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Quantification of Bioactive Caffeic Acid in *Orthosiphon stamineus* Benth Using a Disposable Taste Sensor

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In view of the therapeutic potential and extensive use of herbs, a proper quality control method must be established to ensure the safety, efficacy, and quality of herbal medicines. The therapeutic effects of herbal medicines are not due to their natural origin, but rather to their pharmacological characteristics and dose levels. A disposable screen-printed array sensor strip based on self-plasticized lipid membranes combined with chemometric algorithm was applied to standardize Orthosiphon stamineus Benth extracts. Caffeic acid, a bioactive flavonoid in O. stamineus Benth, was quantified using the sensor system through standard addition technique. The results were compared by high-performance thin layer chromatography (HPTLC) method. Chemometric algorithms, partial least square (PLS) and principal component regression (PCR), were applied to the array sensor for the prediction and validation of results. The analysis showed that both PLS and PCR with three-factor regression model gave almost similar root mean square error of prediction (RMSEP) values, 0.21 and 0.22, respectively. The PLS algorithom showed a better prediction compared to PCR model.

Keywords: *Orthosiphon stamineus*, quantitative, disposable taste sensor, caffeic acid

1. INTRODUCTION

The use of herbal medicine is currently on the rise because of their vast therapeutic potentials [1,2]. These medicine consist of decoctions of several herbs defined in their formulas, and are sold without prescriptions in pharmacies. Therefore, consumers are often confused and uncertain about

their safety, quality, efficacy, and labeling compliance. The therapeutic efficacies of herbal medicines are due to the synergistic effects [3,4] of several chemical compositions, and a single chemical (ginsenosides) has been shown to produce multiple effects[5] on the same tissue. In reality, the overall pharmacology of herbal medicines is complex. Sometimes, misidentification and adulteration lead to serious consequences [2] on people's health. To utilize the full potential of herbal products, a suitable standardization method is required to check their authenticity, quality and purity.

The current technology employed for quality and authenticity evaluations of herbs is largely based on the identification [6] and quantification of one or two biomarkers [7] or pharmacologically active components [8] in herbs. These compounds are believed to be 'active ingredients' with a single mechanism of action. Several well-established analytical instruments, such as high-performance liquid chromatography (HPLC) [9], high-performance thin layer chromatography (HPTLC) [10], gas chromatography–mass spectrometry (GC-MS) [11] and liquid chromatography-mass spectrometry (LC–MS) [12] are used to assess certain ingredients or biomaterials to confirm quality. These approaches do not fulfill the observed value of synergism and are only applicable to materials that have been very well studied in the laboratory. Thus, a holistic quality control approach must be developed to determine the chemical basis [13] of herbal medicines, which in turn are based entirely on herbal extracts.

The use of electronic tongues [14] or biomimetic taste sensors [15-17] offers a promising quality control approach that uses an array of globally selective potentiometric lipid polymer membrane electrodes, together with a multivariate pattern recognition technique, to mimic the human olfactory system. The design concept of the global selective sensor [18] is that each sensor should maximize the overall sensitivity and provide different selectivity profiles over a broad range of target molecules during application. The lipid polymer membrane array sensor is used to mimic [19] the human tongue and sense a wide range of chemical compounds. The array sensor has been characterized in our previous study for the five basic tastes e.g., sweet, sour, bitter, salty and *umami* [2]. Each of the single sensors has a broad sensitivity spectrum toward the bioactive compounds, whereas the array sensor has cross-sensitivity, resulting in co-linearity of the sensor feature [18]. The array of sensors has different sensitivities (non-selective) to the bioactive compounds, where each sensor measures a distinctive property of the sensed chemical [20]. The array sensor will produce a hyperspectral chemical image [21–23] within the multiple-dimensional spectral information, which can be analyzed for a more reliable object characterization. Multivariate pattern recognition techniques, such as principle component regression analysis (PCR) and partial least square (PLS) analyses, are applied to the sensor array [24-26] data for the multi-component analysis in the complex herbal products.

O. stamineus Benth from Lamiaceae family is well known locally in Malaysia as Misai Kucing (Cat Whiskers); it is a popular medicinal plant in Southeast Asia, and is widely used for the treatment of various diseases, such as eruptive fever, hepatitis, hypertension, syphilis, gonorrhea, epilepsy, menstrual disorder and influenza [27]. Several bioactive compounds [28,29], including flavonoids, terpenoids, saponins and caffeic acid derivatives, have been identified in *O. stamineus*. Based on curative property values, quantitative determination of the components in *O. stamineus* is important. In

the present research, caffeic acid, a bioactive compound in *O. stamineus*, was determined by a disposable multi-channel taste sensor and verified with HPTLC.

2. EXPERIMENTAL

2.1 Materials

O. stamineus samples were obtained from different geographical locations in Malaysia. All chemicals and reagents used were of analytical reagent grade and obtained from Merck, Darmstadt, Germany and Sigma Aldrich, USA. Caffeic acid standard was purchased from Indofine Chemical Co., USA. Methanol used as solvent was of HPLC grade.

2.2 Instrumentation

2.2.1 Array sensor system

The array sensor system consist of a National Instrument Data Acquisition Card (NI DAQ, PCMCIA-6024E), an in-house-developed software for data acquisition using Labview 6.1 (National Instruments Corporation, USA), a disposable screen-printed array sensor strip, and a notebook for chemometric data processing. The array sensor were prepared with methacrylate–acrylate polymer blend with lipids. The sensor fabrication is similar to that reported by Maxsim et al. [30].

The array sensor strip consist of eight globally selective [2] electrodes and an Ag/AgCl reference electrode on a PVC base prepared by screen-printing technology. The eight electrodes are connected to analog input channels, and the reference node is grounded. Measurement and Automation Explorer (MAX) is used to access and configure the NI DAQ card. Eight electrodes are connected for the data acquisition, wherein the input signals are ± 1000 mV. Oversampling and averaging are applied in the analog measurements to reduce electrical noise.

2.2.2 HPTLC system

The HPTLC-CAMAG® analysis station includes (1) a semiautomatic applicator (Linomat 5), (2) a densitometer (TLC Scanner 3) including WinCATS (4.05 version) software, (3) horizontal development chamber (Camag), (4) documentation system (CAMAG DigiStore2) and (5) a high-resolution 12 bit CCD camera connected to WinCATS for image acquisition.

The chromatography system consists of pre-coated silica gel 60 F_{254} plate (20 x 10 cm, E-Merck grade), chloroform–glacial acetic acid–methanol–H_2O (61:24:10:5) as mobile phase, and Camag horizontal development chamber.

The TLC Scanner 3 densitometer was used to image the TLC plate for quantification. The device used is computer driven, which operates the specific application WinCATS 4 (Version 4.05). WinCATS® software is used for all data acquisition and optimization procedures, such as baseline smoothing and correction and integration window selection.

2.2.3 Master stock herbal extract preparation

Samples for quantitative analysis in both HPTLC and taste sensor consist of different batches of pilot extracted herbal samples obtained from two suppliers, namely, Hovid Sdn Bhd (HV) and Nusantara Herbs Sdn Bhd (NH). To prepare the herbal samples for analysis, 1.5 g of each extract was weighed and diluted to 100 mL with distilled water in volumetric flasks.

Caffeic acid standard was prepared by dissolving 1.0 mg in 1.0 mL methanol (1000 ppm). The standard was spiked into the sample to build the calibration model.

2.2.4 HPTLC method

Different batches of testing herbal and standard solutions were applied on the HPTLC plate by Linomat 5 applicator for quantification studies. The caffeic acid (200 ppm) standards 1, 3, 5, and 7 ml were used to generate the calibration curve. The HPTLC plates were developed in a Camag horizontal chamber pre-saturated with the above-mentioned solvent system and allowed to run for up to 9 cm. After development, the plate was air dried and scanned based on the R_f value using the densitometer at 365 nm. Quantitative evaluation was based on a comparison of the peak areas of unknown samples with calibration standard chromatographs.

2.2.5. Taste sensor method

Herbals are complex mixture as they contain hundreds of unknown chemical compounds. To prepare a mock herbal standard for the construction of the calibration model, caffeic acid was spiked in the reference standard sample as discussed elsewhere [30]. This method involves the addition of a known standard (caffeic acid) to a known volume of the sample {reference standard solution HVMSDE01 and NHISDE05 (6)}, followed by response potential measurement. Caffeic acid concentrations in the reference standard solutions were determined before spiking-in the standard by HPTLC method. The total caffeic acid concentration and the sensor output were used to train the PLS and PCR algorithms and to construct a calibration model to predict the unknown sample concentration.

The electrical potentials of the solutions were measured at room temperature with the sensor strips, and the data were recorded every 2 s in 2 min measurements. Before the potential was recorded, the strips were washed with distilled water and preconditioned for 1 min in the measurement solutions. After every measurement, the strip was re-washed and the retained water was blotted with filter paper for further use.

3. RESULTS AND DISCUSSION

O. stamineus contains several chemically active constituents, including caffeic acid and sinensetin, which are known bioactive compounds [25]. The aim of this study is to investigate the

quantity of caffeic acid in different batches of *O. stamineus* extracts from two suppliers. The bioactive concentrations were determined by HPTLC method. The results were used to train the PCR and PLS models to set up correlations with the biomimetic taste sensor to predict the unknown concentration.

Although HPLC is a widely used method in quantitative analysis, HPTLC was chosen in this research because of its many advantages. HPTLC is a popularly accepted versatile analytical method that requires less expensive instrumentation and expertise. HPTLC is cost effective and time efficient, allowing simultaneous analysis of up to more than 20 samples depending on the size of HTPLC plates used and requiring easy sample preparation and minimal cleanup procedure. It also uses only about 5% solvents compared with HPLC, and the plates can be scanned to an unlimited number of times through changing the parameters. By contrast, HPLC is limited by extensive sample clean up process, and requires expensive solvents and longer periods of column stabilization.

3.1 Determination of caffeic acid using HPTLC method

The use of HPTLC method offers a way to quantify caffeic acid in several *O. stamineus* samples simultaneously. The *O. stamineus* samples from two suppliers and caffeic acid standards were applied on the silica gel HPTLC plates by the Linomat 5 applicator following two different sequences, as shown in Fig. 1.

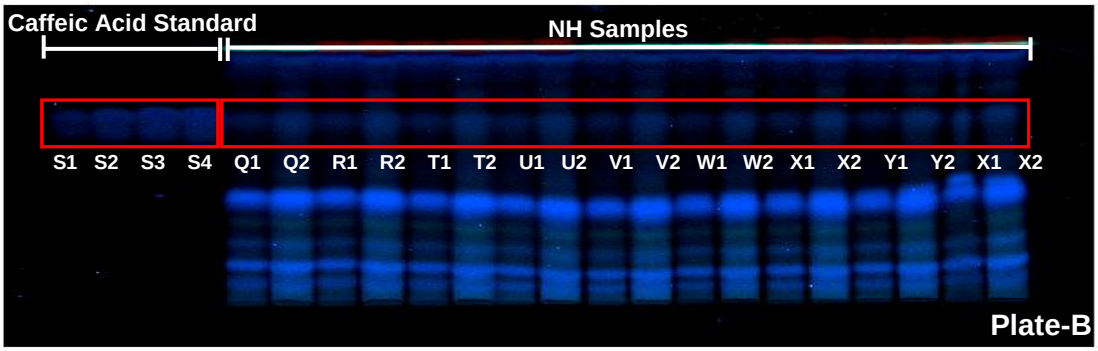

Figure 1. Plate 5.7 HPTLC photograph of caffeic acid separation in Plate B samples under 365 nm UV

The identification of caffeic acid in different samples of herbal extracts was performed by comparing R_f with the corresponding reference standard. The calibration range was set from 200 ng to 1400 ng caffeine, as shown by the increasing intensity of the bluish S1 to S4 band in Fig. 1. Caffeic acid was more visible under 365 nm UV. The three-dimensional integrated pattern of the densitometric peak is shown in Fig. 2. The peaks that matched exactly at R_f 0.67 with the caffeic acid standard indicated the presence of caffeic acid in the herbal samples.

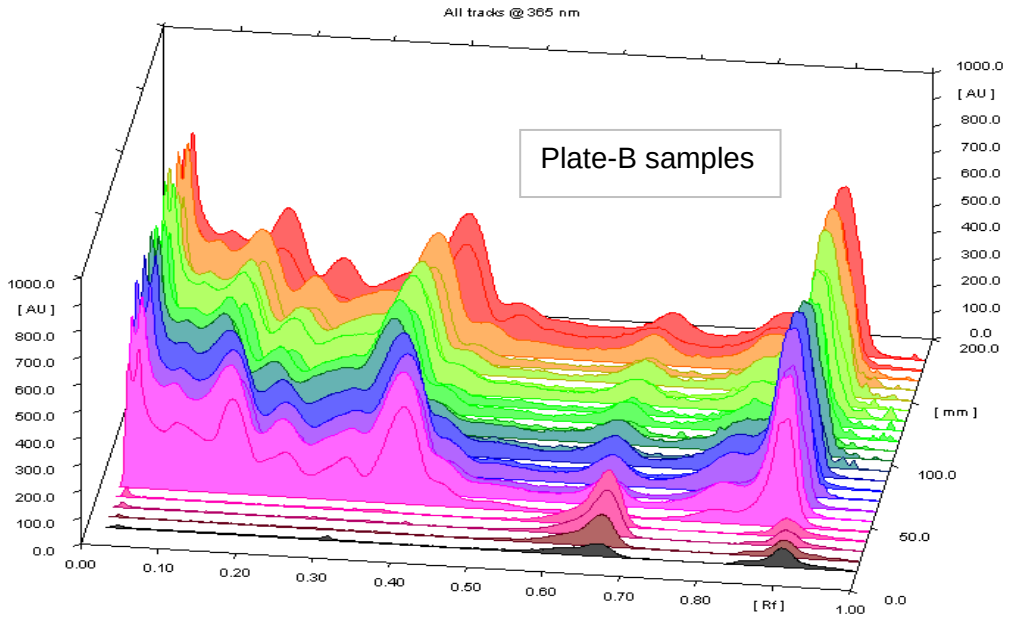

Figure 2. Three-dimensional overlay chromatogram of Plate B samples

Calculations used to determine the concentration of caffeic acid in the *O. stamineus* extract samples involved peak area instead of peak height, as the peak area reflects the overall shape of a specific peak which includes the width and the height, whereas peak height only focuses on the height of a certain peak. Calibration curves were constructed to determine the concentration of caffeic acid in samples from Plate A and Plate B. The calibration plots were based on the linear regression analysis of an equation Y = 533.308 + 7.680X and a correlation coefficient (r^2) 0.97351, as well as an equation Y = 1841.108 + 5.693X and a correlation coefficient (r^2) 0.98785 for Plate A and Plate B, respectively.

3.2 Determination of O. stamineus with disposable taste sensor

The biomimetic array sensor based on the chemometric algorithms was applied to determine the caffeic acid content in different batches of *O. stamineus* extracts from two suppliers. Chemometric algorithms PLS and PCR were chosen as exploratory studies to quantify caffeic acid in the herbal extracts. In addition, a comparative study of the prediction capabilities of these two approaches was undertaken. The final task was to set up a correlation matrix between the array sensor output and the result determined by the HPTLC method.

The detection principle is based on the variation of sensitivities of the sensors to the analytes. Eight global selective lipid membrane electrodes give a signature pattern of the analyte. Multivariate data analysis is used to recognize and quantify the chemical entity.

For quantification purposes, both of PCR and PLS method requires a calibration step in which the relationship between the sensor output and the associated sample concentration area is deduced from a set of training samples, followed by the validation of the generated model. Finally, a prediction

model is set up, in which results of the validated calibration model is used to determine the sample concentration by the sensor output [31].

3.3 Determination of caffeic acid in O. stamineus extracts

For the PCR and PLS trainings, caffeic acid from 12.761 ppm to 21.566 ppm were spiked in the HV sample, whereas that from 11.388 ppm to 20.208 ppm was spiked in the HV and NH sample, respectively. Fig. 3 shows the response pattern of two different herbal extracts analyzed by the biomimetic array sensor system after spiking caffeic acid. Although every sensor in a single strip does not respond linearly toward the target analyte, the slight non-linearity could be overcome by including extra components or factors from the chemometric algorithm [32].

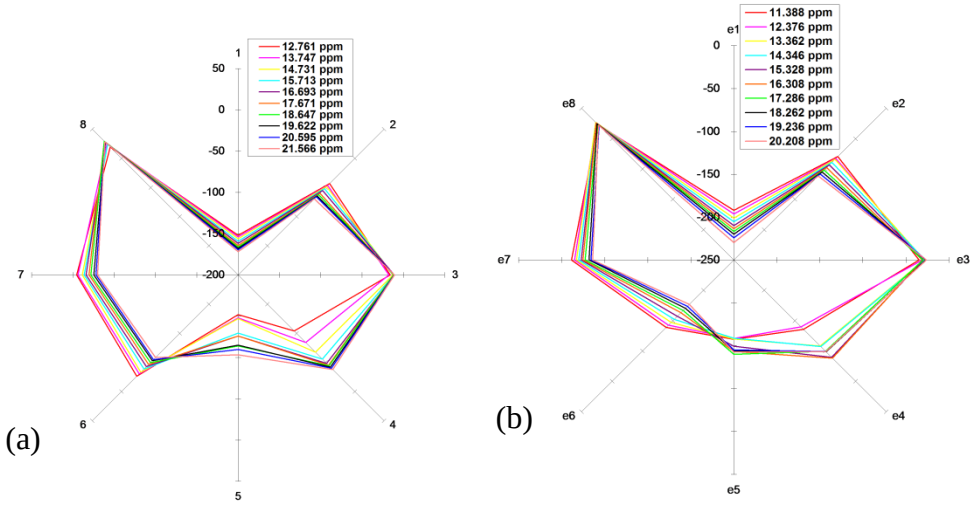

Figure 3. Signal patterns of sinensetin in (a) HVMSDE01 sample and (b) NHISDE05 (6) sample

3.3.1 Calibration and model validation

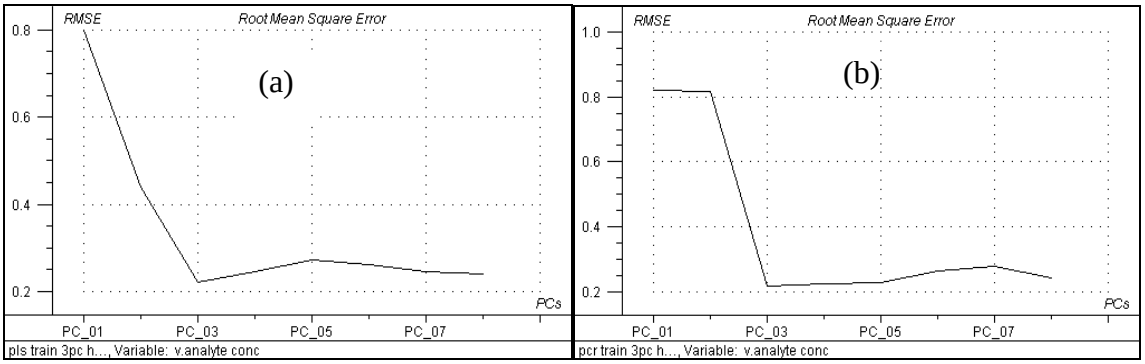

Figure 4. RMSE values vs. the number of factors generated by cross-validation for HV model based on (a) PLS and (b) PCR algorithms

The measured training data set obtained from the array sensor was then used to establish calibration models based on PLS and PCR procedures. PLS and PCR are the factor analysis methods wherein the first step is to determine the number of factors that allows the system to be modeled without over-fitting the concentration data. A cross-validation method, leaving out one sample at a time, was used for both algorithms [33]. The root mean square error of prediction (RMSEP) for each chemometric method was calculated to establish a predictive performance for each calibration.

The calibration models were constructed based on the number of factors that yielded the lowest average prediction error. Three-factor models were chosen for both PLS and PCR methods for the four different calibration models because the lowest RMSEP were yielded in both cases. Graphical presentations of the RMSE values vs. the number of factors generated by cross-validation for the four models are shown in Figs. 4 and 5.

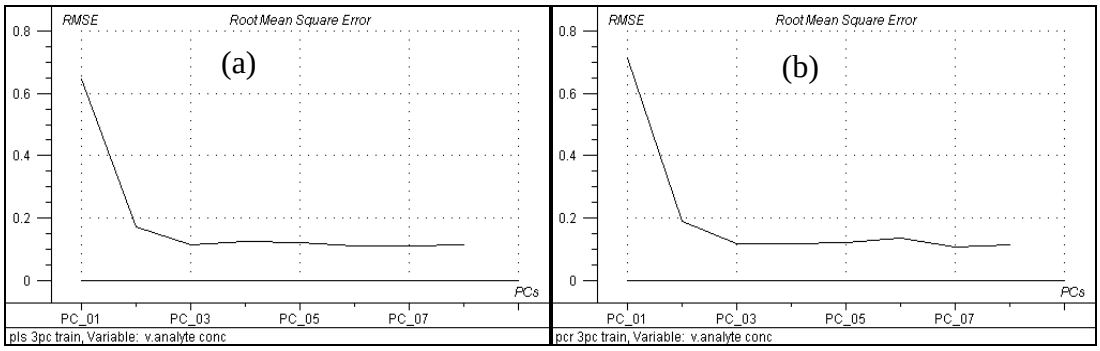

Figure 5. RMSE values vs. the number of factors generated by cross-validation for NH model based on (a) PLS and (b) PCR algorithms

The prediction models based on the factor-chosen calibration outputs were applied to determine the caffeic acid content in the testing data set. The predicting ability of each model toward new samples was evaluated using the average prediction error. Analysis of both graphs showed that the regression on caffeic acid which requires three factors in both PLS and PCR HV models produced similar prediction abilities. The two methods yielded RMSEP values 0.21 and 0.22 ppm, respectively. No significant outliers were observed, and the correlation coefficients for the calibration and validation model were 0.9983 and 0.9970, respectively, for the PLS model and 0.9983 and 0.9969, respectively, for the PCR model. In addition, both PLS and PCR models with three factors explained more than 99.4% of the total concentration variance.

Regression on caffeic acid in both NH models is also shown. No significant difference between the prediction abilities exist for both the three-factor PLS and PCR models, with both yielding an average prediction error of about 0.11 ppm. In both cases, three-factor models explained up to 99.8% of the total concentration variance.

3.3.2 Prediction of caffeic acid in HV extracts

The calibration models were applied in the caffeic acid content prediction in six different HV extracts (in duplicate measurement). The prediction results of both chemometric procedures and standard HPTLC method are listed in Table 1. The results predicted by PLS and PCR methods were almost similar and close to the reference HPTLC method, except for HVMSDE04, HVMSDE05, and HVMSDE06 samples, in which the average relative errors were from 5% to 15%. Poor predictions for these samples were caused by out-of-range of concentrations of the training data set. This phenomenon could be avoided by training the system with a larger concentration range to improve predicting ability.

Table 1. Concentration of caffeic acid in different batches of *O. stamineus* extracts predicted by the array sensor system based on PLS and PCR algorithms

Sample	Percentage of caffeic acid in extract (%)				
	Found by HPTLC	Predicted by PLS, 3 factors	Relative Error (%)	Predicted by PCR, 2 factors	Relative Error (%)
HVMSDE02	0.386	0.383	-0.777	0.383	-0.777
HVMSDE03	0.389	0.388	-0.257	0.390	0.257
HVMSDE04	0.217	0.229	5.530	0.230	5.991
HVMSDE05	0.252	0.266	5.556	0.267	5.952
HVMSDE06	0.146	0.168	15.068	0.169	15.753
HVMSDE07	0.344	0.342	-0.581	0.342	-0.581
NHISDE03	0.252	0.253	0.397	0.254	0.794
NHISDE04	0.418	0.414	-0.957	0.414	-0.957
NHISDE05(7)	0.367	0.362	-1.362	0.361	-1.635
NHISDE05(8)	0.410	0.406	-0.976	0.405	-1.220
NHISDE05(9)	0.257	0.259	0.778	0.259	0.778
NHISDE05(10)	0.221	0.236	6.787	0.237	7.240
NHISDE06(1)	0.280	0.286	2.143	0.287	2.500
NHISDE06(2)	0.200	0.228	14.000	0.228	14.000
NHISDE06(3)	0.391	0.387	-1.023	0.386	-1.279
NHISDE06(4)	0.720	0.622	-13.611	0.621	-13.750

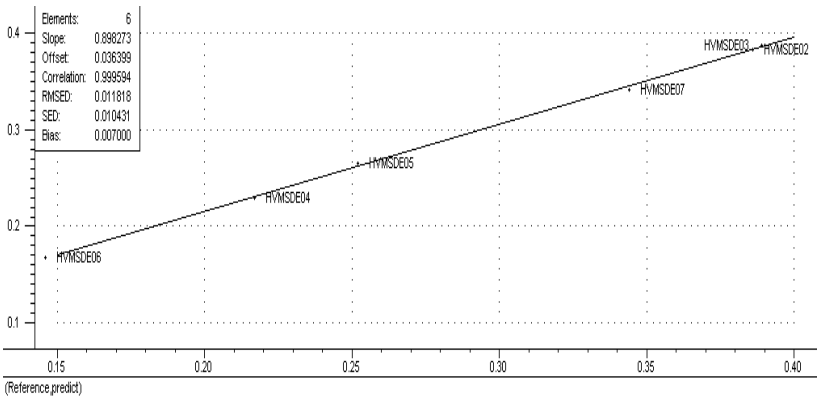

Figure 6. Correlation plot for percentage of caffeic acid found in all six batches of HV extracts by PLS regression with the concentration obtained from the HPTLC method

The PLS and HPTLC comparison result showed a correlation coefficient of 0.99 and an intercept (0.04) near zero, as shown in Fig. 6.

3.3.3 Concentration prediction of caffeic acid in NH extracts

Compared with PCR modeling, the predictions using PLS modeling achieved slightly better predictions, with results nearer the values provided by the reference method. The predicted concentration range of caffeic acid by PLS was within 0.17% to 0.39% for HV samples and within 0.22% to 0.62% for NH samples, respectively. Specifically, within the training concentration range, caffeic acid prediction concentration was well within the average relative error of approximately 2% compared with the reference HPTLC method. For samples which have been predicted with large deviations, the performance can be further enhanced if a wider concentration range is included in the training and modeling procedures [32].

A good correlation was predicted between the caffeic acid concentrations with the array sensor, and those determined with HPTLC method in all 10 extract samples. The established correlation between the two systems, which yielded a correlation coefficient of 0.9955, indicated successful application of the proposed method to determine caffeic acid content in complex herbal samples (Fig. 7).

4. CONCLUSION

The main challenge for the quality control system in a modern and competitive industry is to replace expensive and time-consuming analyses with reliable, fast, and cheaper alternatives. A proper quality control method is needed in the analysis of herbs and their products consist of complex cocktail of herbs. The use of array sensor combined with chemometrics is amongst for handling such complex data. The application of chemometrics techniques to the disposable array sensor strip data has been investigated for both qualitative and quantitative analysis of hebals.

The biomimetic array sensor was used to determine caffeic acid in different batches of O. stamineus extracts from two different suppliers namely HV and NH. All extract samples showed acceptable variation of caffeic acid concentrations using both PCR and PLS model. The PLS scheme provided a better predictions for all samples. On the other hand, the results were in good correlation with given by the HPTLC method. The array sensor system, once calibrated with standard, can be used for fast and economic quantitative purposes.

The biomimetic array sensor could be used as an alternative method for one step quality control system of herbal products. The sensor system presents as a reliable, cheaper and faster QC tool for the analysis of herbs based on the overall properties.

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