Near-infrared spectroscopy to monitor the timing of ovulation in Giant Panda (*Ailuropoda melanoleuca*) based on urinary steroid hormones

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Introduction

The giant panda has been categorised as "Endangered" in the 2009 IUCN Red List of Threatened Species. Thus, captive breeding must be carried out to maintain the population. However, the reproductive efficiency is not good, because females are monoestrous, with spontaneous ovulation during a breeding season. Therefore, accurate monitoring of the estrus cycle to pinpoint the timing of ovulation is critical for successful artificial breeding, such as by artificial insemination (AI). Enzyme immunoassay (EIA) or radio immunoassay has ordinarily been used as the method for monitoring steroid hormones in blood, urine or feces of animals. However, these methods need long processing with expensive reagents and radioactive isotopes. Therefore, the present investigation aimed to assess whether near infrared (NIR) spectroscopy could monitor urinary estrogen during the estrous cycle in female giant panda. In this study, quantitative, or qualitative analyses were applied to investigate whether the changes in the concentration of hormones in urine could be monitored by NIR spectroscopy. The speed of analysis on NIR spectroscopy could be a valuable factor in monitoring the estrous cycle.

Materials and methods

Samples

A female giant panda is accommodated at Kobe Municipal Oji Zoo. Daily urine samples were collected from her from 1st to 25th March 2007 ($n=53$).

Estrogen measurement by EIA

The urinary concentration of E_1G, which is the major estrogen metabolite excreted in the urine during the estrous cycle of giant panda, was measured by EIA. The EIA was carried out using a double antibody method, based on that of Hama *et al.*[1]

Spectra acquisition

NIR transmittance spectra of samples were obtained by a FT-NIR instrument Model MPA (Bruker Optics Inc. Billerica, MA) in the 1010–1880 nm region at 1 nm intervals in a standard 1mm pathlength cell holder. During spectral analysis, each sample was warmed up to 37 °C in a water bath. The NIR instrument recorded 3 consecutive spectra for each sample in order to obtain more robust models as the samples were under temperature and light perturbation during the spectral measurements. Mean centring was applied for all data. All spectra were divided by 2-norm of each spectra, which is known as vector length normalisation for Soft independent modelling of class analogies (SIMCA).

Chemometrics analysis

All chemometrics analyses were carried out by Pirouette (Version 4.0, Infometrix, Bothell, WA) and Matlab (Version 7.1, MathWorks, Inc., South Natick, MA) software programs.

Calibration models were developed using partial least square regression (PLSR) to predict the urinary E_1G concentration. The optimum number of PLS components to be used in regression models was determined by the software program after cross-validation. Then 1 sample (3 spectra) was left from the regression model for internal validation.

During the estrous periods SIMCA was applied to examine the pattern of E_1G excretion series using interclass distances. SIMCA is a classification technique that uses the calculation of principal components for training samples to be recognised as belonging to some classes.[2] The interclass distance was used to investigate the differences from the first day of urine sample collection, i.e. the spectral change over the time.

In this study moving principal component analysis (MPCA)[3] focused on the idea that changing of urinary components can be detected by monitoring of principal components (PC) loadings, using NIRS. A new index was introduced for evaluating changes in direction of each principal component. To calculate principal components successively, a time-window was introduced. To detect a variation of PCs, reference PCs representing the initial condition were defined, and the differences between the reference PCs and PCs representing the current condition were used as indices for monitoring. The following index A_i is proposed for evaluating a change of ith PC,

$$A_i(k)=1-\left|w_i^T w_0\right|$$

where w_i denotes the ith PC calculated step k, and w_{io} denotes the reference of ith PC. Both $w_i(k)$ and $w_{i0}(k)$ are unit vectors. When the ith PC representing a current operating condition is equivalent to its reference, A_i becomes zero. However, A_i becomes one when $w_i(k)$ is orthogonal to $w_{i0}(k)$. In this study, the sample moving window size was set to include three samples (nine spectra). Then, the 1st loading ($i = 1$) was used for calculation of index A to reduce system noise.

Pearson correlation coefficients (r) were calculated to evaluate the relationship between E_1G concentration and the interclass distances of SIMCA, or the index of MPCA, using the software program SPSS Version 10.0 for Windows (SPSS, Chicago, IL, USA). $p < 0.01$ was considered significant.

Results and discussion

E_1G concentrations ranged from 0.22 to 127.88 ng mL^{-1} (n = 53) by EIA. The values showed an increase gradually from 0.80 on March 13th to 127.88 ng mL^{-1} on March 23rd after which a sharp decline was observed.

Raw NIR spectra of all samples are illustrated in Figure.1, which showed that the means of the spectra were very similar.

Figure 2 and 3 show the score plot and the first loading plots for the PCA model, based on the NIR spectra. These results suggested that NIR spectra could see some subtle variation in urine samples. Therefore, the following analyses based on PCA: PLSR, SIMCA and MPCA were proposed.

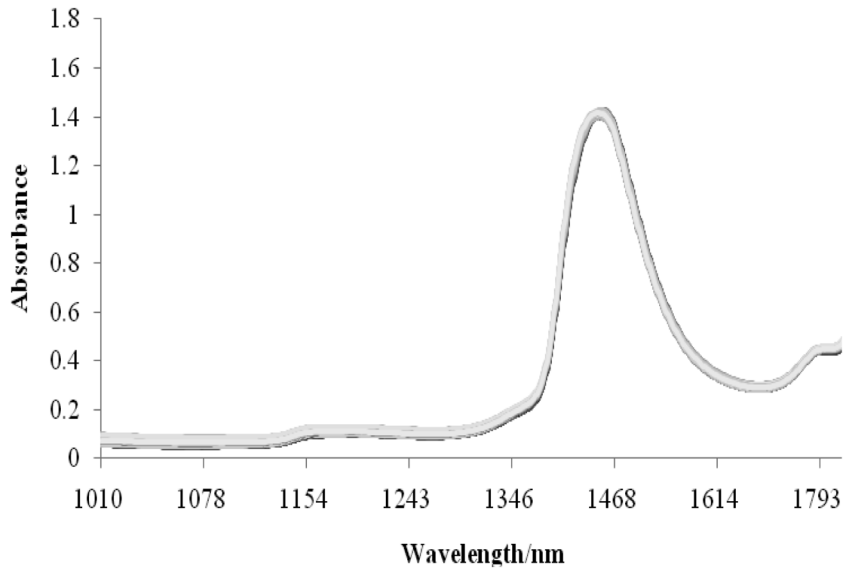

Figure 1. NIR urine spectra in the 1010–1880 nm region.

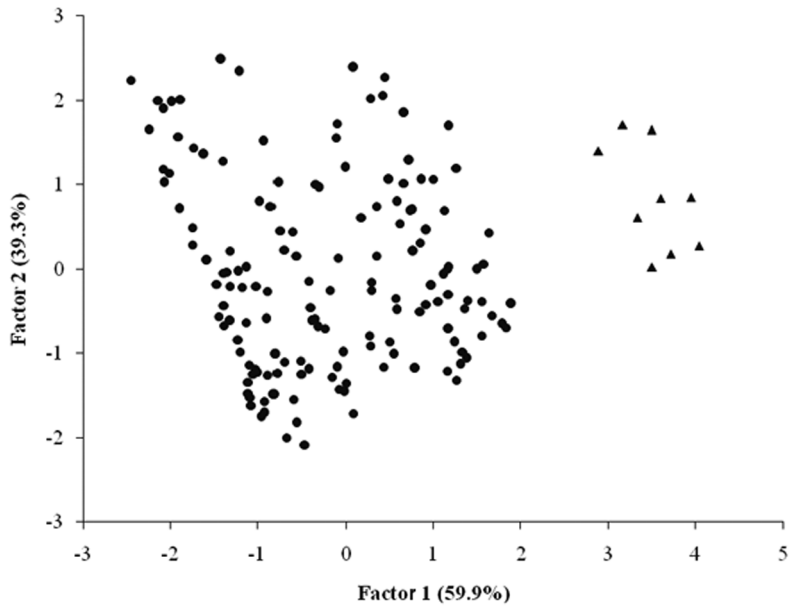

Figure 2. PCA of urine spectral data over the wavelength range from 1010–1880 nm. ▲ are the plots of high E_1G concentrations obtained from March 21th to 23rd, ● are the others.

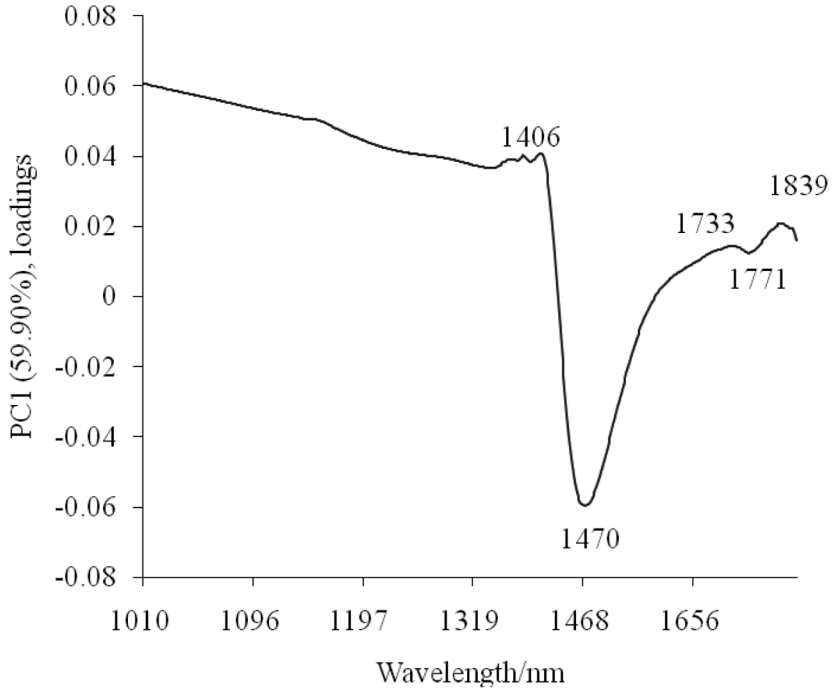

Figure 3. The first loading plots for the PCA model based upon the NIR spectra of urine.

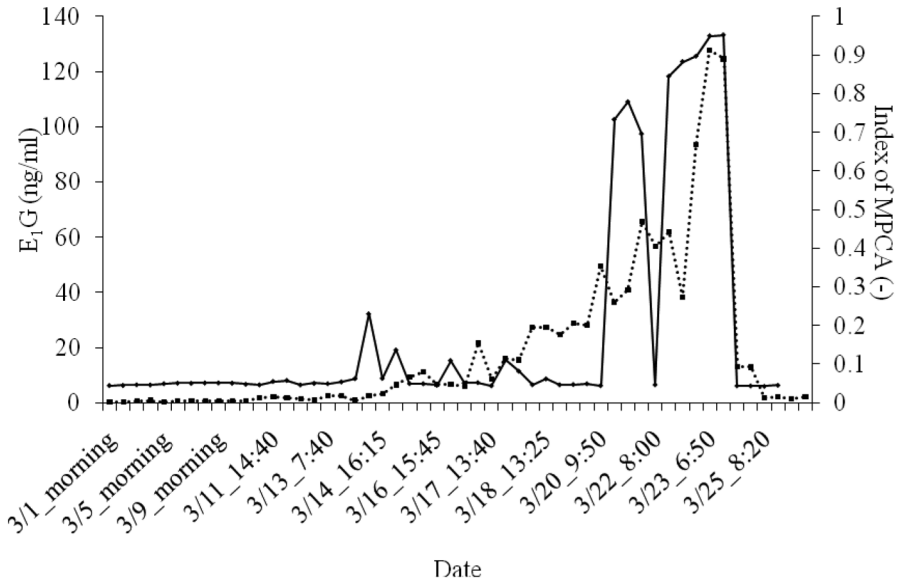

Figure 4. Monitoring results of MPCA index (continuous line) and E_1G concentrations (dotted line) for the same urine samples.

The PLSR using seven PLS factors was applied for the NIR urine spectra, R^2 by internal validation was 0.94 and the standard error of cross validation (*SECV*) was 10.40 ng mL^{-1}. Until this time it was thought that it was difficult to measure a very small amount of components by NIR spectroscopy.[4] Our results suggested that NIR spectroscopy could detect the hormone levels even though they were of the ng mL^{-1} order.

From the SIMCA classification, the interclass distance calculated from the data of the urine spectra showed a tendency of time series as E_1G content ($r = 0.64$, $n = 52$). However, the peak of highest E_1G concentration did not agree with the highest interclass distance. Therefore, further analysis would be needed to propose NIR monitoring for practical use. Meanwhile, MPCA based on the PC1, by which the variance of concentrations was well explained, was consistent with the change of E_1G concentration that followed the next day ($r = 0.81$, $n = 50$) (Figure 4).

The MPCA index showed drastic spectral change on March 14th, when E_1G concentration increased, and a sharp drop on March 24th corresponded to the decrease of the E_1G concentration indicating the ovulation. However, further work must be carried out to verify these observations. The technique must be proved to be reliable for practical use, because artificial breeding needs accurate timing of ovulation. The NIR technique, has good potential, and future efforts could improve NIR spectroscopy to match the accuracy obtained with EIA.

Conclusion

Our results showed a good agreement between E_1G values predicted by NIR SPECTROSCOPY to actual E_1G concentration, as determined by EIA, using PLSR with seven PLS factors ($R^2 = 0.94$,

$SECV = 10.04\,ng\,mL^{-1}$). The results of quantitative analysis by SIMCA and MPCA were thought to be reasonable results ($r = 0.64$, 0.81 respectively ($p < 0.01$)). MPCA in particular showed changes on the day that E_1G concentration fluctuated. Therefore, the spectral data were considered to contain information of the E_1G present in the urine. This study suggested that the NIR spectra of urine has the potential to estimate the estrus state in female giant pandas.

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