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Multiplicative effects model with internal standard in mobile phase for quantitative liquid chromatography–mass spectrometry

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ABSTRACT

Liquid chromatography–mass spectrometry assays suffer from signal instability caused by the gradual fouling of the ion source, vacuum instability, aging of the ion multiplier, etc. To address this issue, in this contribution, an internal standard was added into the mobile phase. The internal standard was therefore ionized and detected together with the analytes of interest by the mass spectrometer to ensure that variations in measurement conditions and/or instrument have similar effects on the signal contributions of both the analytes of interest and the internal standard. Subsequently, based on the unique strategy of adding internal standard in mobile phase, a multiplicative effects model was developed for quantitative LC–MS assays and tested on a proof of concept model system: the determination of amino acids in water by LC–MS. The experimental results demonstrated that the proposed method could efficiently mitigate the detrimental effects of continuous signal variation, and achieved quantitative results with average relative predictive error values in the range of 8.0–15.0%, which were much more accurate than the corresponding results of conventional internal standard method based on the peak height ratio and partial least squares method (their average relative predictive error values were as high as 66.3% and 64.8%, respectively). Therefore, it is expected that the proposed method can be developed and extended in quantitative LC–MS analysis of more complex systems.

1. Introduction

Mass spectrometry (MS) coupled with high performance liquid chromatography (HPLC) has become a widely used analytical technique with its high sensitivity and high specificity [1–3]. The role of the LC is to separate almost any mixture that can be dissolved, while the MS is to provide identification or quantitative determination by ionizing the separated peak. Currently, the main application areas of LC–MS are in the field of pharmaceutical, environmental and biochemical analysis [4]. Déglon et al. [5] established an automated system applied to the pharmacokinetic study of flurbiprofen (FLB) and its metabolite in human whole blood without sample processing. Manfio et al. [6] developed a method for simultaneous detection of sufentanil and morphine. Bassan et al. [7] quantitatively determined 43 common drugs contained in human serum. Due to its strong separation and structural analysis capabilities, liquid chromatography tandem mass spectrometry has also been widely applied to drug metabolism [8].

Even though LC–MS has many excellent features, it also has its own weak points. The interference of chemical background ions (chemical noise), signal drift, ion suppression, and the signal instability limit its application in routine quantitative analysis. Many efforts have been made to overcome these problems. For examples, Guo et al. [9] used exclusive ion/molecule reactions with dimethyl disulfide (DMDS) to reduce chemical noise. Autry et al. [10] coated a layer of gold on the surface of ion source to improve signal stability. Annesley [11] confirmed that enhanced specimen cleanup, chromatographic changes, reagent modifications, and effective internal standardization could minimize or correct ion suppression. Nevertheless, the application of LC–MS in routine quantitative analysis is still challenging.

In quantitative analysis using LC–MS, the number of ions detected by mass spectrometry must be proportional to the amount of the analytes of interest injected. Hence, signal stability is of utmost importance for quantitative analysis using LC–MS. However, variations in either instrumental parts or measurement conditions can significantly influence the signal stability. It is well known that the key factor contributing to the signal instability in LC–MS is the ion source of mass spectrometer which is responsible for ionizing the injected analytes and further pushing the selected ions into the mass analyzer. The gradual fouling of the ion source, vacuum instability, and aging of the ion multiplier are likely to change the ionization efficiency of the analytes of interest at different times, and hence lead to signal instability. It was

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observed in election impact ionization (EI)–quadrupole MS that a continuous loss of signal happened in 10 repeated injections with a more than 50% decrease in peak areas after 10 injections. A maximum 95% of the lost signal might be recovered after a time interval of at least 32 h. It should be emphasized that the above observation is a regular occurrence, rather than an occasional phenomenon [10]. Continuous signal variations could invalidate the calibration models established for quantitative LC–MS analysis, if no proper measures have been taken to correct their detrimental effects. Consequently, calibration models need to be rebuilt frequently to ensure acceptable quantitative accuracy and precision. It is rather costly and time consuming.

To overcome or minimize the detrimental effects of continuous signal variations on quantification results of LC–MS, an internal standard method [12,13] is generally adopted, where peak height ratios (or peak area ratios) of the peak of the analytes of interest to that of an internal standard are calculated and used in quantitative analysis. However, the presence of possible baseline drift and background interferences complicates the application of internal standard method. In addition, the difficulty in finding proper internal standards for complex systems also limits its application. Multiplicative calibration transfer [14,15] developed in area of NIR spectroscopy has been successfully utilized by Pavón et al. to rectify baseline drift and sensitivity changes over long periods of time in mass spectrometers [16]. But one shortcoming of this method is that an extra set of samples must be analyzed at regular intervals. Therefore, the routine quantitative application of LC–MS still calls for more advanced methods which can eliminate the influence of baseline drift and sensitivity changes at minimum cost.

In this paper, based on the multiplicative effects model developed by Chen et al. [17–20] for quantitative spectroscopic analysis of complex systems involving solids, a unique method was proposed to address the problems caused by baseline drift and sensitivity changes, and hence realize the long term applicability of calibration models for quantitative LC–MS analysis.

2. Novel quantitative strategy for LC/MS-multiplicative effects model with internal standard in mobile phase (MEM_{IS})

For quantitative LC–MS analysis with continuous signal variations (i.e. variations in sensitivity), the mass spectrum ($\mathbf{x}_i$, row vector) of the ith sample measured at the peak of the chromatographic elution curve of the target analyte can be expressed as follows:

$$\mathbf{x}_i = b_i c_{\mathrm{targ},i}\mathbf{s}_{targ} + \mathbf{d}_i,\ i = 1, 2, \ldots, N \quad (1)$$

Here, $\mathbf{s}_{targ}$ and $c_{\mathrm{targ},i}$ are the pure mass spectrum and concentration of the target analyte in the ith sample, respectively; $\mathbf{d}_i$ represents the possible baseline and background interferences in $\mathbf{x}_i$; N denotes the number of samples; b_i accounts for the effects of variations in sensitivity on the mass spectrum of the ith sample, due to changes in measurement conditions (e.g. vacuum degree and environmental temperature) and/or ion suppression. Obviously, the relationship between $c_{\mathrm{targ},i}$ and $\mathbf{x}_i$ does not follow a linear model because of the presence of the multiplicative parameter b_i which varies across samples. To determine $c_{\mathrm{targ},i}$ accurately, the confounding effect of b_i must be eliminated.

The multiplicative effects model developed by Chen et al. [18] for quantitative spectroscopic analysis of complex systems reveals that multiplicative effects confounding with the concentrations of the target analyte can be estimated by optical path-length estimation and correction method—OPLEC [19,20] or its modification version [18,19] as long as another coexistent analyte with constant concentration underwent the same multiplicative confounding effects simultaneously. In quantitative LC–MS assays, one possible way to satisfy the above prerequisite is to add a small amount of certain internal standard chemical compound into the mobile phase. The internal standard added should not be retained on the solid phase of LC, and can be ionized and detected by mass spectroscopy. Consequently, the mass spectrum ($\mathbf{x}_i$) of the ith sample measured at the peak of the chromatographic elution curve of the target analyte contains the contributions of both the target analyte and internal standard in the mobile phase:

$$\mathbf{x}_i = b_i \cdot (c_{targ,i} \cdot \mathbf{s}_{targ} + c_{stand}\mathbf{s}_{stand}) + \mathbf{d}_i,\ i = 1, 2, \ldots, N \quad (2)$$

Here, c_{stand} is the concentration of the internal standard added in the mobile phase, which is constant across samples; $\mathbf{s}_{stand}$ denotes the pure mass spectrum of the internal standard. The multiplicative parameter vector $\mathbf{b}$ ($\mathbf{b}=[b_1;b_2;\ldots;b_N]$) for N calibration samples can be estimated from their mass spectra $\mathbf{X}_{cal}$ ($\mathbf{X}_{cal}=[\mathbf{x}_1;\mathbf{x}_2;\ldots;\mathbf{x}_N]$) by OPLEC or its modified version. Two calibration models can then be built by multivariate linear calibration methods such as partial least squares (PLS) [21]. The first model is between $\mathbf{X}_{cal}$ and $\mathbf{b}$, and the other is between $\mathbf{X}_{cal}$ and $\mathrm{diag}(\mathbf{c}_{targ})\mathbf{b}$ ($diag(\mathbf{c}_{targ})\mathbf{b} = [b_1\mathbf{c}_{targ,1}; b_2\mathbf{c}_{targ,2}; \ldots; b_N\mathbf{c}_{targ,N}]$):

$$\mathbf{b} = \alpha_1\mathbf{1} + \mathbf{X}_{cal}\boldsymbol{\beta}_1;\ diag(\mathbf{c}_{targ})\mathbf{b} = \alpha_2\mathbf{1} + \mathbf{X}_{cal}\boldsymbol{\beta}_2 \quad (3)$$

where $\mathbf{1}$ is a column vector and its elements equal unity. For simplicity, the same number of latent components is generally used in the above two PLS calibration models. Once the mass spectrum of a test sample at the peak of the chromatographic elution curve of the target analyte has been recorded, the multiplicative confounding effects caused by the variations in sensitivity can be removed by dividing the prediction of the second calibration model by the corresponding prediction of the first calibration model, and accurate concentration prediction for the target analyte in the test samples is therefore readily achieved according to Eq. (4):

$$c_{targ,test} = \frac{\alpha_2 + \mathbf{x}_{test}\boldsymbol{\beta}_2}{\alpha_1 + \mathbf{x}_{test}\boldsymbol{\beta}_1} \quad (4)$$

3. Experimental

3.1. Reagents and chemicals

Nicotinamide (98.5%) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Tyrosine (Tyr, 99%) and Tryptophan (Trp, 98%) were from Sigma-Aldrich (Shanghai, China). Phenylalanine (Phe, 98%) was purchased from Shanpu Chemical Co., Ltd. (Shanghai, China). HPLC grade methanol was from Oceanpak Alexative Chemical, Ltd. (Beijing, China). All of these products were used as received without further purification. Stock solution (0.1600 μg/L) of each amino acid was prepared by dissolving an appropriate amount of corresponding amino acid in ultrapure water in 25 ml volumetric flasks at room temperature and stored at 4 °C. A milli-Q system from Aquapro (Taiwan, China) was applied to produce ultra-pure water used throughout the experiment.

3.2. Sample preparation for the determination of amino acids in water

Appropriate amounts of Tyr, Trp and Phe stock solutions were mixed and diluted with ultrapure water to prepare seven calibration samples and five test samples (hereinafter to be referred as "test set 1"). Among the calibration samples, the concentrations of Tyr and Trp ranged from 0.0100 μg/ml to 1.0000 μg/ml, while in the test samples, the concentrations of Tyr and Trp were in the range of 0.0400–0.8600 μg/ml. The concentrations of Phe in both

the calibration and test samples were kept constant (0.5000 μg/ml). One week after the above twelve mixture samples were analyzed by LC–MS, another five test samples (hereinafter to be referred as "test set 2") with different concentrations of Tyr and Trp were prepared and analyzed. The concentrations of Phe in the samples of test set 2 were also kept at 0.5000 μg/ml.

3.3. HPLC–MS /MS analysis

All the samples were analyzed by a 1200 HPLC system (Agilent Technologies) equipped with a C_{18} reversed-phase column (2.1 mm i.d. × 150 mm length)packed with 3.5 μm particles (Agilent, USA). The column was maintained at 25 °C. The mobile phase used consisted of water containing 0.1% formic acid and 0.0500 μg/ml nicotinamide (eluent A), and methanol (eluent B). The volume ratio of eluent A to eluent B was 50%:50%. The flow rate was 0.2 ml/min. For each sample, a volume of 10 μl was loaded onto the column via an auto-sampler from a 96-well sample tray. Mass spectra of samples were collected by an Agilent G6400 Triple Quadrupole mass spectrometer (Agilent Technologies) with electrospray interface (ESI) operated in the positive mode using the following settings: nebulizer pressure=15 psi, capillary voltage=4000 V, drying gas flow rate=11 L/min; and drying gas temperature=300 °C. The MS detector was operated in multiple reaction monitoring (MRM) mode at a rate of 1.23 cycle/s. The chosen precursor ions and product ion transitions for Tyr, Trp and Phe were provided in Tables S1 and S2 (Supporting information). All the samples were analyzed three times.

3.4. Data analysis

The estimation of the multiplicative parameter vector **b** in MEM_{IS} for the calibration samples was estimated by $OPLEC_m$ [19]. The performance of MEM_{IS} was compared with the conventional internal standard method carried out by the quantitative analysis software provided by the HPLC–MS/MS instrument and the PLS calibration models in terms of root-mean-square error of prediction (RMSEP,$RMSEP=\sqrt{\sum_{i=1}^{N}(c_{targ,i}-\hat{c}_{targ,i})^2/N}$), where $\hat{c}_{targ,i}$ is the predicted concentration for the target analyte in the *i*th sample) and the average relative prediction error (ARPE,$ARPE(\%)=100\sqrt{\sum_{i=1}^{N}|c_{targ,i}-\hat{c}_{targ,i}|/c_{targ,i}}$).

4. Results and discussion

Fig. 1a shows total ion chromatogram (TIC) of four samples measured on the same day. It can be seen that the peak heights of Tyr and Trp increase along with the increase of their concentrations. However, the peak height of Phe also shows significant variation, despite its concentrations in the four samples being constant, which suggests that LC–MS signals are unstable even within very short time intervals. Increase in time interval resulted in obvious variations in both peak height and retention time of TIC (Fig. 1b). More importantly, the degree of variations in peak heights of difference species differed significantly. The peak height of Phe saw a prominent decrease, while that of Trp showed a slight increase.

To overcome the lack of signal stability in quantitative LC–MS assays, the convention internal standard method was tried. Phe was chosen as the internal standard as its concentration was constant across samples. The raw mass spectra of the analytes of interest (i.e., Trp and Tyr) recorded at the peaks of their chromatographic elution curves were divided by signal intensity at the mass to charge ratio of 120.1 (which is most intense fragment ion of Phe). And then univariate calibration models were established between the signal intensity ratios at the most intense fragment ions of the analytes of interest and their concentrations in mixture samples. Table 1 shows RMSEP and ARPE values for the calibration and test sets obtained by the univariate calibration models based on the conventional internal standard method. For the test samples analyzed on the same day as the calibration ones, the univariate calibration models gave rather accurate predictions, suggesting that the conventional internal standard method efficiently corrected the influence of signal instability within relatively short time intervals. However, as can be seen in Table 1, the univariate calibration models failed to provide satisfactory predictions for test set 2 analyzed one week later. The concentration

Table 1
Recovery RMSEP and ARPE values for the calibration and test sets obtained by the univariate calibration models based on the conventional internal standard method.

Comp.	Calibration set		Test set 1		Test set 2	
	RMSEP (μg/ml)	ARPE (%)	RMSEP (μg/ml)	ARPE (%)	RMSEP (μg/ml)	ARPE (%)
Trp	0.0012	2.1	0.0021	1.1	0.2621	66.3
Tyr	0.0057	4.2	0.0085	3.7	0.1278	28.3

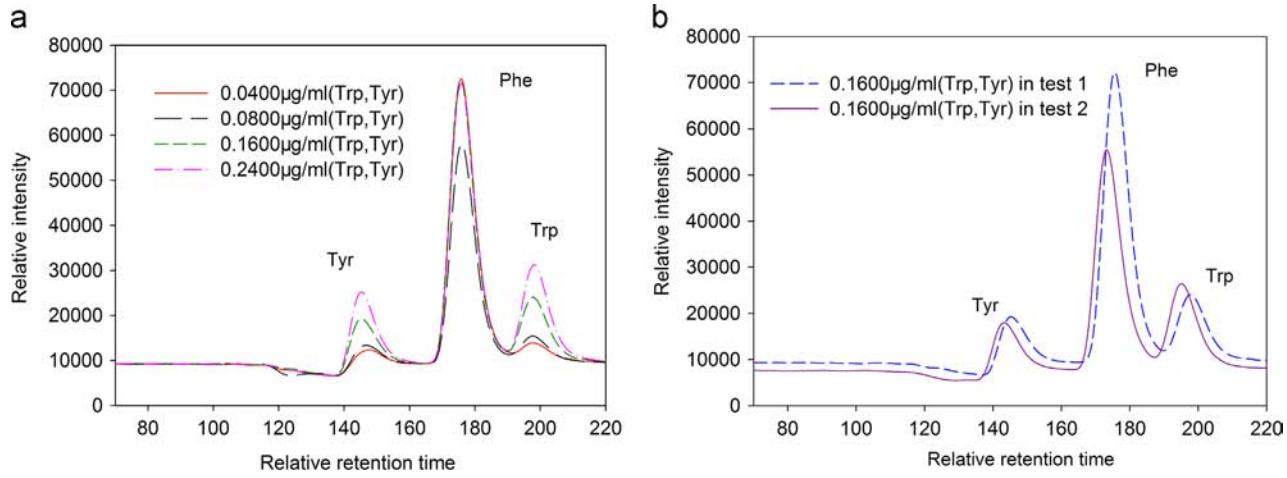

Fig. 1. a) Total ion chromatograms (TICs) of four samples measured on the same day and b) TICs of the same sample measured on two different days with a time interval of a week. The concentrations of Phe in all samples were 0.5000 μg/ml.

predictions for Trp and Tyr in test set 2 severely deviated from the actual values, with ARPE values of 66.3% and 28.3%, respectively. The failure of the conventional internal standard method can be explained by the differences in the degree of variations in peak heights of difference species. Therefore, the conventional internal standard method is unable to solve the problem of signal instability in quantitative LC–MS assays.

To address the problem caused by the differences in the degree of variations in peak heights of difference species, nicotinamide with greater polarity than those of the analytes of interest was added into the mobile phase, ionized and detected together with the analytes of interest by the mass spectrometer. Variations in measurement conditions and/or instrument would then have similar effects on the signal contributions of both the analytes of interest and nicotinamide. Therefore, the mass spectra of samples measured at the peak of the chromatographic elution curve of the target analytes follow the MEM_{IS} model in Eq. (2). The two multiplicative parameter vectors **b** for Tyr and Trp in the calibration samples were estimated by $OPLEC_m$ and are displayed in Fig. 2. Clearly, different samples had significantly different multiplicative parameters reflecting the signal instability across samples. More interestingly, for most of the samples, the multiplicative parameters calculated for Tyr were different from the corresponding ones calculated for Trp, which further confirmed that variations in measurement conditions and/or instrument might have different degrees of influence on the signals of different analytes with different retention times.

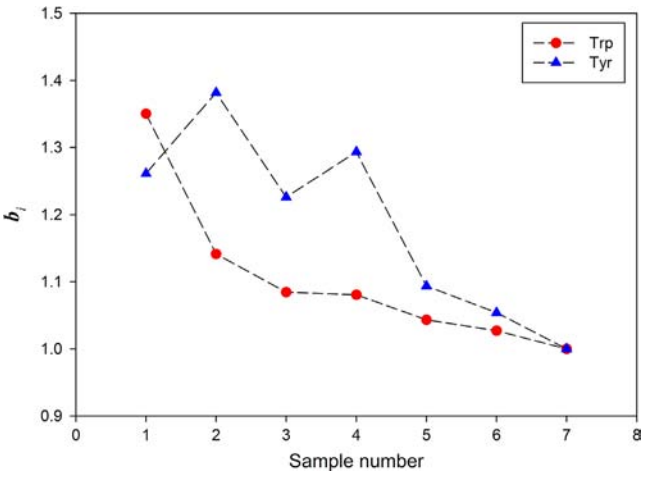

Fig. 2. The multiplicative parameter vectors **b** for Tyr and Trp in the calibration samples estimated by $OPLEC_m$.

Table 2
RMSEP and ARPE values for the test sets obtained by MEM_{IS} and PLS models.

Comp.	MEM_{IS}				PLS			
	Test set 1		Test set 2		Test set 1		Test set 2	
	RMSEP (μg/ml)	APRE (%)	RMSEP (μg/ml)	APRE (%)	RMSEP (μg/ml)	APRE (%)	RMSEP (μg/ml)	APRE (%)
Trp	0.0258	9.6	0.0236	14.6	0.0318	23.7	0.0661	64.8
Tyr	0.0155	8.1	0.0425	10.6	0.0225	10.2	0.1116	29.9

Table 2 shows the results of PLS and MEM_{IS} calibration models. The RMSEP values of PLS calibration models for test set 2 are far higher than the corresponding values for the test set 1. Large deviations can readily be observed in the concentration predictions for both Trp and Try in some samples (Figs. 3a and 4a). These results suggest that the PLS method is also not an effective way to deal with the problem of signal instability in quantitative LC–MS assays. Though the RMSEP values of MEM_{IS} for test set 2 are still somewhat larger than the corresponding values for test set 1, the predictive precision of MEM_{IS} for test set 2 is much more accurate in comparison with PLS. The concentrations of Tyr and Trp in the two test sets predicted by MEM_{IS} are rather close to their expected values (Figs. 3b and 4b). Such results are quite satisfactory, and demonstrate the effectiveness of MEM_{IS} in correcting the influence of signal instability on quantitative results.

5. Conclusions

LC–MS suffers from signal instability. Due to the differences in the degree of variations in signal intensities of difference species, the conventional internal standard method based on the peak height ratio (or peak area ratio) of the peak of the analytes of interest to that of an internal standard is unable to solve the problem of signal instability in quantitative LC–MS assays. With a view to effectively address the problem of signal instability, an internal standard which is not retained on the solid phase was added into the mobile phase, ionized and detected together with the analytes of interest by the mass spectrometer to ensure that variations in measurement conditions and/or instrument have similar effects on the signal contributions of both the analytes of

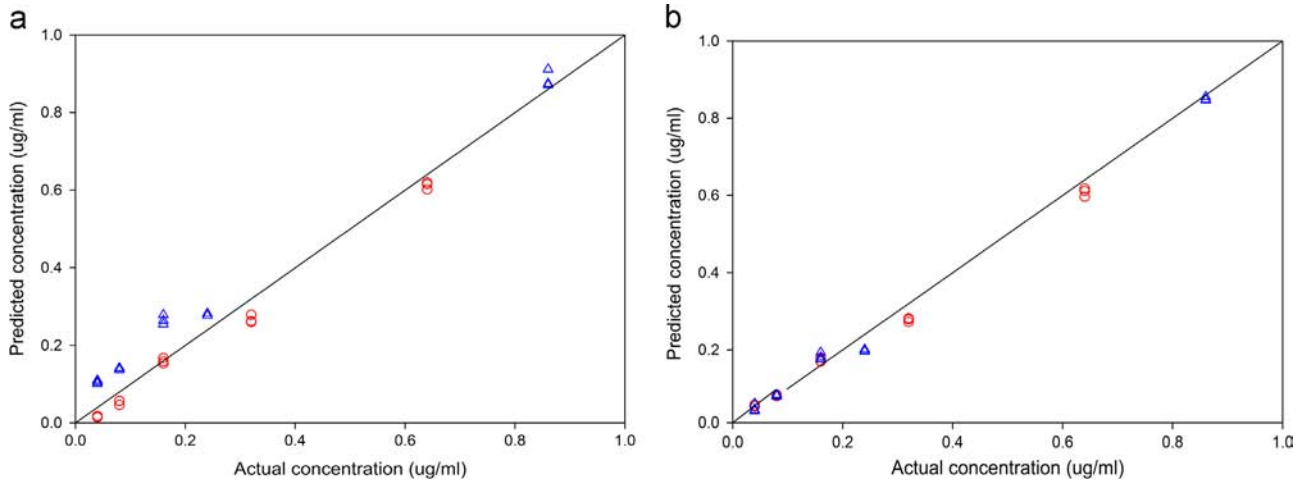

Fig. 3. Concentration predictions for Trp in both the test set 1 (red circle) and test set 2 (blue triangle) obtained by PLS (a) and MEM_{IS} (b) calibration models. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

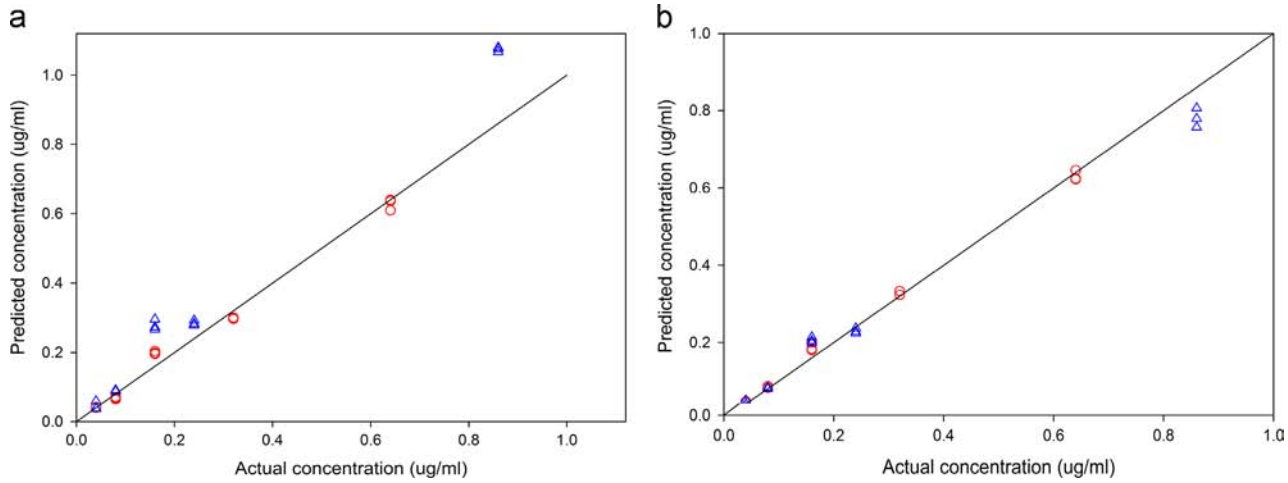

Fig. 4. Concentration predictions for Tyr in both the test set 1 (red circle) and test set 2 (blue triangle) obtained by PLS (a) and MEM_{IS} (b) calibration models. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

interest and the internal standard. Based on the above unique strategy, a multiplicative effects model with internal standard in mobile phase (MEM_{IS}) was developed for quantitative LC–MS assays. The experimental results on the determination of amino acids in water by LC–MS demonstrated that the continuous signal variation in LC–MS analysis could significantly deteriorate the precision and accuracy of both the conventional internal standard method and partial least squares (PLS). The average relative predictive error values of conventional internal standard method and PLS were as high as 66.3% and 64.8%, respectively. In comparison, MEM_{IS} could efficiently mitigate the detrimental effects of continuous signal variations, and achieved quantitative results with average relative predictive error values in the range of 8.0–15.0%. Therefore, it is reasonable to expect that MEM_{IS} would become a promising alternative for quantitative LC–MS analysis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.03.028.

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