Slamet Widiyanto et al./Animal Production 16(3):193-201, September 2014
Accredited by DGHE No. 81/DIKTI/Kep./2011. ISSN 1411-2027

Stress Estimation of Pre-Slaughter and Slaughtered by Means of Fourier Transform Infrared Spectroscopy Analysis Through Measurement of Cortisol and Catecholamine Level in Female Cattle Urine

Slamet Widiyanto[1)*], Irkham Widiyono[2)], Prabowo Purwono Putro[2)] and Pudji Astuti[2)]

[1)]**Department of Animal Physiology, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia.**
[2)]**Department of Physiology, Faculty of Veterinary, Gadjah Mada University, Yogyakarta, Indonesia**
[*]**Corresponding author email: slametbio@ugm.ac.id; slametbiougm@yahoo.com. Phone: 08122966114**

Abstract. This study was aimed to analyze the levels of catecholamine and cortisol as stress indicator by invasive and non-invasive methods. Twelve female cattle PO were used in this study and were collected from slaughter house in Yogyakarta. Catecholamine and cortisol level of urine were measured by Enzyme linked Immunoassay (EIA) method and Fourier Transform Infrared (FTIR), were statistically analyzed to determine the difference between pre slaughter and slaughter conditions. FTIR spectra were analyzed using chemometrics software. These results showed that the concentration of urinary cortisol were 2.12±1.68 ng/dl of pre-slaughter and 7.58±3.89 ng/dl of slaughtered respectively. The levels of urinary catecholamine in pre slaughter and slaughter were 3.07±2.05 ng/dl and 4.15±2.68 ng/dl respectively. In FTIR analysis showed the spectral separation between the different quadrants before and during slaughter. The result suggested the correlation between the results of the analysis using the EIA and FTIR spectra. It is assumed that the separation of the FTIR spectrum in line with the increased levels of cortisol catecholamine and samples. It can be concluded that FTIR can be used to analyze the status of stress in animals, especially in cattle.

Key words: catecholamine, cortisol, non-invasive, FTIR

Abstrak. Penelitian ini bertujuan untuk menganalisis kadar katekolamin dan kortisol sebagai indicator stress secara invasif maupun non invasif dan mengembangkan metode deteksi stress pada sapi. Penelitian menggunakan sampel urin sapi Peranakan Ongole (PO) yang diambil dari rumah potong hewan di Yogyakarta. Analisis kadar katekolamin (CA) dan kortisol (CO) dilakukan dengan menggunakan metode EIA dan FTIR. Data konsentrasi kortisol maupun katekolamin hasil pengukuran menggunakan metode EIA dilakukan analisis statistik untuk mengetahui pengaruh stres terhadap konsentrasi CA dan CO urine. Hasil penelitian ini menunjukan rata-rata konsentrasi kortisol urin adalah 2.12±1.68 ng/dl dalam kondisi prapenyembelihan dan 7,58±3,89 ng/dl pada saat penyembelihan. Sedangkan konsentrasi katekolamin urin pada saat prapenyembelihan dan penyembelihan secara berurutan adalah 3,07±2,05 ng/dl dan 4,15±2,68 ng/dl. Analisis FTIR menggambarkan pemisahan spektral pada quadran berbeda antara sebelum dan saat penyembelihan. Berdasarkan hasil ini menunjukkan korelasi antara hasil analisis dengan menggunakan EIA dan FTIR, baik konsentrasi katekolamin dan kortisol dengan spektra FTIR dari sampel. Perbedaan konsentrasi CA dan CO urin prapenyembelihan dan saat penyembelihan sejelan dengan pemisahan spektra FTIR. Hal ini diyakini bahwa pemisahan spektrum FTIR disebabkan adanya peningkatan kadar katekolamin dan kortisol dari sampel. Dapat disimpulkan bahwa FTIR dapat untuk menganalisis status stres pada hewan khususnya pada sapi.

Kata kunci : katekolamin, kortisol, non invasif, FTIR

Introduction

Animal welfare is a major consideration in meat production and is based upon the belief that animals also feel during the sacrifice. Welfare may be considered in terms of the subject experiences of animals (measured using preference testing) or in terms of biological functioning (measured using reactions to stress including plasma levels of glucocorticoids, catecholamine, prolactin and endorphins, as well as heart rate and brain levels of neurotransmitters) (Manteca, 1998).

Stress is the inevitable consequence of the process of transferring animals from farm (pre-slaughter activities) to slaughter. The pre-slaughter phase includes the conditions and practices that apply during the period when the animal is moved or mustered on-farm to the slaughterhouse or knocking box. These activities and processes take place on the farm, during transportation, marketing and at the slaughter plan (Ferguson and Warner, 2008). During this period, animals possible to expose to a range of challenging stimuli including: (i) handling and increased human contact; (ii) transport; (iii) novel/unfamiliar environments; (iv) food and water deprivation; (v) changes in social structure (i.e. through separation and mixing), and (vi) changes in climatic conditions. As a consequence of these pre-slaughter challenges, animal may experience fear, dehydration and hunger, increased physical activity and fatigue also physical injury. Moreover, the inability to adequately resolve some of these conditions (e.g. dehydration or fatigue) may invoke further psychological distress (Ndou et al., 2011; Rushen, 2008).

The activation and regulation of the neuroendocrinal response to fear-eliciting stimuli has been studied extensively (Chrousos, 1998; Moberg, 2001; Steckler, 2005). The two central integrated processes include the autonomic nervous system and hypothalamic–pituitary–adrenal (HPA) axis. An autonomic response is typically initiated in reaction to acute stress that require a rapid response. Obvious physiological changes include tachycardia, increased respiration rate, elevated body temperature and redistribution of visceral blood volume towards skeletal muscle and the brain. Behavioural changes are also evident including heightened alertness, immobilisation, aggression and escape/avoidance. The sympatho-adrenal component of the autonomic response is mediated by catecholamines (epinephrine and norepinephrine). Activation of the HPA axis is manifested by the release of glucocorticoids (e.g. cortisol) from the adrenal cortex. It is also worth noting that the HPA axis operates independently of stressful situations in a circadian manner (Chrousos, 1998). The secretion of catecholamine resulted significant changes in energy metabolism including lipolysis, glycogenolysis in muscle and gluconeogenesis (Kuchel, 1991). Several physiological indicators that are measured cortisol and catecholamine levels as an indicator of stress. Concentrations of plasma and urine both of cortisol and catecholamine have been widely used to quantitatively assess the stress response in animals (Tessa et al., 1997).

The quantitative methods that commonly used to measure stress hormones and their metabolites are Enzyme linked Immunoassay (EIA) and Radioactive Immunoassay (RIA). Both methods produce accurate data. However, the materials and equipment that they need are expensive. Both methods are also not practical to be used on small number of samples and have relatively short expired period of their materials. RIA even has a high risk of radiation due to its destructive radioisotope (Maryam, 2007). Fourier Transform Infrared (FTIR) is one of safe and applicable alternative methods to measure compound level of samples both natural and artificial. The method has been used to measure protein level of food and drink, such as milk (Suseno and Firdausi, 2008). Protein and glucose in plasma (Petibois et al., 2001), as well as blood analysis of kidney failure patients (Renuga et al., 2009), urea level of urine measurement as well (Ohnishi et al., 2000; Sjahfirdi et al., 2010; Sjahfirdi et al., 2011).

Recently the role of infra red methods is greatly increased in biomedical analysis of hormones. The FTIR imaging play an important role in the study of the structure-activity relationship for hormones (Minaeva et al., 2008). FTIR has been used as universal

instrument to analyse varieties of samples due to its ability to identify functional group of chemical compounds, such as carbohydrate and ester, as well as inter atom chemical bonds. Every organic molecule of the sample absorbs in the mid-infrared region (4000–500 cm^{-1}) (Petibios et al., 2000). FTIR has high accuracy level in identifying process (Syahfirdi et al., 2012).

Therefore, the infrared spectrum was the fingerprint of a molecule (Smith, 1979). The FTIR would then identify a sample on functional group level. The different bindings such as C-C, C=C, C-C, C-O, C=O, O-H and NH have their own characteristic frequencies as absorption bands in infrared spectrum. These bindings would be identified on different wave numbers according to the absorption bands in infrared spectrums (Suseno and Firdausi, 2008). Measurement of stress physiological parameters such as cortisol and catecholamine level with non-invasive method based methods FTIR is expected to provide results more quickly and accurately.

Materials and Methods

The experimental protocol was approved by the Animal Ethics Committee of The Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta Indonesia, according to number 115/KEC-LPPT/VII/2013, dated July 30, 2013.

Animals. Twelve female cattle (*Bos indicus),* with 400-600 kg of body weight were used in this research. All cattle were housed in a cage house with good standard, with 12 hours photoperiod cycle and were given food and water.

Sample Collections. Urine samples (10 ml) were collected using urine tube from pre-slaughter and slaughter and stored at -80^{0}C before the measurement with FTIR and ELISA. Pre-slaughter urine samples (as control sample) were collected from female cattle with continuous stroking of the skin just below the vulva to induce urination and from the bladder at the time of slaughter to evaluate the possibility of stress due to slaughter. The urine were immediately analyzed or stored at -80^{0}C for further analysis.

Analysis. Spectrum-One ABB MIRAcle Type MB3000 FTIR Spectrophotometer was used in this research. The spectrum recorded in the mid-infrared region of 4000-650 cm^{-1}. FTIR spectra for all samples were measured using FTIR equipped with a deuterated triglycine sulfate detector and KBr beam splitter is connected to the computer operating system software. Using micro pipette sample was placed in the contact section of the FTIR instrument with Horizontal Attenuated Total Reference (HATR) elements at a controlled ambient temperature (25^{0}C). FTIR spectra were collected in the region of 4000-650 cm-1 from the Min-infrared by adding 32 scans and at a resolution of 4 cm^{-1}. These spectra were subtracted from reference spectrum of air acquired by collecting a spectrum from the cleaned blank HATR crystal before the measurement of each sample replication. At the end of every scan, the surface of HATR crystal was cleaned with hexane twice and dried with soft tissue following the collection of each spectrum.

Level of urinary cortisol (CO) and catecholamine (CA) were assayed using commercial KITS products by DRG Instruments GmbH, German with Enzyme Immuno Assay (EIA) method. The data of Urine CO and CA level also FTIR spectra were calculated and presented as mean ± SD. The anova test was used to determine the reliability of the differences between the tested parameters levels. Differences considered significant at $P<0.05$. Duncan's correlation coefficient was used for statistical association between parameters.

Results and Discussion

Level of cortisol and catecholamine. At the first step of the research was conducted to find optimal characteristic data of CA and CO urine concentration by EIA method, to create further a model for stress estimation of female cattle of pre-slaughter and slaughtered.

In general, the urine and blood CO in pre-slaughter condition, found in this experiment, ranged between 0.44-4.79 ng/dl. After transport to the slaughter house and slaughtered, CO of urine increased in range between 2.51–13.46 ng/dl. The cortisol data are summarized in Figures 1 and Table 1. The average level of cortisol urine were 2.16±1.68 µg/dl in pre-slaughter condition and 6.98±3.26 µg/mg in slaughter plan. An increase in the concentration of cortisol in urine both pre slaughter and slaughter plan. The mean percentage increase of urinary cortisol was 222.58%. According to Peter and Bosu (1987) on bovine serum cortisol levels increased 100 times during high stress, based on it can be predicted that the animal handling of slaughterhouse led to a very stressful time.

CA secretion is a primary neural response to stress stimuli through activation of the sympathetic nervous system thoroughly. The hypothalamus will help to prepare the body to fight due to stress stimuli. Stimulation of the sympathetic nerves to the medulla adrenaline causes the release of large amounts of epinephrine and norepinephrine into the blood circulation, and both hormones are then carried in the blood to all tissues of the body (Guyton, 2000; Sherwood, 1996)

Table 1. Urinary cortisol (CO) level (ng/dl) of female cattle for pre-slaughter and slaughter condition

Sample Code	Pre-slaughter CO	Slaughtered CO
2	4.79±1.05	5.59±2.21
12	1.36±0.07	4.50±0.44
18	0.44±0.21	3.00±0.00
22	0.64±0.16	10.40±4.64
30	3.03±1.55	7.86±1.95
31	4.47±8.66	9.39±2.46
49	1.77±1.32	2.51±2.04
50	2.13±0.84	6.00±0.58
51	0.74±0.01	7.49±1.34
52	0.56±0.01	9.21±2.33
53	4.79±1.71	13.46±3.23
54	1.25±0.21	4.31±0.01

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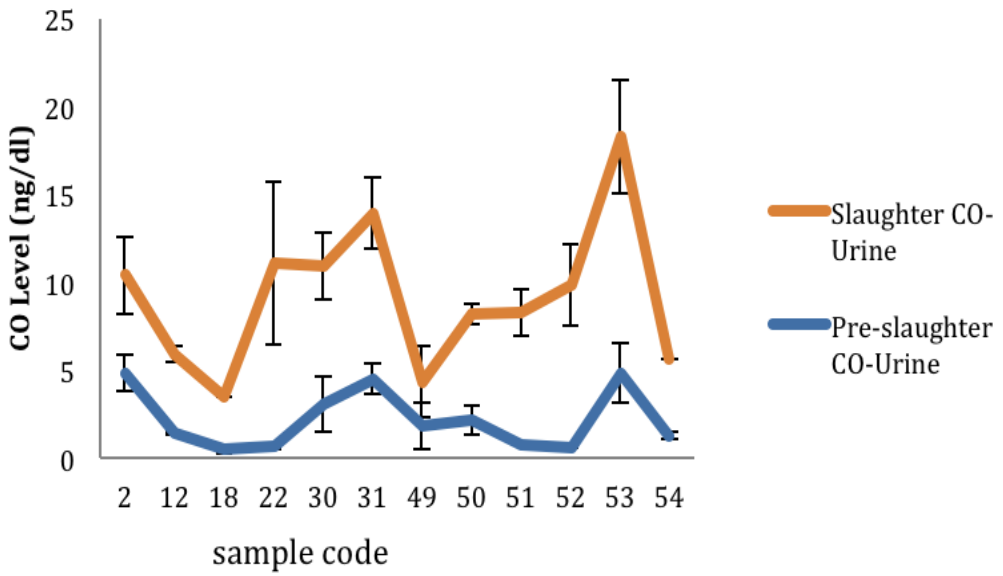

Figure 1. Comparison level of urinary CO between pre-slaughter and slaughter time conditio

The results of measurements of the urinary catecholamine levels were show in Figure 2 and Table 2. Urinary catecholamine level during the pre- slaughter is lower than the level of slaughter time. The levels of urinary catecholamine in pre slaughter and slaughter were 3.07 ± 2.05 ng/dl and 4.15 ± 2.68 ng/dl respectively. Based on these data showed that an increase in the levels of urine cortisol and catecholamine more than 100% in the slaughter than pre-slaughter condition.

Table 2. Catecholamine (CA) level (ng/dl) of female cattle urine for pre-slaughter and slaughter condition

Sample Code	CA pre-slaughter	CA slaughtered
2	5.88±0.93	7.90±0.41
12	6.64±0.41	8.17±0.15
18	3.20±0.09	6.08±0.43
22	5.06±0.66	5.72±0.28
30	2.49±0.33	2.93±0.19
31	3.64±0.57	4.79±1.67
49	0.74±0.09	0.85±0.41
50	2.93±0.19	3.09±0.49
51	3.04±0.53	3.15±1.15
52	0.35±0.09	0.97±1.33
53	2.71±0.53	5.83±0.90
54	0.19±0.19	0.31±0.28

FTIR spectra of urine. In this step was conducted to obtain the characteristic urine spectra by FTIR spectroscopy, to create further a model for stress estimation of pre-slaughter and slaughter time using calibration analysis with urine cortisol and catecholamine levels. Fourier Transform Infrared (FTIR) is a universal instrument that has been used to analyze organic/inorganic samples. The advantages of using FTIR are accurate, safe, rapid, and sensitive (Smith 1979; Rintoul et al., 1998).

Based on the principle works FTIR can identify specific functional groups within a component. Special on CA and CO, FTIR can identify the component methyl group (CH3), ketone (=O), methylene (NH2) and OH. Each functional group can be recorded in a specific wavelength.

Samples were identified by FTIR could not be prepared evenly in terms of the thickness. The different thickness has caused functional group spectrums gave different absorbance although they had the same concentration level. Therefore, same number of compounds in each samples needed to be determined first. Cortisol and catecholamine had the difference concentration level in urine of pre and slaughter condition in this research.

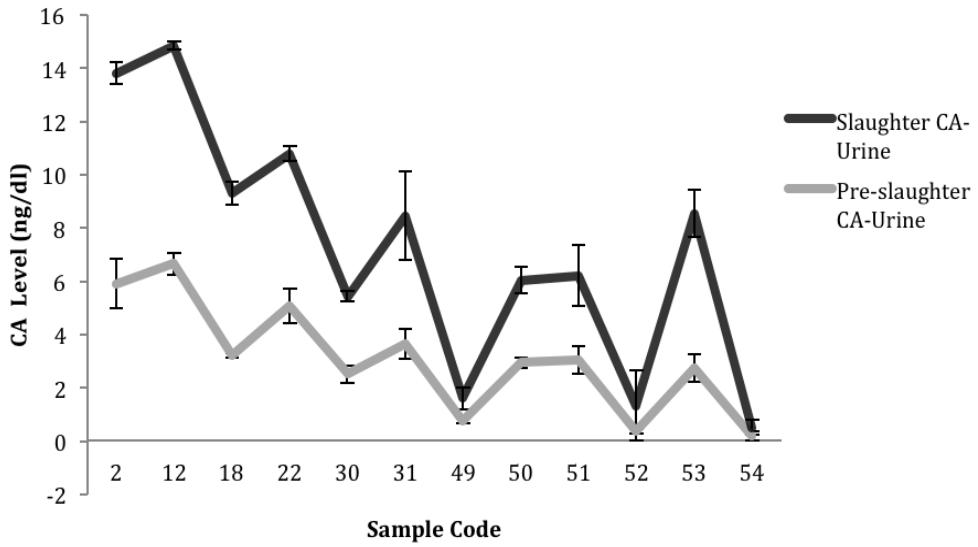

Figure 2. Urine Catecholamine level of pre-slaughter and slaughter time condition.

Figure 3 showed FTIR spectrums of cattle urine during pre-slaughter and slaughter time. Similar spectrums were also obtained from serum during pre-slaughter and slaughter time. FTIR spectrum of pre-slaughter and slaughter both in urine and serum showed separate spectra.

It was shown in Figure 3 the essential peaks of urine and serum, which were on 500-900, 1500-1650, and 3000-3650. In the Figure, shows that the different spectra between pre-slaughter and at the time of slaughter. Each represented compound functional groups, including hormone metabolites and catecholamine in urine and serum.

Urine contains many compounds each having their own absorption spectrum in the mid-IR region. Thus, the IR spectrum of urine is a superposition of all these individual spectra and the intensities of the absorption bands in this spectrum are proportional to concentrations of the components (functional group level) (the Bouguer- Lambert-Beer law). The urine spectrum contains several absorption bands in the mid-IR region (4000–550 cm^{-1}) which are typical for biological samples (Figure 1). The peaks with wave numbers 1300-900 cm^{-1}, 1630-1530 cm^{-1}, 1720-1600 cm^{-1} and 2880-2850 cm^{-1} are usually identified in functional group materials and characterize C-O (lactate, glycerol, saccharide), NH_2 (Amine), C-H (Amide) and CH_3 bending vibrations, respectively (Petibios et al., 2000).

The obtained spectra were used to demonstrate the stress condition of pre-slaughter and slaughter time via chemo-metrical analysis (Spectrum-One ABB MIRAcle Software). This software is used to view the differences between the spectra of the sample group (pre-slaughter and slaughter time). Based on the results of chemo-metrics analysis, spectra were classified using Principle Component Alnaysis software.

In Figure 4 showed the result of FTIR spectra analysis using chemo-metric software in 2880-2850, 1720-1600, 1630-1530 and 900-1300 CM^{-1}, which shows that the spectra of pre-slaughter and slaughter urine separately on different quadrant.

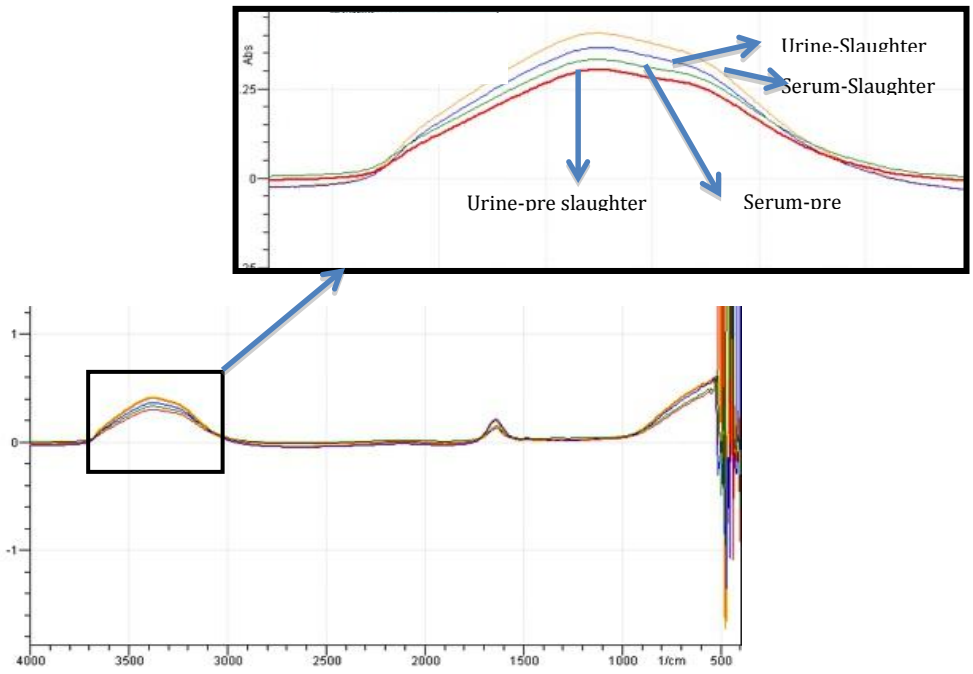

Figure 3. FTIR spectra pattern of cattle female urine and serum for pre-slaughter and slaughter time in region 4000 – 500 CM^{-1}

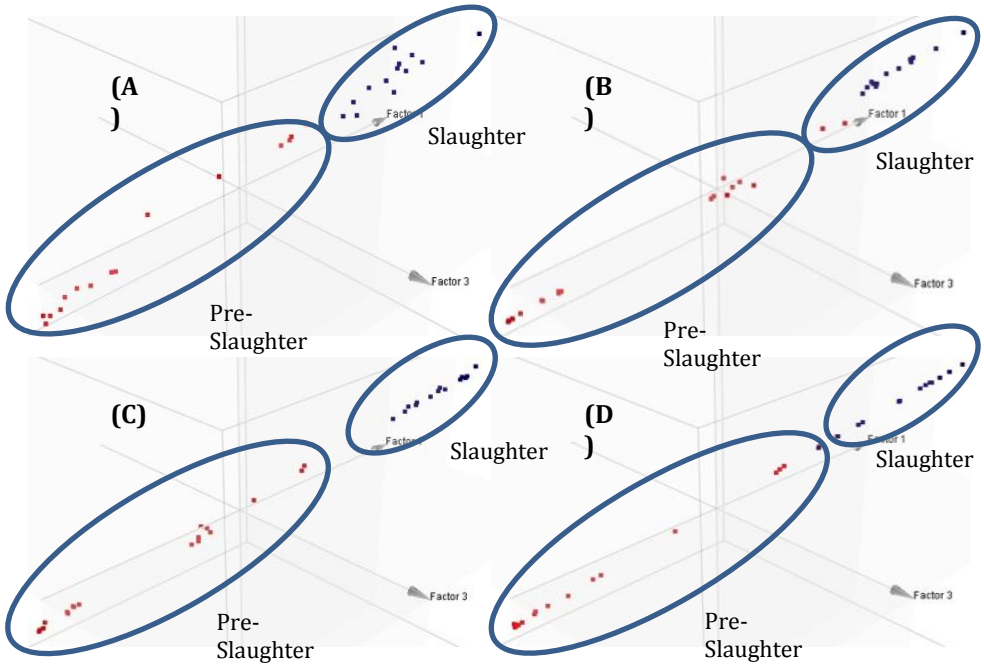

Figure 4. FTIR spectra of female cattle urine
(a) 900-1300 CM^{-1}, (b) 1630-1530 CM^{-1}, (c) 1720-1600 CM^{-1}, and (d) 2880-2850 CM^{-1}.

Correlation. In recent years there are an increased number of studies on the relationships between the dynamics of change in CA and CO as indicators under different stress conditions (Linares et al., 2007; Khaustova et al, 2010; and Astuti et al., 2014). Most of these studies were done by measuring multiple parameters with the invasive method. Integrated analysis of three major regulatory systems biomarkers in the urine of cattle during slaughter by using FTIR performed for the first time.

Both CA and CO levels are indices of stress hormonal systems, but the extent to which these 2 systems are correlated is unclear. Using ELISA and FTIR, we examine how the relationship between the concentration of CA and CO in urine and serum Cattle for pre and time of slaughter. CO is a relatively long term effector: it has a half-life measured in hours, and its effects last even longer. CA is an immediate-response hormone: it has a short half-life, and its effects disappear rapidly if the hormone is no longer present. This difference is a consequence of the mechanism by which the hormones act. CO increases (or decreases) the amount of a given enzyme. Epinephrine acts by modulating the activity of existing enzymes (Elizabeth and Naomi, 2004).

CA and CO concentration measurements using ELISA method in urine samples showed the difference between the pre-slaughter and slaughter time. Has an increase in the concentration of CA and CO at the time of slaughter in urine samples. In FTIR analysis of the wave numbers 1300-900 cm^{-1}, 1630-1530 cm^{-1}, 1720-1600 cm^{-1} and 2880-2850 cm^{-1} are usually group-identified in functional materials and characterize CO (lactate, glycerol, saccharide) NH_2 (Amine), CH (Amide) and CH_3 bending vibrations, respectively (Petibios et al., 2000) illustrates the spectral separation occurs at different quadran between pre and time of slaughter. Based on these results demonstrate compatibility between the results of the analysis using ELISA and FTIR, both at the levels of CA and CO from the sample. It is assumed that the separation of the FTIR spectra in line with the increased levels of CA and CO from the sample.

In result, the urine cortisol in pre slaughter condition, found in this experiment, ranged between 0.5-4.7 µg/dl. After transport to the slaughterhouse CO of urine increased in range between 3-14.1 µg/dl. The mean levels of urinary CA in pre slaughter and slaughter were 3:07±2:05 µg/dl and 2.68±4.15 µg/dl respectively. Based on these results it can be concluded that FTIR can be developed as an instrument that can be used in the determination of the physiological status of particular stress in cattle.

Conclusions

Pre-slaughter handling at the slaughter house animals can cause stress as indicated by increased levels of urinary cortisol and catecholamine, that are 1.68±2:12 and 3:07± 2:05 ng/dl in the pre-slaughter conditions and 07:58±3.89 and 2.68±4.15 ng / dl in the slaughter of each plan. Accordingly, the results of the analysis of chemo-matrick cattle urine FTIR spectrum of wave numbers 1300-900 cm^{-1}, 1630-1530 cm^{-1}, 1720-1600 cm^{-1} and 2880-2850 cm^{-1} resulted in a difference quadrant between the pre-slaughter and slaughtered spectrum. Based on these results it can be concluded that FTIR can be developed as an instrument that can be used in the determination of the physiological status of a particular stress on the cattle.

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