Estrus Period Determination of Female Rats (*Rattus norvegicus*) by Fourier Transform Infrared (FTIR) through Identification of Reproductive Hormones Metabolites in Urine Samples

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***Abstract*— Recently, some fertility determination methods by determining estrus cycle through analysis of the existence and level of estradiol hormones metabolites in urine such as estrone conjugate (E_1C) and pregnanediol 3-glucuronide (PdG) have been developed. Those hormone determination analyses were using destructive methods, which were complicated and highly-cost. Therefore, a fertility analysis was conducted in this research by determining the E_1C and PdG level using the FTIR method. The samples in this research were urine of 3 estrus and non-estrus periods of the cycle from 10 rat. Those samples were analysed by 2 methods: qualitative method with vaginal smears which observing proportion of mature and immature epithelial cells of vagina, and FTIR method. The FTIR analysis results were spectrums with absorbancy numbers which then been used to determine E_1C and PdG level. The absorbancy values were compared relatively with similar creatinine values throughout the cycles. Creatinine was identified on ketone peak number 1730 cm^{-1}. The low SD values showed that creatinine had constant values throughout the cycles. Functional group absorbance represented E_1C and PdG were compared to creatinine. PdG was identified on aldehyde peak number 1639 cm^{-1}, carboxyl acid number 1745 cm^{-1} and alkyl 1379 cm^{-1}. E_1C was identified on alkyl peak number 416 cm^{-1}, aromatic 709 cm^{-1} and hydroxyl 3468 cm^{-1}.**

***Index Term* – FTIR, functional group, vaginal smear, pregnanediol 3-glucuronide, estrone conjugate, estrus cycle.**

I. INTRODUCTION

Nowadays, observing the frequency of female steroid reproductive hormones are essentially important and needed for learning and checking the variations of an individual or population, as well as knowing its relation with demography, health, environment, socio-culture and biological condition of them (O'Connor et al. 2003: 1139).

The fertility detection and measurement methods that are commonly used work by measuring steroid reproductive hormones, such as estrogen and progesterone. Those methods need to use blood, serum or plasma samples that are collected invasively. Nowadays, the non-invasive sample collection has been developed using saliva, urine or fecal by measuring the derivatives of steroid hormones as an alternative (Monfort et al. 1987: 832). This research used urine as samples due to its excellence compared to fecal. Urine collection is easier and infection-free. The donors are able to collect it on their own as well. Urine can be tested directly after the collection, need no special extraction treatments and can be kept in room temperature for certain period of time (Monfort 1987: 833; O'Connor et al. 2003: 140).

Estrone conjugate (E_1C) and pregnanediol 3-glucuronide (PdG) are steroid metabolites of estrogen and progesterone in blood. E_1C and PdG are excreted through urine as metabolites of estrogen and progesterone which have been inactivated in the liver by saturating their bonds and adding them with sulphate group or glucuronide to make them dissolvable in water so that able to be excreted through urine (Nalbandov 1990: 228-229).

The quantitative methods that commonly used to measure reproductive hormones and their metabolites are EIA (Enzyme linked Immunoassay) and RIA (Radioactive Immunoassay). Both methods produce accurate data. However, the materials and equipments 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: 19--21).

FTIR (Fourier Transform Infrared) is one of safe and applicable alternative methods to measure compound level of samples. FTIR is commonly used for compound identifying, both natural and artificial. The method has been used to measure protein level of food and drink, such as milk (Suseno & Firdausi 2008: 23). It also has been applied to measure protein and glucose in blood plasma (Petibois et al. 2001:49), as well as blood analysis of kidney failure patients (Renuga Devi dkk. 2009: 49). FTIR has been used in crystal and urea level of urine measurement as well (Ohnishi et al. 2000: 299, Sjahfirdi et al 2010: A 003 & Sjahfirdi et al 2011(in press))

In FTIR, two chemically different compound molecules have also different infrared spectrums. It is due to the difference in their binding type and vibratory frequency. Although they have the same binding type, the vibratory frequency would be different if they were two different compounds. Therefore, the infrared spectrum was the

fingerprint of a molecule (Smith 1979: 3 & 123). 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 & Firdausi 2008: 24).

Analysis of estrone conjugate (E_1C) and pregnanediol 2-glucuronide (PdG) using FTIR method has not been applied. Functional group structures of E_1C consisted of aromatic ring, C=O (ketone) and R-CH_3 (methyl). Each of this functional group would be identified on different wave number or frequency according to its absorbance spectrums. The absorbance result would describe E_1C and PdG. The E_1C level would be determined by comparing it with creatinine that has constant level and specific structure and wave number on the samples. Fluctuation of E_1C level found in urine then being used to analyse the fertility cycles.

II. METHODHOLOGY

A. Animals

Ten female rat (*Rattus norvegicus*) strain Sprague-Dawley 3-month old with 150-200 g of weight were used. Every 2 rats were housed in a cage (30x20x10) cm^3 with woodchip bedding, 23^oC of temperature with 12 hours photoperiod cycle and were given food and water by *ad libitum*.

B. Sample Collections

Urine samples were collected from estrus and non-estrus periods of the cycles. Samples were collected using metabolic cage. One ml of urine was collected inside a tube and stored at 8^oC before the measurement with FTIR conducted.

C. Analysis

Vaginal smears were taken before the collection of urine samples to know the difference between estrus and non-estrus period, also the phase happened in each rat. The vaginal smears always took place before every sample collections.

FTIR type IRPrestige-21 SHIMADZU was the type of FTIR used in this research. Urine samples were first smeared on to object glass made of ZnSe, then scanned on 300--400 cm^{-1} wave number. Other equipments used were light microscope, vaginal smearing kit, and animal cage.

E_1C and PdG would be described as the peaks on graphic results. The E_1C and PdG levels were obtained from comparing the specific peaks of E_1C and PdG with creatinine.

III. RESULTS AND DISCUSSION

A. FTIR Spectra of Urine

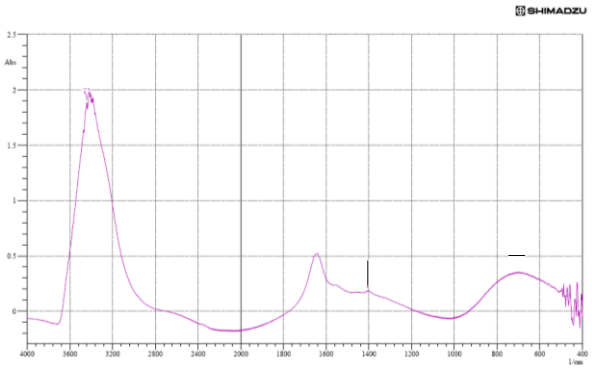

Fig. 1. FTIR spectrums of rat urine

Figure 1 showed FTIR spectrums of rat urine during estrus period. Similar spectrums were also obtained from blood during non-estrus period. The similarity of estrus and non-estrus spectrums caused difficulty in detecting the functional groups that represented E_1C and PdG. Therefore, the statistic method was used in determination of this research.

It was shown in Figure 1 the essential peaks of urine, which were on 400, 1400, 1700, 2400 and 3300. Each represented compound functional groups, including reproductive hormone metabolites in urine. The determination of E_1C and PdG contained in urine can be described as following.

B. Determination of Functional Groups

1. Creatinine

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. Creatinine had the same concentration level in urine of every condition, estrus and non-estrus, in this research. Creatinine was the derivative product of phosphate creatine in muscles and commonly was produced in constant level by the body depending on muscle mass (Fischback & Dunning 2009: 274).

When functional group absorbance in urine was compared relatively to creatinine absorbance, the functional group concentration would be obtained as relative absorbance value. The validation with concentration gained from measurement of blood samples would produce the same functional group concentration as estradiol and progesterone hormones.

As shown in Figure 1 about urine FTIR spectrums, there were many peaks represented various compounds of urine. A more detailed identification showed thousands of peaks were in the spectrums. With the assumption that creatinine had constant concentration level throughout cycle, the creatinine absorbance would be the same. Out of thousands of peaks data, the peaks that represented creatinine would

therefore have the smallest average absorbance and deviation standard, as shown in Table I.

Table I
Peak absorbance 1730 cm^{-1}

Estrus		Non-estrus	
Animals	Wave number (cm^{-1}) 1730 (Ketone)	Animals	Wave number (cm^{-1}) 1730(Ketone)
Rat 1	0.467	Rat 1	0.227
Rat 2	0.121	Rat 2	0.404
Rat 3	0.147	Rat 3	0.221
Rat 4	0.297	Rat 4	0.352
Rat 5	0.189	Rat 5	0.388
Rat 6	0.214	Rat 6	0.204
Rat 7	0.120	Rat 7	0.363
Rat 8	0.131	Rat 8	0.133
Rat 9	0.111	Rat 9	0.227
Rat 10	0.530	Rat 10	0.146
Average	0.233	Average	0.253
SD	0.152	SD	0.097

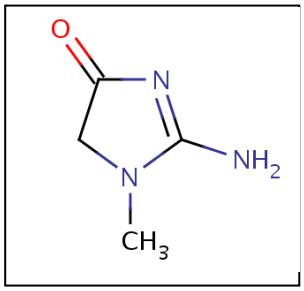

Fig. 2. Structure of creatinine

Table 1 showed the average and deviation standard of peak absorbance on 1730 cm^{-1}. The small average difference between estrus and non-estrus, as well as small deviation standard showed that the peak represented creatinine. The 1730cm^{-1} peak was the ketone peak of creatinine (Fig 2). Ketone was sharply identified on 1705--1730 cm^{-1} wave numbers (Smith 1979: 304).

2. Pregnanediol 3-glucuronide

The PdG urine determination was based on fluctuation of its concentration during estrus and non-estrus period. Concentration fluctuation of PdG and E_1C throughout cycle theoretically (Shimizu 2005: 3) could be shown in figure below.

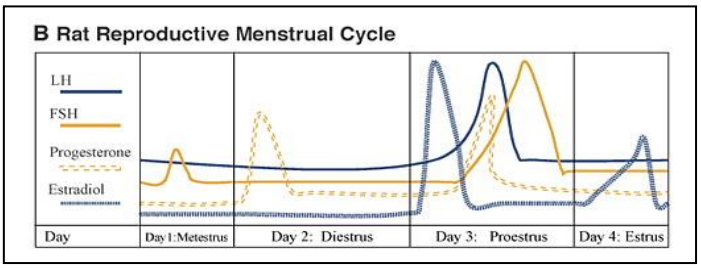

Fig. 3. Estrogen and progesterone hormones cycle of rat

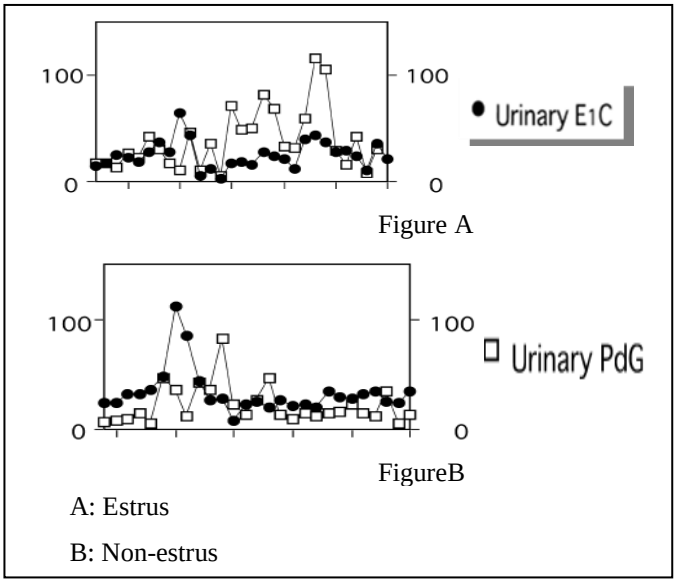

Fig. 4. Estrone conjugate (E_1C) and pregnanediol 3-glucuronide (PdG) reproductive hormones cycle

Figure 3 and 4 showed that PdG had very low concentration during estrus compared to non-estrus. Figure 5 showed the structures of PdG and E_1C.

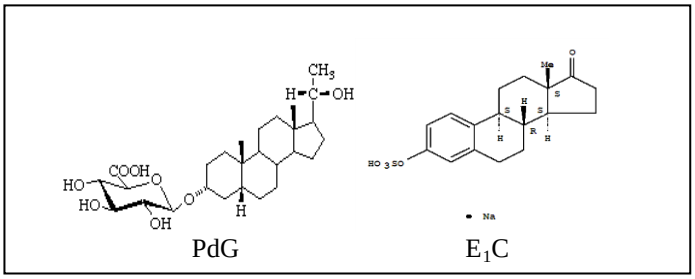

Fig. 5. Structures of PdG and E_1C

PdG on FTIR spectrum showed some spectrums represented functional groups, such as aromatic, carboxyl acid, alkyl, etc. The absorbance levels of these functional groups were very low during estrus, but very high during non-estrus. Table 2 showed the FTIR of PdG peak relative absorbance..

Table II
PdG represented peak relative absorbance

Animals	Wave number (cm^{-1})/Creatinine					
	1639 (aldehyde group)		1379 (alkyl group)		1745 (carboxyl acid group)	
	Estrus	Non-estrus	Estrus	Non-estrus	Estrus	Non-estrus
Rat 1	3.110	4.696745	0.743368	1.886704	0.201	0.6242
Rat 2	2.437	8.24291	0.443964	1.038861	0.289	1.1373
Rat 3	3.574	5.429483	0.552188	0.933282	0.401	0.5100
Rat 4	4.129	5.070132	0.720279	1.387236	0.308	0.9199
Rat 5	3.885	8.24291	0.841497	1.083934	0.252	0.6242
Rat 6	3.187	6.931745	1.117607	1.635465	0.157	0.4262
Rat 7	4.089	6.002493	1.245361	2.375038	0.279	0.5403
Rat 8	2.629	3.975897	1.081485	3.247119	0.159	0.3278
Rat 9	2.555	4.696745	1.343877	1.886704	0.583	1.0115
Rat 10	2.174	3.175426	1.452649	3.247119	0.621	1.1373
Average	3.177	5.646449	0.954227	1.872146	0.322	0.7259
SD	0.718	1.710808	0.343479	0.851318	0.163	0.2995

Table II showed relative absorbencies of peaks represented PdG in urine. These peak absorbencies were relative to creatinine. The 1639 cm^{-1} peak showed estrus had lower average value than non-estrus. The same condition also applied to 1379 cm^{-1} and 1745 cm^{-1} wave number had much lower average value on estrus than non-estrus. Therefore. all peaks could be used as indicator represented progesterone hormone. The 1639 was representative of aldehyde (1600--1820), the 1745 cm^{-1} peak was representative of carboxyl acid group (1735--1750 cm^{-1}). whereas the 1379 cm^{-1} peak was representative of alkyl which is methyl (CH_3) (alkyl was sharply identified on 1375--1450)cm^{-1} wave number). Both were the functional group of PdG. Thus. the presence of PdG could be indicated by the presence of 1639 cm^{-1} aldehyde peak. 1379 cm^{-1} alkyl peak and 1745 cm^{-1} carboxyl acid peak.

Progesterone concentration had been validated by the estrogen and progesterone standard determination method. The FTIR analysis indicated similar results of rat urine. both estrus and non-estrus on the same functional group.

Table III
Hormone concentrations

Wave number	Estrus		Non-estrus	
	Urine relative absorbancy average (PdG)	Blood(Progesterone)	Urine relative absorbancy average (PdG)	Blood(Progesterone)
1639 (aldehyde)	3.17737	0.8781028	5.646449	1.03868490
1379 (alkyl)	0.954227	1.08357330	1.872146	1.16394660
2877 (carboxyl acid)	0.325574	1.09466650	0.725926	1.17258250
Average	1.48572367	1.018780867	2.58861	1.12507133
SD	1.49834964	0.121956974	2.68078855	0.07493735

Table III showed validation of PdG to progesterone. The result showed PdG average absorbance in urine had much higher than progesterone in blood sample. in estrus phase validation between PdG and progesterone was 1.5:1 and in non-estrus phase validation was 2:1

3. Estrone conjugate (E_1C)

The condition applied in E1C determination was in contrary to PdG. As shown on Figure 4. the level of E_1C was very high during estrus. and vice versa lower in non-estrus. Table 4 showed the relative absorbance of E_1C-represented peaks.

Table IV
Relative absorbance of peaks represented E1C

Animals	Wave number (cm^{-1})/Creatinine					
	416 (alkyl group)		709 (aromatic group)		3468 (hydroxyl group)	
	Estrus	Non-estrus	Estrus	Non-estrus	Estrus	Non-estrus
Rat 1	1.829	0.221202	6.121141	5.145728	14.29494	12.09691
Rat 2	1.961	1.725724	8.95156	6.570912	12.87161	10.11673
Rat 3	2.448	2.095309	6.581922	5.108139	10.57251	4.247241
Rat 4	1.953	0.395482	4.726148	3.605537	14.04677	8.815036
Rat 5	2.203	1.725724	4.488815	4.319917	10.83834	10.11673
Rat 6	1.567	0.324979	3.81124	3.605537	11.00524	8.817355
Rat 7	1.284838	0.986159	3.557497	3.355503	12.03104	8.815036
Rat 8	1.193716	0.20872	3.304788	3.179599	10.61143	5.403922
Rat 9	1.017829	0.221202	3.255485	3.157222	10.0312	9.454803
Rat 10	1.037101	0.369992	4.70632	3.748895	10.20586	5.91596
Average	1.649371	0.827449	4.950492	4.179699	11.6509	8.379972
SD	0.478173	0.708004	1.80072	1.110688	1.496592	2.314759

As seen on Table 4 the 416 cm^{-1}. 709 cm^{-1} and 3468 cm^{-1} wave numbers had higher relative absorbencies during estrus cycle than non-estrus. These wave numbers were qualified as E_1C representatives due to their high level during estrus cycle. Therefore. E_1C could be identified with the presence of 416 cm^{-1} alkyl. 709 cm^{-1} aromatic and 3468 cm^{-1} hydroxyl peaks. It was shown on Figure 5 that the alkyl. aromatic and hydroxyl were functional groups of E_1C.

i. Estrus condition determination

In this research. FTIR was expected to be able to determine estrus and non-estrus condition of rat female based on E_1C and PdG level in urine. Therefore. the determination of E_1C maximum level and PdG minimum level during estrus was needed to be held. Figure 6 below showed hormone level clustering based on PdG relative absorbance. Figure 7 applied for E_1C.

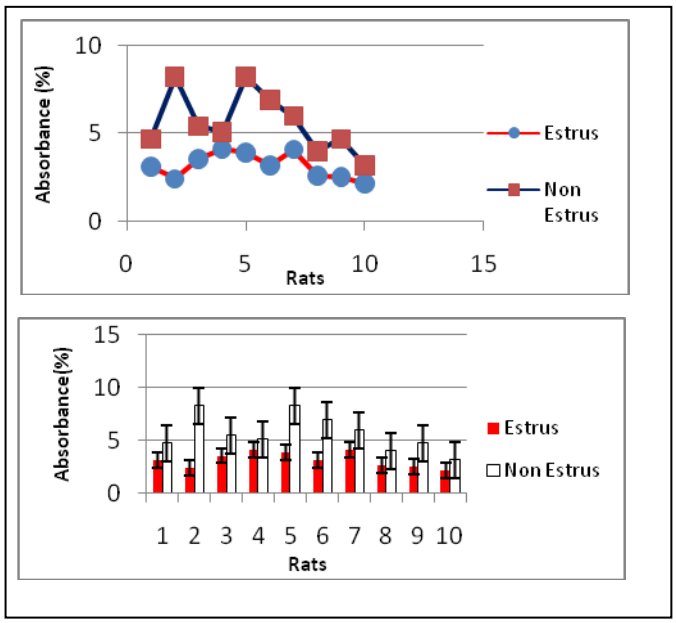

Fig. 6A. PdG relative absorbance on 1639 cm^{-1} wave number

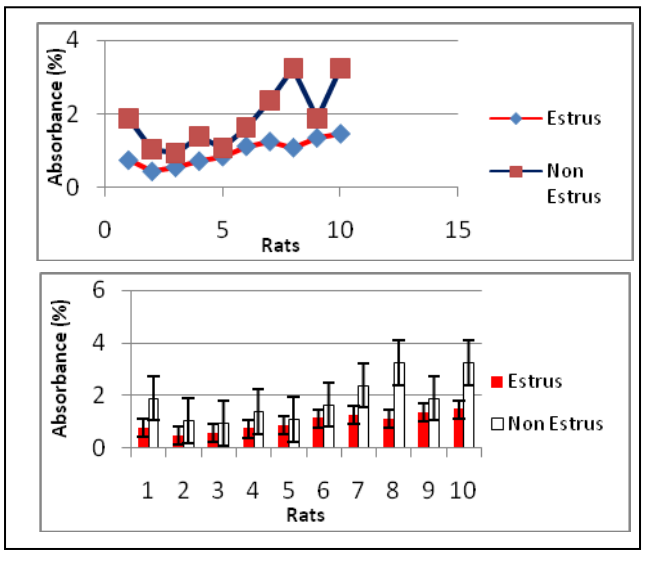

Fig. 6B. PdG relative absorbance on 1379 cm^{-1} wave number

As seen on Figure 6. PdG levels were low during estrus. and high during non-estrus. However. as shown by the hormone level cycle on Figure 4 hormone levels could have similar low values during estrus and non-estrus due to long period of fluctuation. Thus. the estrus period must have been determined on PdG maximum level which could guarantee that the level happened on estrus condition. Estrus state occurs when the relative absorbance PDG that are indicated by the average value of 1.48572367, and when non-estrus by 2.58861.

From Figure 7 the condition of estrus is indicated by absorbance values relative to creatinine of at least 6.083588 and when non-estrus at 4.462373333.

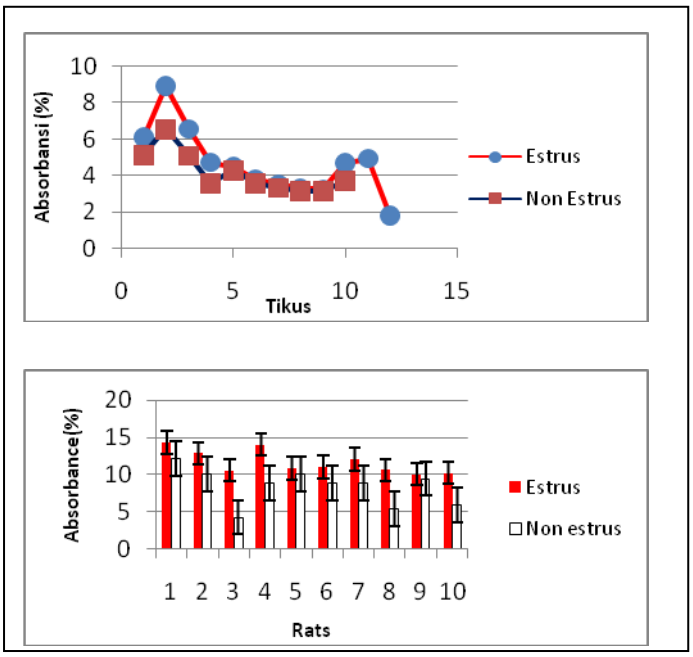

Fig. 7A. Relative absorbance of estrone conjugate (E_1C) on wave number 709 cm^{-1}

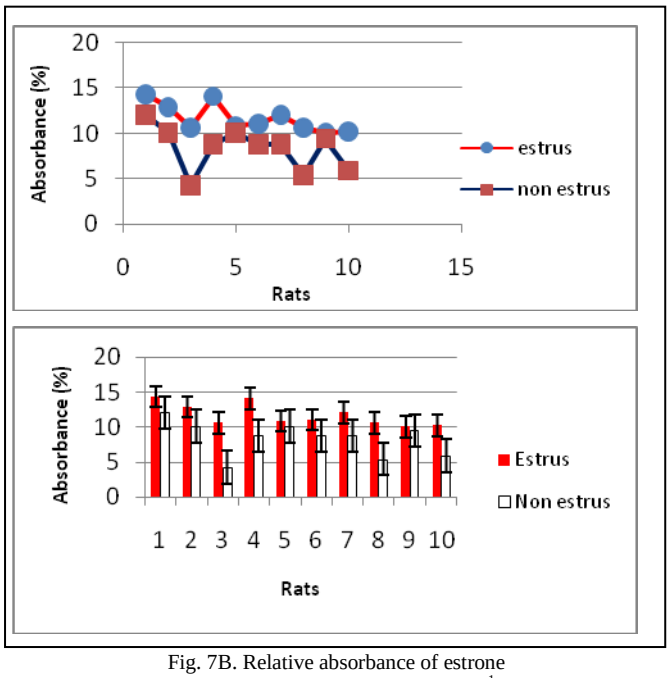

Fig. 7B. Relative absorbance of estrone conjugateon wave number 3468 cm^{-1}

IV. CONCLUSION

1. Pregnanediol 3-glucuronide (PdG) was identified by peaks of 1639 cm^{-1} aldehyde. 1745 cm^{-1} carboxyl acid and 1379 cm^{-1} alkyl. Whereas estrone conjugate (E_1C) was identified by 416 cm^{-1} alkyl. 709 cm^{-1} aromatic and 3468 cm^{-1} hydroxyl.
2. Hormon metabolite concentration of urine was shown by relative absorbance which is the comparison of each functional groups represented hormone metabolites to creatinine absorbance on 1730 cm^{-1} wave length.
3. Estrus state PDG achieved if the relative absorbance of creatinine at 1.48572367 and non-estrus at 2.58861 and the relative absorbance of creatinine at the time of E1C to estrus at 6.083588 and when non-estrus by 4.4623733333.

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