

## Zeranol Residue Detected by HPLC in Bovine Meat from Three Different Cities in Java Island

R. Widiastuti\* & Y. Anastasia

Indonesian Research Center for Veterinary Science  
Jalan RE Martadinata 30, Bogor 16114, Indonesia

\*Corresponding author: [widiastuti.raphaela@gmail.com](mailto:widiastuti.raphaela@gmail.com)

(Received 17-12-2019; Revised 04-05-2020; Accepted 20-05-2020)

### ABSTRACT

Zeranol is one of non-steroidal hormonal growth promoters (HGP) that is still permitted to be used in some countries such as Australia, the United States, and others to increase weight gain. However, this non-steroidal HGP is not permitted in Indonesia. The use of zeranol to increase the growth of livestock can cause the occurrence of residue in livestock tissues and organs, having a great dangerous potential for human health. This study aimed to investigate the presence of zeranol residues in 105 samples of bovine meat collected from Jakarta, Surabaya, and Malang cities and analyzed by high-performance liquid chromatography (HPLC). The samples were added with the deproteinizing extractant of 0.2% metaphosphoric acid and acetonitrile (6:4, v/v), and purified using SAX SPE cartridge. Zeranol was then analyzed by HPLC using Shimp-pack VP-ODS (4.6x250 mm) column with a mixture of acetonitrile-water (40:60, v/v) as the mobile phase and detected on photo diode array detector at 262 nm. The recoveries of the method of 3 different concentrations (2, 5, and 10 ng/g) of zeranol were 73.96% to 103.48%. The detection limit and quantification limits were 0.54 ng/g and 1.80 ng/g, respectively. Zeranol residues were detected in 12 (11.43%) out of 105 samples at the concentration of 1.67 to 33.29 ng/g and 7 among them exceeding 2.0 ng/g. The results obtained in this study indicated that zeranol was still being used to increase cattle-meat production. Therefore, strict control must be implemented at all stages, from production to consumption, regarding the application of this HGP in livestock.

**Keywords:** bovine meat; zeranol; growth promoter; residue; HPLC

### INTRODUCTION

The production of animal origin foods, especially meat and meat products, has substantially increased throughout the world to meet the increased demand. Zeranol or zearanol ( $\alpha$ -zearalanol), is a synthetic non-steroidal estrogen of the resorcylic acid lactone group produced by *Fusarium* species, have been used to increase the live weight gain in food animals. Because of its carcinogenic potential and endocrine-disrupting biological activity, zeranol had been banned since 1981 within the European Union (EU) and the Member States.

Zeranol is still approved to be used as a growth promoter in several countries like Australia and the USA under the trade name of RalGro® in the USA. Zeranol and its derivatives (zearalenone and taleranol) can accumulate in humans consuming food containing zeranol regularly (Bircher *et al.*, 2015). Zeranol residues in meat consumed cause adverse effects on human health, such as disruption in human hormone balance, causing developmental problems, interfering with the reproductive system, and can even lead to the development of breast, prostate, or colon cancer (Kumar *et al.*, 2018). Therefore, FAO/WHO through Codex Alimentarius,

set the maximum residue level of zeranol is 2 ng/g in muscle and 10 ng/g in the liver (CX/MRL 2-2018, 2018). In Indonesia, zeranol residue is one of the residues which must be tested for imported livestock products entering Indonesia, based on the Decree of The Head of Agriculture Quarantine Agency Number 2464/Kpts/KR.120/K/11/2018 (Badan Karantina Pertanian, 2018).

Zeranol residue was found in many different types of samples such as bovine meat, liver, and kidney of cattle (Mor *et al.*, 2011, Yücel *et al.*, 2018), chicken meat, liver and kidney of chicken (Hemmat *et al.*, 2018), and bovine urine (Matraszek-Zuchowska *et al.*, 2013). Fortunately, zeranol residue is not stable during storage and cooking treatment (Kukhtyn *et al.*, 2020). Frozen temperature (-18°C) for 6 months would decrease by 33.2% of the zeranol content, whereas cooking up to 60 min would decrease 32% of the zeranol content.

The development of a simple, rapid, sensitive, and specific method to detect zeranol residue in animal food products is required. Several detection methods for determination of zeranol had been reported, and most of the methods applied are high-performance liquid chromatography (HPLC) (Lee *et al.*, 2018), enzyme-linked immunosorbent assay (ELISA) (Khadijah *et al.*,

2015), liquid chromatography-mass spectrometry-mass spectrometry (LC-MSMS) (Matraszek-Zuchowska *et al.*, 2013), and gas chromatography mass-spectrometry (GC-MS) (Matraszek-Zuchowska *et al.*, 2012). Mass-spectrometry methods are good in specificity and sensitivity. Still, they are costly, complicated for routine clinical studies, and not available in most laboratories. In contrast, ELISA exhibits good results and rapid screening but has the main drawback of providing incorrect results occurred from cross-reactions (Yücel *et al.*, 2012).

This study was the first investigation report in Indonesia aiming to monitor the presence of zeranol residue in bovine meat of imported (premium and non-premium) beef meat from Jakarta, fattening beef meat from Malang, and domestic beef meat from Surabaya which were analyzed by HPLC with PDA detection. A previous study in Indonesia reported the presence of zeranol residue in imported Australian and New Zealand beef meat (Khadijah *et al.*, 2015) with the use of ELISA detection.

## MATERIALS AND METHODS

### Beef Meat Collection

In this study, a total number of one hundred and five (105) beef meat samples (approximately 100 to 250 g) were collected from three different cities (Jakarta, Malang, and Surabaya). Four types of beef meat samples collected were: (1) 5 premium beef meat samples collected from Soekarno Hatta Airport Agricultural Quarantine Centre, (2) 50 non-premium imported beef meat samples collected from Tanjung Priok Seaport Agricultural Quarantine Centre, both in Jakarta, (3) 28 fattening beef meat samples collected from a slaughterhouse in Malang City (Malang has the largest and most populated for beef cattle in East Java Province), and (4) 22 domestic beef meat samples bought from traditional and supermarkets in Surabaya.

### Chemicals and Reagents

Zeranol standard was of high purity grade (~98%, HPLC) and was supplied by Sigma (Sigma-Aldrich, Darmstadt, Germany). Both reagents and solvents used were of analytical or HPLC grade quality and purchased from Merck (Darmstadt, Germany). Ultrapure (UP) water was generated by a water purification system of Milli Q Direct 8/16 System (Millipore SAS, 67120 Molsheim, France). Samples were purified through SAX SPE (Bond Elut LRC-SAX 500 mg, Agilent, USA). Technical nitrogen gas was produced by the nitrogen generator unit (Claind, Leino-Italy).

### Standard Solutions Preparation

Zeranol stock standard solution with a concentration of 1 mg/mL (1000 µg/mL) was made by diluting 25 mg zeranol standard powder in 25 mL methanol (HPLC grade) in 25 mL volumetric flask and put in the ultrasonic bath for 5 min. The stock standard solution was

stored in amber glass vials at 4°C in the absence of light. Working standard solutions were prepared weekly by diluting stock standard solutions with methanol and stored in the refrigerator.

### Sample Extraction and Zeranol Detection

The extraction method was adapted from a method developed by Horie & Nakazawa (2000). A 1 g fat-free beef meat sample was placed in a 50 mL polypropylene tube and added with 10 mL of the deproteinizing extractant consisted of 0.2% metaphosphoric acid and acetonitrile (6:4, v/v), and vortexed for 2 min. The filtrate was purified through a SAX SPE that conditioned previously with 5 mL of acetonitrile. After the application to the SAX column, the barrel was then rinsed using 5 mL of 20% acetonitrile. The zeranol was eluted with 5 mL of acetonitrile and the residue was evaporated under a stream of nitrogen gas at 45°C until dry. The residue was dissolved in 1 mL of 40% acetonitrile and passed through a 0.45 µm PVDF syringe filter unit prior to injecting it into the HPLC. The injection volume was 20 µL.

### Instrumentation and Chromatographic Condition

The HPLC instrumentation used was a Shimadzu Prominence (Kyoto, Japan) consisted of LC-20AD pump and SPD-M20A photo diode array (PDA) detector. The wavelength was set at 262 nm. The analytical separation was conducted using a reversed-phase column Shimp-pack VP-ODS (4.6x250mm) (Shimadzu), equipped with a guard column (5x4.6 mm) containing the same packing material. The mobile phase prepared daily consisting a mixture of acetonitrile-UP water (40:60, v/v), filtered through a 0.45 µm PVDF filter (Whatman, Maidstone, UK), and sonicated prior to use and carried isocratically (Liu *et al.*, 2007) at a flow rate of 1 mL/min.

### Method Validation

Half method validation for analysis of zeranol in beef meat was assessed in this study that was guided by ICH (2005) and FDA (2019) with the following parameters of specificity, matrix-matched calibration curve, recovery, detection limit, and quantitation limit.

**Specificity.** The selectivity test was performed by observing the chromatogram of zeranol standard, unfortified (blank) samples, and spiked samples.

**Linearity and range.** A matrix calibration curve was prepared from a series of zeranol standards at different concentrations from 5 to 20 ng/mL in the blank meat sample, and plot the peak areas versus concentration, to obtain the correlation coefficient ( $r^2$ ).

**Recovery and precision.** The recoveries of the method were determined by fortified the zeranol standards (prior to the extraction step) into the blank meat samples at 2, 5, and 10 ng/g. The accuracy of the method was determined

## RESULTS

### Method Validation

by assessing the agreement between the measured and known concentrations of the fortified samples. The intra-day precision of the method was determined three times on the same day by calculating the relative standard deviation (% RSD) for the repeated measurements of zeranol.

**Sensitivity.** The sensitivity of the method was evaluated from the detection limit (LOD) and quantification limit (LOQ) that were obtained by adding 3 and 10 times, respectively, standard deviations of 10 blank samples analyzed to the mean blank value. The detection limit (LOD) is the concentration that provides a signal-to-noise ratio of about 3:1, while the quantification limit (LOQ) is the concentration that provides a signal-to-noise ratio of about 10:1. The calculation is mean + 3.3 SD blank for the LOD, and mean + 10 SD blank for LOQ.

The first important step of this research was the selection of HPLC conditions, referring that mostly the detection method available for zeranol in meat samples was performed by LC-MS. Figures 1, 2a, and 2b are a chromatogram of zeranol standard, a chromatogram of unfortified (blank) meat sample, and a chromatogram of meat sample with positive zeranol residue, respectively. Zeranol is eluted approximately at 3.9 minutes and revealed the absence of co-eluting peaks at the retention time of zeranol interference. This result indicates that the identified peak of the analyte was pure and confirmed. All parameter studies of the half validation method are summarized in Table 1 and Table 2.

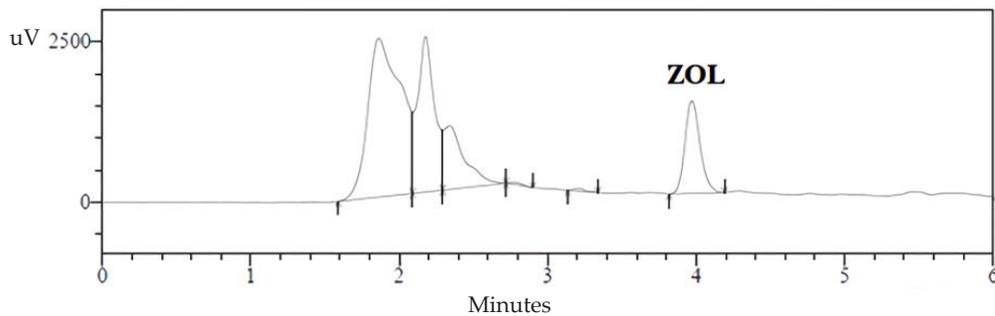


Figure 1. Chromatogram of 50 ng/mL zeranol (ZOL) standard detected using a mobile phase of acetonitrile-DI water (40:60, v/v), at a flow rate of 1 mL/min and a wavelength of 262 nm.

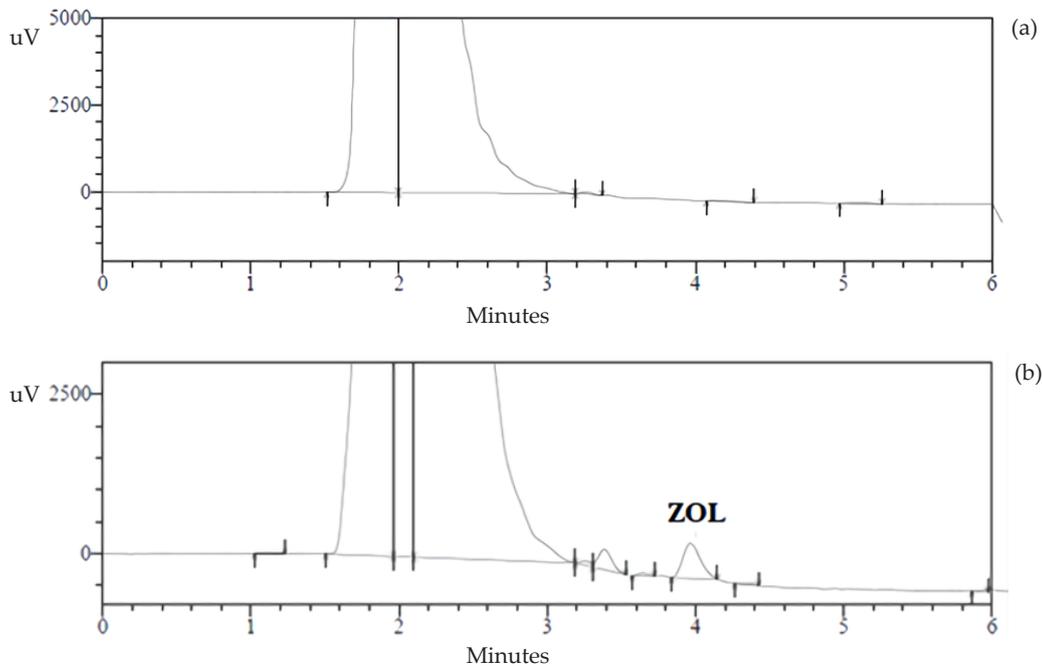


Figure 2. The chromatogram of (a) unfortified (blank) meat sample, and (b) meat sample with positive zeranol (ZOL) residue.

Table 1. Regression and validation parameters of the HPLC method for determination of zeranol

Parameters	Results
Linearity range (ng/g)	5 to 20
Correlation coefficient (r <sup>2</sup> )	0.9933
Recovery (%)	73.96 to 103.48
LOD (ng/g)	0.54
LOQ (ng/g)	1.80

Note: LOD= detection limit; LOQ= quantification limit.

Table 2. Precision of analytical method in detecting zeranol in beef meat

Spike level (ng/g)	Recovery (%)	Intraday RSD (%)*
2	103.48± 4.31	4.16
5	73.96±11.10	15.01
10	77.51±10.26	13.24

Note: \*Intraday RSD (relative standard deviation) (n= 9), average of three different concentrations repeated three times within a day.

Table 3. Zeranol residues in beef meat samples collected from different locations

Sampling locations	Types of samples	N samples	n positive samples (%)	Concentration range (ng/g)	n sample ≥ 2 ng/g
Jakarta	Premium imported meat	5	ND	ND	ND
Jakarta	Non-premium imported meat	50	7 (14.00)	1.67-33.29	2
Malang	Fattening meat	28	3 (10.71)	2.40-5.37	3
Surabaya	Domestic meat	22	2 (9.09)	2.70-5.21	2
	Total	105	12 (11.43)	1.67-33.29	7

Note: ND= not detected

### Detection of Zeranol in Meat Samples

Table 3 summarizes the results on monitoring the occurrence of zeranol residue in this study. Zeranol was positively detected in 7 (14%) out of 50 non-premium meat samples, 3 (10.71%) out of 28 fattening-beef meat samples from a slaughterhouse in Malang, and 2 (9.09%) out of 22 domestic meat samples bought in markets in Surabaya with the concentration range of 2.70-5.21 ng/g. There were 7 among 105 samples having zeranol concentration above the MRL (2 ng/g) and the highest level (33.29 ng/g) also was found in the meat sample from Jakarta. None of the zeranol was detected in 5 of those premium imported meat samples.

### DISCUSSION

The half method validation results presented in Table 1 and Table 2 were obtained for the lowest laboratory validation level with the simplest level of validation requirements and are appropriate for confirmation within the limited application, which are achievable and repeatable. Linearity is typically demonstrated via least-square regression, which often judged by examining the correlation coefficient and y-intercept, and residual sum of squares. A correlation coefficient of 0.9933 met the criteria of more than 0.99, which is generally acceptable. The mean recovery (n= 3) of the analytes was found by experiment to lie between 73.96% and 103.48% and meet the requirement of 70%-110%.

The C<sub>18</sub> non-polar sorbent reversed-phase column is the most frequently used for veterinary drug analysis, including zeranol. In this study, zeranol eluted at 3.9 minutes with the use of Shimp-pack VP ODS column (4.6 mm×15 mm) and run isocratically at a flow rate of 1 mL/min. Liu *et al.* (2007) applied the same mobile phase on Waters Sphrisorb® S5 ODS column (4.6 mm×20 mm), which run at a flow rate of 1.2 mL/min, and revealed

that zeranol eluted at 12.2 minutes. Therefore, the results show that the HPLC method presented in this study can be considered suitable for the analytical determination of zeranol in bovine meat, owing to quick, easy to perform, having linearity in the concentration range used, and precision and adequate accuracy at the concentrations studied.

The results in Table 3 showed that the presence of zeranol residue in non-premium imported meat indicates that this substance probably might be from animals that may have been treated shortly before slaughtered in the country of origin (Australia or New Zealand) or the animals were slaughtered before reaching the withdrawal time (60-65 days) after treatment (Kart *et al.*, 2008), and the live animals imported to Indonesia did not all go through a quarantine process (Danial *et al.*, 2015). Whereas for fattening, positive samples indicate that those animals may have been treated shortly before shipping or after arrival in the country and slaughtered before reaching the withdrawal period.

Even though zeranol is banned from being used in Indonesia, there were 2 domestic meat samples also contaminated by zeranol, that might arise from illegal used or interconversion (see Figure 3) from feed contaminated with mycotoxin zearalenone produced by *Fusarium* sp which metabolized to zeranol and are formed naturally in urine and bile after animals consume the zearalenone (Kleinoca *et al.*, 2002). Therefore, it is necessary to study further on the source of zeranol contamination in animal products (meat), using the appropriate methods (Matraszczek-Zuchowska *et al.*, 2012). For compliance reasons, control laboratories should be able to differentiate the presence of zeranol resulting from administration and natural exposure of zearalenone using an analytical tool such as a GCMSMS (Dusi *et al.*, 2009).

The prevalence of the residue presence obtained in our study (detection by HPLC) of 11.43% at concentration levels of 1.67 to 33.29 ng/g, showed higher preva-

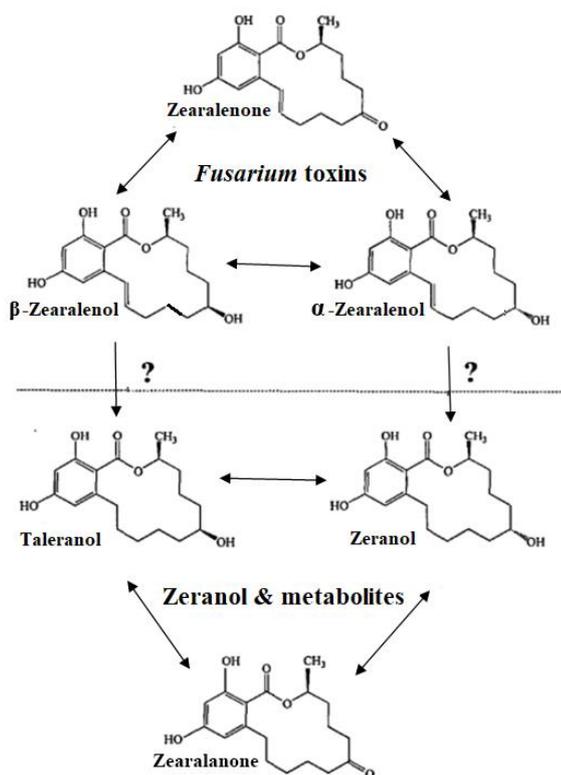


Figure 3. The possible metabolic interconversion of zearanol and its metabolites (zeranol, taleranol, and zearalanone) and the *Fusarium* spp. toxins (zearalanone,  $\alpha$ -zearalenol, and  $\beta$ -zearalenol) (adopted from Kennedy *et al.*, 1998).

lence and higher contamination levels compared to the result of the previous study in Indonesia conducted by Khadijah *et al.* (2015) (detection by ELISA method), i.e., 5.08% at concentration levels of 0.53 to 0.92 ng/g. The present results indicate the possibility of misuse of this HGP, which in the future may adversely affect human health.

For comparison, the ban of zearanol in Europe did not mean the absence of zearanol in beef meat samples analyzed. Mor *et al.* (2011) detected zearanol in 11 out of 30 samples collected from a slaughterhouse in Burdur (Turkey) at concentration levels of 100 to 500 ng/g. Şevik & Ayaz (2017) found zearanol residue at the levels of 0.10, 0.09, and 0.61 ng/g among 200 cattle meat samples from the butchers and supermarket in Kocaeli, Turkey. Yücel *et al.* (2018) detected zearanol in 24 among 80 cattle meat samples within the concentration range of 0.30-0.50 ng/g, with the highest concentration of 1.81 ng/g and noticed that the highest values were found to be that of September and October and Kukhtyn *et al.* (2020) still found zearanol residue on 29.8% of beef samples collected from meat processing enterprises of the Western region in Ukraine. On the other hand, Salata (2018) nor Cayci *et al.* (2019) did not detect zearanol residue in cattle meat.

## CONCLUSION

Zeranol residue had been detected in 12 (11.43%) samples (non-premium and domestic beef samples) at

the concentration of 1.67 to 33.29 ng/g among 105 bovine meat samples that were analyzed by HPLC. There were 7 samples among them had the concentrations above the MRL of 2 ng/g. The results obtained in this study indicate zeranol was still being used to increase meat cattle production. Therefore, strict control must be implemented at all stages from production to consumption, regarding the application of this HGP in livestock.

## CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript. Both authors have also contributed equally.

## ACKNOWLEDGEMENT

We would like to acknowledge the invaluable thank to the Ministry of Agriculture through the Indonesian Agency for Agricultural Research and Development, for supporting this study by the Indonesian Research Center for Veterinary Science (IRCVS) for funding this study. This work was conducted with the permissions and help from the staffs from Soekarno-Hatta Agricultural Quarantine Center and Tanjung Priok Agricultural Quarantine Center, the Head of Livestock Services in Malang and Surabaya City. Part of this article had been displayed at the Eighth International Symposium on Hormone and Veterinary Drug Residue Analysis, Ghent, Belgium, 22-25 May 2018.

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