:50-13 min 0% A, 13-20 min 90% A. The electrospray capillary voltage was 5000V. Nitrogen was used as curtain and collision gas. Auto sampler, MS/MS and screening parameters are shown in *Table 1*, *Table 2* and *Table 3*, respectively.

Table 1. Auto sampler parameters**Tablo 1.** Otomatik örnekleme parametreleri

| Parameter | Value |
|--------------------------|-----------|
| Injection volume | 50 µl |
| Syringe speed | 8.0 µl/sn |
| Washing volume | 1000 µl |
| Washing speed | 250 µl/sn |
| Auto sampler temperature | 10°C |
| Column temperature | 40°C |

Table 2. MS/MS parameters**Tablo 2.** MS/MS parametreleri

| Parameter | Value |
|-----------------------------|--------------------|
| Ionization mode | ESI + |
| Screening | SRM |
| Spray voltage | 5023 |
| API nebulizing gas pressure | 55 |
| Drying gas pressure | 2.0 |
| Aux gas pressure | 15 |
| Drying gas temperature | 400°C |
| Quad MS/MS bias | -1.0 |
| Screening time | 0.01 sec |
| SIM width | 0.7 amu |
| Needle | + 5000 V |
| Shield | + 500 V |
| Dedector | + 1700 V |
| CID gas pressure | 1.30 mTorr |
| Spray chamber temperature | 65°C |
| Mass peak width in Amu | Q1=0.7 Q3=0.7 |

Table 3. MS/MS screening parameters**Tablo 3.** MS/MS tarama parametreleri

| Analyte | MS MH+ (m/z) | MS-MS (m/z) | Fragmentation Energy |
|---|-----------------------------|-------------|----------------------|
| 2-NP-SEM | 209.00 | 166.00 | 13 |
| 2-NP-SEM | 209.00 | 192.00 | 11 |
| 2-NP-SEM-13C ₁₅ N ₂ | 212.00 | 168.00 | 12 |
| 2-NP-AHD | 249.00 | 134.00 | 14 |
| 2-NP-AHD | 249.00 | 104.00 | 19 |
| 2-NP-AOZ | 236.00 | 134.00 | 15 |
| 2-NP-AOZ | 236.00 | 104.00 | 18 |
| 2-NP-AOZ-D4 | 240.00 | 134.00 | 15 |
| 2-NP-AMOZ-D4 | 340.00 | 296.00 | 11 |
| 2-NP-AMOZ | 335.00 | 262.00 | 19 |
| 2-NP-AMOZ | 335.00 | 291.00 | 12 |
| I. Segment | 335 | | |
| II. Segment | 209.00-249.00-236.00-240.00 | | |
| Screening mode | SRM | | |
| Segmentation energy | 10 | | |

Preparation of Matrix Standard and Validation Procedure

Five pieces of 2 g blank tissue samples were taken into 50 ml centrifuge tubes. The external standard solution was added on these samples (50, 100 and 150 µl for 0.5, 1 and 1.5 µg/kg, respectively). Then they were extracted like the other samples which are described in the sample extraction section extracted like the other samples that was described in the part of sample extraction. The method validation was done according to the criteria laid down in Commission Decision No. 2002/657 EC [26].

Calibration Curves and Results

We used blank egg samples from nitrofurans AOZ negative layer flocks for the calibration process and calculation value of CC_α and CC_β. Calibration curves, used in validation process, were based upon responses (analyte/internal standard peak area ratio versus concentration of analyte) from four concentration levels ranging from 0 to 1.5 µg/kg (0, 0.5, 1 and 1.5 µg/kg), were linear. The recovery values (Rec) ranged between 88-97.9%, and repeatability (CV) ranged between 3 and 4.3%. Calibration curves, used in calculation process, were made by spiking blank matrix samples of egg with nitrofurans AOZ corresponding to concentrations of 0.5, 1 and 1.5 µg/kg.

Statistical Analysis

SPSS 17.0 programme was used for calculations. Results are expressed as mean (x) and standard error (S.E.). Differences between groups were evaluated using Wilcoxon test.

RESULTS

During the validation process, different egg samples were analysed by the same instrument and the same operator. No significant differences were found between the curves. The calibration curves were linear, with the corresponding correlation coefficients (R^2) values higher than 0.998. The decision limit ($CC\alpha$) and detection capability ($CC\beta$) were calculated for m/z 236 \rightarrow 134, ion transition for AOZ. These values were calculated using eighteen calibration curves (at four levels 0.0, 0.5, 1.0 and 1.5 $\mu\text{g}/\text{kg}$) from six different experiments in a day, on different egg samples and 3 different days. All curves, in validation and in calculation of residues, were constructed using analyte/internal standard peak area ratio versus concentration of analyte. Linearity was proved between 0.5-1.5 $\mu\text{g}/\text{kg}$, $CC\alpha$ was 0.70, $CC\beta$ was 0.77 and the recovery values ranged between 88-97.9% and CV was between 3-4.3%.

The within-laboratory reproducibility was calculated in different spiked egg samples at concentration of 1 $\mu\text{g}/\text{kg}$ (MRPL level). They were analysed for three different days, with the same instrument and different operators. The within-laboratory reproducibility was found satisfactory for the nitrofuran AOZ. For the calculation of the residue levels we used one calibration curve (Fig. 1) with four spike

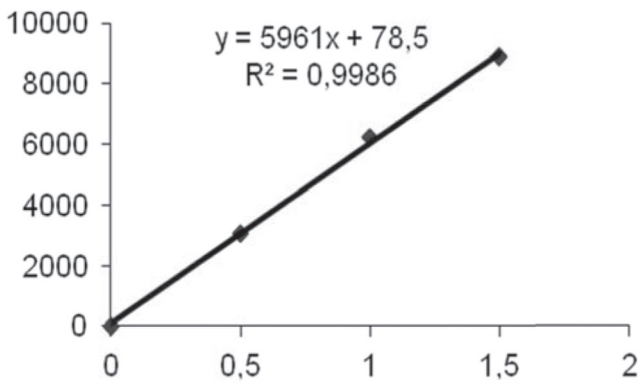


Fig 1. Calibration curve and R^2 , spiked with 0.5, 1 and 1.5 $\mu\text{g}/\text{kg}$ concentrations for uncooked eggs

Şekil 1. 0.5, 1 ve 1.5 $\mu\text{g}/\text{kg}$ düzeyinde yüklenmiş pişmemiş yumurtaların kalibrasyon eğrisi ve R^2 değeri

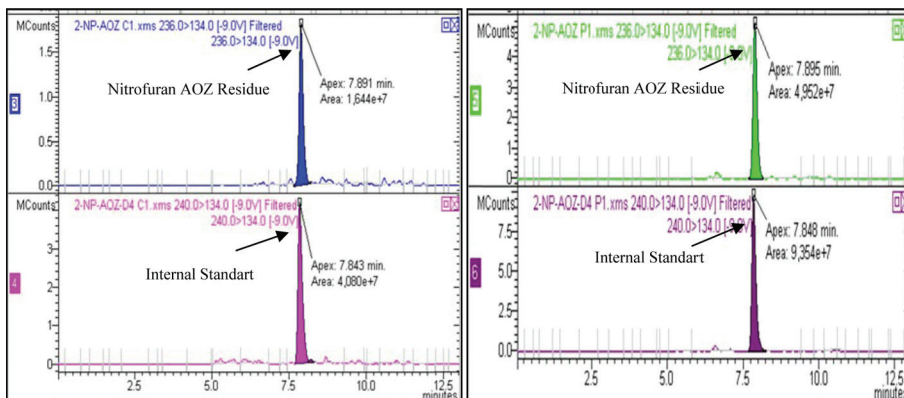


Fig 2. LC-MS/MS analysis of Nitrofuran AOZ residues and internal standart concentrations of first sample before (0.82 $\mu\text{g}/\text{kg}$) and after boiling (2.44 $\mu\text{g}/\text{kg}$) respectively

Şekil 2. Birinci örneğin haşlama öncesi (0.82 $\mu\text{g}/\text{kg}$) ve sonrası (2.44 $\mu\text{g}/\text{kg}$) LC-MS/MS analizi ile ölçülen Nitrofuran AOZ kalıntı değeri ve iç standart konsantrasyonları

concentrations (0.0, 0.5, 1.0 and 1.5 $\mu\text{g}/\text{kg}$). The validation results were in accordance with the performance method of the European Commission Decision 2002/657/EC and the method was successfully applied to confirm and quantify nitrofuran AOZ in eggs [26].

The detected avarage nitrofuran AOZ residue level in 13 uncooked eggs was 0.86 ± 0.017 $\mu\text{g}/\text{kg}$. The range of detections was between 0.76-0.97 $\mu\text{g}/\text{kg}$ for uncooked eggs. After boiling, residue level was increased to 2.42 ± 0.037 $\mu\text{g}/\text{kg}$ (Table 4). The range of detections in cooked eggs was between 2.04-2.55 $\mu\text{g}/\text{kg}$. Thus it was observed that the residue level of nitrofuran AOZ was higher ($P<0.001$) in boiled eggs than in uncooked eggs (Fig. 2 and 3).

DISCUSSION

Improperly and illegal administration of nitrofurans may produce residues in various foodstuffs such as eggs. The presence of these residues arises as a serious public health issue including mutagenic, carcinogenic and genatotoxic effects, induces allergic reactions in humans

Table 4. Nitrofuran AOZ residue levels before and after boiling in eggs

Tablo 4. Yumurtalarda kaynamadan önce ve sonra Nitrofuran AOZ kalıntı düzeyleri

| Sample No | Before Boiling ($\mu\text{g}/\text{kg}$) | After Boiling ($\mu\text{g}/\text{kg}$) |
|-----------|--|---|
| 01 | 0.82 | 2.44 |
| 02 | 0.81 | 2.04 |
| 03 | 0.97 | 2.54 |
| 04 | 0.76 | 2.31 |
| 05 | 0.8 | 2.39 |
| 06 | 0.87 | 2.49 |
| 07 | 0.93 | 2.55 |
| 08 | 0.88 | 2.49 |
| 09 | 0.94 | 2.53 |
| 10 | 0.85 | 2.42 |
| 11 | 0.83 | 2.41 |
| 12 | 0.82 | 2.46 |
| 13 | 0.9 | 2.42 |

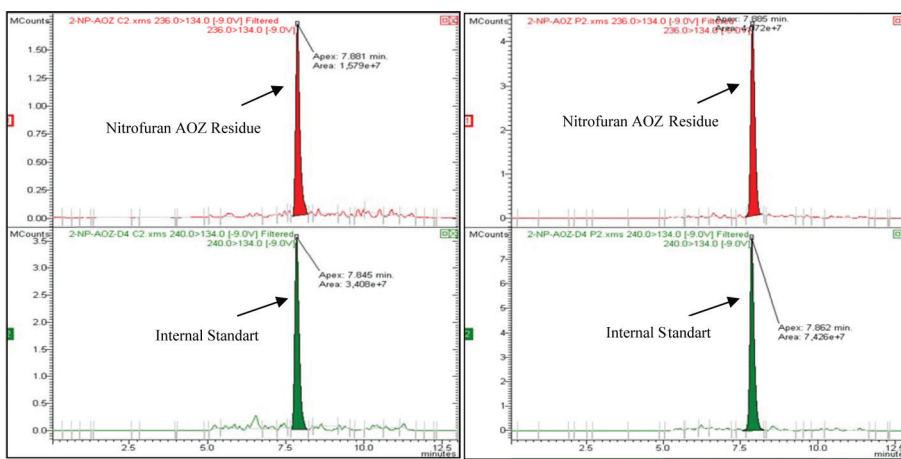


Fig 3. LC-MS/MS analysis of Nitrofurantoin AOZ residues and internal standart concentrations of second sample before boiling (0.81 µg/kg) and after boiling (2.04 µg/kg) respectively

Şekil 3. İkinci örneğin haşlama öncesi (0.81 µg/kg) ve sonrası (2.04 µg/kg) LC-MS/MS analizi ile ölçülen Nitrofurantoin AOZ kalıntı değeri ve iç standart konsantrasyonları

and give rise to an increase in the antibiotic resistance of pathogenic bacteria. Due to all these hazardous and severe problems, application of efficient detection and quantification methods and elimination of these drug residues from food matrices are important issues for consumers.

Our results run counter to the claim that heat process in general should decrease various antibiotic levels in food. In general, antibiotics in various tissues such as amphenicols residues in chicken meat [1], sulfanomides residues in chicken meat [27], ormetoprim (OMP) and sulfadimethoxine (SDM) in channel catfish muscle [28], malachite green (MG) and its major metabolite, leucomalachite green (LMG) in carp muscles [29], OTC in black tiger shrimp [30] and Japanese eel [31], chlorotetracycline and oxytetracycline in canned pork [32] are reduced by several heat process. On the contrary, some residues, such as clenbuterol in food [33] and AOZ in liver kidney and muscle [10] are stable during several heat process. Similarly, Cooper and Kennedy [34] also reported that Nitrofurantoin AOZ, 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), 1-aminohydantoin (AHD) and semicarbazide (SEM) residues remained after cooking techniques in muscle and liver of pigs and continue to pose a health risk. Furthermore, Mccracken and Kennedy [10] also reported that grilling increased the extractable AOZ content, at the expense of bound AOZ residues in both liver and kidney. The present study also showed that nitrofurantoin AOZ levels in eggs were significantly increased after boiling agreement with that reported by Mccracken and Kennedy [10]. These studies have also shown that the heat stability of drug residues in foodstuffs depend on type of drugs.

In conclusion, the occurrence of nitrofurantoin AOZ residues in eggs in Turkey was determined. Results of this study showed that nitrofurantoin AOZ residues were still present in the boiled eggs. In this study, the boiling procedure could not degrade residual nitrofurantoin AOZ in eggs. Moreover, nitrofurantoin AOZ residues increased with boiling. Therefore, boiling process might increase possible pharmacological and/or toxic effects of these compounds. The observed

increase ($P < 0.001$) in nitrofurantoin AOZ levels in boiled eggs relative to uncooked eggs may be due to enhanced efficiency of extraction in boiled samples. For this reason, boiled eggs should be used for analysis of nitrofurantoin AOZ levels in order to obtain more reliable and more predictive results.

So, it may not be assumed that heating of nitrofurantoin residues in food will result in a safe product for human consumption. Alternative management options such as vaccinations should be applied that could reduce the frequency of antibiotics usage in laying hens, emergence of drug residues in eggs and spread of antibiotic-resistant bacteria. To ensure efficient food safety and accurate monitoring of compliance with the ban, periodic analysis to monitor the drug residues in different foodstuffs should be made more frequently and more effectively by national legal authority.

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