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Effect of different matrices on physiological amino acids analysis by liquid chromatography: evaluation and correction of the matrix effect

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Abstract

Free amino acids in plasma, muscle and liver tissues were determined using the Pico-Tag method which involves HPLC and pre-column derivatization. A study of the accuracy in the determination is carried out by means of external calibration, standard-added calibration, Youden calibration and analyte recovery. It is conclusively shown that the method is affected by a proportional systematic error due to matrix effects, but not by a constant one. A new function named matrix-corrected calibration is proposed to correct for proportional bias when free analyte matrices for matrix-matched addition is unavailable.

Keywords: Matrix effect; Correction of matrix bias; Amino acids

1. Introduction

An accurate (true and precise) [1] determination of free amino acids in biological fluids is needed to improve our knowledge of amino acid metabolism in humans and animals. It is of particular importance in the diagnosis and monitoring of the inherited disorders of amino acid metabolism. The nutritional significance and potential utility of changes in plasma and tissue free amino acids in the evaluation of protein quality, the assessment of protein nutritional status and the evaluation of requirements of essential amino acids have been appreciated for a number of decades [2–4]. Therefore, the availability of reliable methods for amino acid analysis of plasma and tissues becomes important for the precise prediction of nutritional status.

The classical analytical technique uses automated amino acid analyzers. However, this method requires expensive dedicated equipment due to the post-column derivatization of the amino acids, long assay times and large samples volumes. More recently, reversed-phase high-performance liquid chromatography (RP-HPLC), which employs pre-column derivatization procedures, has developed. Numerous pre-column derivatization reagents have been introduced for the analysis of amino acids and all of them show pros and cons. The most frequently used for free amino acid determination are 9-fluoroenylmethyl-chloroformate (FMOC-Cl) [5], *o*-phthaldehyde (OPA) [6], 4-dimethylaminoazobenzene-sulfonyl chloride (DABSCl) [7], 6-*N*-aminoquinolyl-*N*-hydroxysuccimidyl carbamate (ACQ) [8] and phenylisothiocyanate (PITC) [9]. OPA and PITC are the most widely used in clinical and nutritional studies and PITC has the additional advantage of reacting with secondary amino acids. Hence, we chose PITC as the derivatizing agent, following the Pico-Tag method developed by Waters [10].

The aim of the present study was to evaluate the suitability of the Pico-Tag method to analyze free amino acids, in different common physiological samples, namely plasma, muscle homogenate and liver homogenate. These samples present analytical problems due to the complex matrix that may introduce an analytical bias [11]. Sample components different from the analyte, grouped together under the generically designation of sample matrix, may be the cause of a disturbance that affects either the measurement system or

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the analytical signal generated and produce a systematic error in the measurement. As a consequence, the matrix effect can increase the level of random errors by diminishing the analytical resolution and/or introduce systematic errors, which can be constant, if the analytical blank is affected, or proportional to the analyte concentration, if the method sensitivity is modified. The matrix effect influences the accuracy of the results because the precision is modified (if random errors are increased) and/or the trueness is affected (if systematic errors are introduced). The detection and correction of constant and proportional systematic errors have been extensively studied by Cardone [12–15] and a statistical protocol has been proposed [16,17]. Those studies require the establishment of a standard-added curve (by application of the standard addition method) to avoid proportional errors [18,19] and the measurement of the true sample "blank" from the Youden curve to evaluate the constant errors [20]. Nevertheless, the lack of statistical studies in these methodologies implies insufficient information to establish the level of signification of the conclusions.

The usual way to quantitate amino acids by HPLC is the utilization of a simple external calibration with or without internal standard, without considering the sources of error above mentioned. The aim of this work is the evaluation and correction of possible matrix effects on plasma, muscle and liver-free amino acids analysis using the Pico-Tag method. A statistical protocol based on the standard addition methodology and the Youden curve [18] was used. A new calibration function is proposed to correct the matrix effect when it is not possible to have an amino acids-free matrix (plasma, muscle or liver). This function was validated using a recovery study.

2. Experimental

2.1. Reagents and standards

The physiological acidic and neutral, and basic amino acid standard solutions (in 0.l M HCl), phenylisothiocyanate, norleucine and glutamine were purchased form Pierce (Rockford, IL, USA). HPLC-grade methanol and acetonitrile were purchased from Scharlau Chemie S.A. (Barcelona, Spain) and sodium acetate (HPLC grade, trihydrate), ethylenediamminetetracetic acid disodium salt (EDTA), sample eluent (phosphate buffer, pH 7.40) and triethylamine (TEA) were from Sigma (St Louis, MO, USA). Chemically pure acetic acid glacial was purchased from Panreac (Barcelona, Spain).

High purity water (>18 MΩ/cm) was obtained by a Milli-Q purification system (Millipore, Milford, MA, USA) and used to prepare mobile phases.

The Pico-Tag physiological free amino acid analysis column, pre-column filter, ultrafiltration devices (Ultrafree MC microcentrifuge device, Millipore P/N SK lP44V7), reaction vials, samples diluent were purchased from Waters (Milford, MA, USA).

2.2. HPLC configuration

A Waters (Milford, MA, USA) HPLC system was used. The apparatus consisted of a temperature control module, two Model 510 solvent delivery systems, a model 710B Wisp autosampler, a Lambda-Max Model 481 multi-wavelength absorbance detector (controlled at 254 nm filter) and an Interface module all from Waters. Analytical method development, data collection and data integration were performed using a Maxima 820 Chromatography software (Dynamic Solution, Division of Millipore) run on a PC. The column was a Pico-Tag physiological free amino acid analysis C_{18} reversed-phase (300 mm × 3.9 mm i.d.) also from Waters. The column temperature was set at 46 ± 1 °C. Samples were injected in volumes of 10 μl.

2.3. Analytical solvents and gradient composition

Derivatized amino acids were eluted from the column using a binary gradient formed from eluent A (aqueous buffer of 70 mM sodium acetate containing 2.5% acetonitrile and 1 ppm EDTA titrated to pH 6.45 with 10% glacial acetic acid) and eluent B (organic phase containing acetonitrile–methanol–water, 45:15:40 v/v/v). Eluents were under an inert N_2 atmosphere. The gradient employed in the separation started with eluent B rising from 3–34% in 60 min. After a washing step of 10 min with 100% B, the column was re-equilibrated for 20 min with 100% A. A constant flow rate of 1 ml/min was maintained.

2.4. Plasma and tissue preparation

Blood, liver and biceps muscle were obtained from growing chickens slaughtered under general anaesthesia. Plasma was obtained by heparinized blood centrifugation at 1500 × *g* for 15 min. Fresh biceps muscle and liver were homogenized (Omni 2000, CO, USA) in hydrochloric acid (0.1 M) keeping the tube in ice, and centrifuged at 2000 × *g* for 20 min. Pellets were discarded and supernatants containing the free amino acids used for analysis. After addition of Norleucine as internal standar, and amino acid standard solution when necessary, plasma, muscle and liver homogenates were deproteinized by ultrafiltration (3000 × *g*, 20 min).

2.5. Derivatization of amino acids with PITC

Twenty five μl of deproteinized specimen, standard solution of amino acids or specimen spiked with standard solution of amino acids, were dried under vacuum (<45 mmHg; 1 mmHg = 133.332 Pa) by means of a thermo-regulated centrifuge (Gyrovap Howe Inc., USA). The samples were reconstructed with 10 μl of 1 M sodium acetate-methanol-TEA (2:1:1) solution, dried at 45 mmHg, and dissolved in 20 μl of derivatization solution (methanol–water–TEA–PITC, 7:1:1:1). Derivatization of

both primary and secondary amino acids occurred in 20 min at 25 °C and produced the corresponding phenylthiocarbamoyl (PTC) derivatives. The samples were then dried until a constant vacuum of 45 mmHg was obtained. Finally, the dried samples were dissolved in 150 μl of sample eluent and injected.

2.6. Calibration

Three calibration experiments are required to obtain the data set necessary to carry out the statistical protocol for evaluation of the method. The complete analytical procedure above mentioned was applied to all the calibration samples. Statgraphics Plus 5.0 software package [21] was used for regression analysis (lineal model). Alamin software package [22] was used for the statistical analysis of data.

2.6.1. External calibration (EC)

A set of calibration standards was prepared from a commercial standard solution (see above) containing 32 physiological amino acids at a concentration of 1 mM in 0.1 M HC1, except for cystine, cystathionine-1, cystathionine-2, hydroxylisine-1, hydroxylisne-2 (0.5 mM) and proline (5 mM). Glutamine was added fresh daily (1 mM final concentration) to the standard mix to prevent degradation in acidic solution. A nor-leucine solution 4 mM (in 0.1 M HCl) was added to standard solutions prior to derivatization as internal standard.

The standard mixture was diluted with 0.1 M HC1 to cover a wide range of final concentrations (0.1–1.0 mM final concentration, 0.1 mM increments). Additionally, a blank containing just 0.1 M HC1 and internal standard followed the same procedure than amino acids standard solutions. The standard solutions were dried under vacuum and reconstructed for derivatization as previously described. Two experimental replicates and three instrumental replicates of different concentrations of analyte standard solutions, including the blank, are necessary for evaluating the linear model by least square regression analysis [23].

2.6.2. Standard-added calibration (AC)

Standard-added calibration was obtained by addition of different amounts of amino acid standard solution to a constant volume of sample. It is not necessary to carry out replicates for each addition. The value of "zero" addition should be included. Increasing amounts of the amino acids standard solution (0.00–0.40 mM final concentration, 0.04 mM increments) and internal standard nor-leucine was added to 100 μl of plasma, muscle homogenate (9.3 mg/ml) or liver homogenate (8.4 mg/ml). Aliquots (450 μl) of each sample were deproteinized, and derivatized following the previously mentioned analytical procedure. Linear least square regression of analytical signal versus amount of amino acid added for each amino acid and each matrix were established and slope, intercept, and residual standard deviation for each curve were calculated.

2.6.3. Youden calibration (YC)

A Youden calibration curve was established with increasing amounts of sample. In this curve, the value that corresponds to sample volume "zero" is not included and replicates are not necessary. Aquaeus dilutions [0.1–1.0 (sample volume/total volume), 0.1 increments] with milliQ water of plasma, muscle homogenate or liver homogenate were prepared; nor-leucine was added as internal standard and 450 μl of each dilution was subjected to the whole analytical procedure as previously described. Linear least square regression curves of analytical signal on sample dilution were established.

3. Results and discussion

Matrices can affect the analyte response by two different mechanisms. Some of the matrix components present at the time of measuring can somehow modify the analyte response. Or some sample treatment, indispensable because of the matrix presence, can affect the response; any operation to which the sample is subjected prior to the measurement (and that are not necessary during EC, e.g. ultrafiltration) can result in a matrix effect.

3.1. Evaluation of matrix effect

3.1.1. Contribution of external calibration (EC) to the evaluation of matrix effect

Table 1 shows the statistical parameters obtained after applying least square lineal regression analysis to external calibration for each amino acid. The extrapolation of responses to amount zero, i.e. the regression intercept (a_{EC}; Fig. 1), measured in the presence of finite amounts of analyte is an estimate of the standard blank or reactive blank. For all amino acids, intercept was statistically not different from

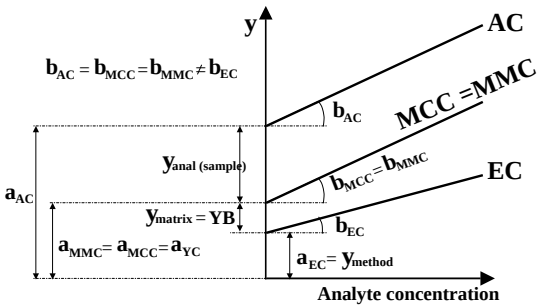

Fig. 1. External calibration (EC), standard-added calibration (AC), matrix-matched calibration (MMC) and matrix-corrected calibration (MCC) curves. a_{EC}, a_{AC}, a_{MMC} and a_{MCC} are the corresponding intercepts and b_{EC}, b_{AC}, b_{MMC} and b_{MCC}, the slopes. The analytical signal due to the method blank (y_{method}) is the intercept of the EC curve (a_{EC}) and $y_{anal(sample)}$ is the signal due to the initial fraction of analyte in the actual test sample portion. y_{matrix} is the signal due to the matrix blank, that is, the Youden blank. For details, see text.

Table 1
Intercepts (a_{EC}) and slopes (b_{EC}) of amino acids from external calibration, and slopes from standard-added calibration (b_{AC}) in different matrices: plasma, muscle and liver

Amino acid[a]	a_{EC}	b_{EC}	b_{AC}		
			Plasma	Muscle	Liver
CREA	−0.001	0.077	0.092	0.131	–
SER(P)	0.006	0.387	0.517	0.460	0.423
ASP	0.008	0.680	0.922	0.878	0.885
GLU	0.009	0.402	0.552	0.569	0.540
AAD	0.007	0.381	0.515	0.505	0.508
HYP	0.008	0.399	0.550	0.560	0.538
PEA	0.000	0.404	0.552	0.561	0.543
SER	0.001	0.428	0.628	0.602	–
ASN	0.002	0.193	0.280	0.273	–
GLY	0.004	0.433	0.667	0.629	0.614
GLN	0.010	0.472	0.691	0.683	0.630
β-ALA	0.010	0.555	0.736	0.730	0.713
TAU	0.008	0.435	0.620	0.611	0.617
HIS	0.000	0.340	0.587	0.579	0.574
THR	0.003	0.398	0.588	0.573	0.563
ALA	0.009	0.426	0.660	0.593	0.546
CAR	0.000	0.400	0.519	0.662	–
PRO	0.014	0.422	0.649	0.599	0.592
1MHIS	0.009	0.457	0.638	0.635	0.649
ANS	−0.002	0.416	0.380	1.277	0.541
ETN	0.001	0.381	0.563	0.534	0.531
ABU	0.010	0.435	0.532	0.576	0.579
TYR	0.006	0.462	0.615	0.611	0.608
VAL	0.001	0.468	0.533	0.543	0.547
MET	0.008	0.482	0.717	0.684	0.662
CYS	−0.003	0.260	0.380	0.357	0.331
ILE	0.015	0.493	0.923	0.738	0.720
LEU	0.001	0.474	0.696	0.689	0.703
PHE	0.000	0.493	0.628	0.655	0.625
TRP	0.004	0.469	0.657	0.637	0.562
ORN	0.000	0.848	1.142	1.143	1.093
LYS	0.021	0.743	1.141	1.171	1.125

[a] Amino acids abbreviations: CREA, Creatinine; SER(P), (*O*-3-phosphonoserine); ASP, aspartic acid; GLU, glutamic acid; AAD, α-aminoadipic acid; HYP, 4-hydroxyproline; PEA, phosphoethanolamine; SER, serine; ASN, asparagine; GLY, glycine; GLN, glutamine; β-ALA, β-alanine; TAU, taurine; HIS, histidine; THR, threonine; ALA, alanine; CAR, carnitine; PRO, proline; MHIS, 1-methyl-histidine; ANS, anserine; ETN, ethanolamine; ABU, α-aminobutyric acid; TYR, tyrosine; VAL, valine; MET, methionine; CYS, cysteine; ILE, isoleucine; LEU, leucine; PHE, phenylalanine; TRP, tryptophan; ORN, ornithine; LYS, Lysine.

zero [24] that is, there is no standard blank and the reagents do not contribute to analytical signal.

The lack-of-fit test was applied to check the linearity of the calibration graphs according to the Analytical Method Committee [25]. It determines whether the linear model is adequate to describe the observed data and it is performed by comparing the variability of the current model residuals to the variability between observations at replicate values of the independent variable (amount of amino acid in the standard solution). Since the *P*-value for lack-of-fit test was greater or equal to 0.10 for all amino acids, the linear model appears to be adequate for the observed data (0.0–1.0 mM).

Table 2
Comparisons of slopes from external calibration (b_{EC}) and slopes from addition calibration (b_{AC}) in different matrices: plasma, muscle and liver, using appropriate *t*-test: calculated *t*-value (t_b) and significance level (*P*-value) associated

Amino acid	Plasma		Muscle		Liver	
	t_b	*P*-value (%)	t_b	*P*-value (%)	t_b	*P*-value (%)
CREA	3.0	6.1×10^{-3}	9.1	4.8×10^{-9}	–	–
SER(P)	10.2	1.1×10^{-8}	5.5	3.9×10^{-5}	2.3	3.7×10^{-2}
ASP	10.9	5.8×10^{-11}	8.7	6.6×10^{-9}	8.3	9.2×10^{-9}
GLU	12.4	1.2×10^{-11}	10.7	3.6×10^{-10}	10.7	2.2×10^{-10}
AAD	9.3	1.4×10^{-9}	8.7	8.0×10^{-11}	8.8	3.7×10^{-9}
HYP	7.9	2.2×10^{-8}	8.6	4.5×10^{-9}	7.4	7.8×10^{-9}
PEA	6.2	2.2×10^{-6}	7.7	5.8×10^{-8}	7.6	6.0×10^{-8}
SER	19.1	1.2×10^{-15}	16.4	8.1×10^{-14}	–	–
ASN	19.5	3.3×10^{-16}	16.9	7.8×10^{-15}	–	–
GLY	18.0	8.1×10^{-14}	14.8	3.0×10^{-12}	12.7	4.8×10^{-11}
GLN	15.6	5.1×10^{-13}	16.6	3.4×10^{-13}	12.0	1.2×10^{-10}
β-ALA	6.7	3.0×10^{-7}	6.5	5.5×10^{-7}	5.9	2.6×10^{-6}
TAU	15.2	1.0×10^{-11}	15.1	1.1×10^{-11}	14.8	1.5×10^{-11}
HIS	30.4	1.4×10^{-17}	37.6	2.7×10^{-19}	30.1	4.1×10^{-18}
THR	9.7	8.5×10^{-11}	8.8	9.0×10^{-10}	7.2	3.7×10^{-8}
ALA	10.4	2.8×10^{-11}	7.5	3.3×10^{-8}	5.4	7.1×10^{-6}
CAR	6.8	6.1×10^{-7}	9.2	3.5×10^{-9}	–	–
PRO	14.3	2.9×10^{-11}	9.9	5.9×10^{-9}	11.6	4.9×10^{-10}
1MHIS	14.9	2.8×10^{-12}	15.0	1.0×10^{-12}	14.4	2.4×10^{-12}
ANS	5.9	8.4×10^{-6}	6.7	1.3×10^{-6}	14.5	9.6×10^{-12}
ETN	11.1	3.9×10^{-11}	10.9	5.7×10^{-11}	10.4	1.4×10^{-10}
ABU	9.6	6.9×10^{-10}	14.3	7.8×10^{-14}	12.4	3.5×10^{-12}
TYR	17.9	8.6×10^{-14}	22.8	8.5×10^{-16}	17.9	8.8×10^{-14}
VAL	5.8	1.1×10^{-5}	7.7	1.4×10^{-7}	7.1	5.3×10^{-7}
MET	15.4	6.5×10^{-14}	15.9	1.4×10^{-14}	12.6	6.8×10^{-8}
CYS	13.5	1.6×10^{-10}	10.9	4.6×10^{-9}	9.6	8.4×10^{-8}
ILE	18.4	1.9×10^{-16}	11.9	5.4×10^{-12}	13.5	3.1×10^{-13}
LEU	10.6	4.3×10^{-8}	10.9	1.6×10^{-8}	10.2	6.2×10^{-7}
PHE	22.2	1.9×10^{-18}	26.8	2.8×10^{-18}	16.7	2.3×10^{-14}
TRP	14.9	1.2×10^{-12}	16.8	2.8×10^{-13}	7.3	4.4×10^{-7}
ORN	14.0	8.8×10^{-11}	16.9	4.7×10^{-12}	17.0	1.5×10^{-12}
LYS	11.8	1.1×10^{-8}	12.6	5.0×10^{-9}	12.7	1.0×10^{-8}

3.1.2. Contribution of AC to the evaluation of matrix effect

Slopes obtained in the standard-added calibration for each amino acid studied and for each matrix are shown in Table 1. Proportional bias is estimated comparing the slopes of the straight lines of external calibration and addition calibration. If the slopes are similar, then a component of proportional bias is not involved. To check the similarity of the EC and AC slopes (b_{EC} and b_{AC}, respectively; Fig. 1), a Student's *t* test was applied [16,26]. The calculated *t*-value (t_b) and the significance level (*P*-value) associated are shown in Table 2. The alternative hypothesis was accepted for a significance level less than 1%. For all amino acids and matrices (plasma, muscle, liver) considered the significance level is less than 1%, which implies a second order analyte/matrix effect, that is, there is a proportional bias involved in the analysis of amino acids using Pico-Tag HPLC method.

3.1.3. Contribution of YC to the evaluation of matrix effect

A difference between the intercepts of the curves EC and YC (a_{EC} and a_{YC}, respectively; Fig. 1), indicates a constant

bias due to sample matrix effect. Because both intercepts are obtained from different independent variables (amino acid final concentration and sample dilution, respectively, for EC and YC), it is not possible to establish a comparison test. However, it can be considered that a difference between both values exists if the value of the Youden calibration intercept (a_{YC}) is not included within the confidence interval value of the external calibration intercept (a_{EC}) [16]. If a_{YC} is not included within the a_{EC} confidence interval, the true sample "blank" (Youden blank) is calculated using the following expression:

$$YB = a_{YC} - a_{EC} \quad (1)$$

The true sample blank is the difference between the intercepts of Youden calibration and external calibration (reagent blank) regressions. Intercepts from Youden Calibrations (a_{YC}) for plasma, muscle and liver for each amino acid were not significantly different from zero, which implies that there is no constant bias due to matrix effect in the analysis of amino acids using Pico-Tag HPLC method.

3.2. Correction of matrix effect in sample amino acid composition calculation

If there is a constant error and/or a proportional error due to the matrix nature it should be considered the correction of this effect when calculating sample analyte concentration. In sample composition calculations gross errors can be made when analysis is attempted by combining a single sample signal and a external calibration ignoring constant and proportional matrix errors:

$$c_{x,EC} = \frac{R_x - a_{EC}}{b_{EC}} \quad (2)$$

Where, $c_{x,EC}$ is the concentration of the analyte x estimated from EC in the test portion, R_x is the measured response of the sample test portion, a_{EC} is the intercept of the external calibration curve and b_{EC} is the slope of the external calibration curve.

Sample composition calculations should be made combining both AC and YC features, to correct for the matrix effect [16]:

$$c_{x,AC} = \frac{(a_{AC} - YB) - a_{EC}}{b_{AC}} = \frac{a_{AC} - a_{YC}}{b_{AC}} \quad (3)$$

where $c_{x,AC}$ is the concentration of analyte x estimated from AC in the test portion, a_{AC} the intercept of the AC curve, YB the Youden blank as previously described, a_{EC} the intercept of the EC curve, b_{AC} the slope of the AC curve and a_{YC} is the intercept of the YC curve.

Constant error is corrected deducting the intercept of YC curve (a_{YC}) from the intercept of the AC curve (a_{AC}), and when this figure is expressed relative to the slope of the AC curvet (b_{AC}), proportional error is taken into account as well.

In our study only the presence of proportional bias was detected ($b_{EC} \neq b_{AC}$) and ordinates of external calibration (a_{EC}) and Youden calibration (a_{YC}) were equal to zero, therefore, the analyte concentration in the test portion can be calculated according to the following formula:

$$c_{x,AC} = \frac{a_{AC}}{b_{AC}} \quad (4)$$

Table 3 shows the differences in three amino acids concentration for plasma, muscle and liver matrices when calculations are attempted considering ($c_{x,AC}$) or not ($c_{x,EC}$) the matrix effect. $c_{x,EC}$ was calculated from the EC curve and $c_{x,AC}$ was calculated from theAC curve. Failure to consider the matrix effects grossly underestimate the real free amino acid concentrations in the three matrices considered. Therefore, wrong conclusions could be derived from nutritional and metabolic studies where free amino acid analysis are performed in complex matrices without taking into account constant and or proportional errors introduced by the matrices.

3.3. New methodology for correction of matrix effects

3.3.1. Matrix-corrected calibration (MCC)

Matrix-matched calibration (MMC) [18,27] is a calibration procedure where an analyte-free matrix is spiked with known amounts of the standard. For amino acids analysis it is not feasible the use of amino acid free plasma, muscle or liver matrices for MMC. The approach we propose is a simulation of a MMC by means of an empirical calibration curve obtained using the YC and AC curves regression parameters. This new calibration function, named MCC, is a linear curve where the independent variable is the analyte concentration (in the presence of matrix) and the dependent variable is the analytical signal (Fig. 1).

The MCC slope is defined as:

$$b_{MCC} = b_{AC} \quad (5)$$

where b_{MCC} is the slope of the MCC curve and b_{AC} is the slope of the AC curve.

The MCC intercept is calculated from:

$$a_{MCC} = a_{AC} - y_{anal(sample)} = a_{AC} - (a_{AC} - a_{YC}) = a_{YC} \quad (6)$$

where a_{MCC} is the intercept of the MCC curve, a_{AC} is the intercept of the AC curve, $y_{anal(sample)}$ is the contribution to the signal due to the initial fraction of analyte present in the test sample portion, and a_{YC} is the intercept of the YC curve. Both standard blank a_{EC} and Youden blank (YB) can potentially contribute to a_{YC} (see Fig. 1).

The proposed expression for MCC function is:

$$y = a_{MCC} + b_{MCC} \cdot x = a_{YC} + b_{AC} \cdot c_{x,MCC} \quad (7)$$

where y is the measured total signal from the test sample portion, and $c_{x,MCC}$ is the (unbiased) estimated analyte concentration in the test sample portion.

Eqs. (5)–(7) are exclusive for each type of matrix (each matrix has its own AC curve) and are valid only provided

Table 3
Differences in amino acid concentration[a] for plasma, muscle and liver real samples when calculations are attempted from AC ($c_{x,AC}$) or from EC ($c_{x,EC}$) (the matrix effect is not considered in the last one), and recovery from samples spiked with three different levels of amino acids

Matrix	Parameter	THR (mM)	VAL (mM)	ALA (mM)
Plasma	$c_{x,EC}$ (mM)	0.288	0.165	0.270
	$c_{x,AC}$ (mM)	0.615	0.439	0.605
	c_{added} (mM)	0.120, 0.240, 0.360	0.120, 0.240, 0.360	0.120, 0.240, 0.360
	c_{found} (mM)	0.122, 0.239, 0.362	0.124, 0.237, 0.357	0.117, 0.250, 0.365
	Recovery (%)	101.6, 99.5, 100.6	103.3, 98.7, 99.2	97.5, 104.2, 101.4
	$\bar{\Re}$[b]	100.6	100.4	101.0
	P-value[c]	0.518	0.840	0.702
Muscle	$c_{x,EC}$ (mM)	0.058	0.036	0.034
	$c_{x,AC}$ (mM)	0.152	0.099	0.164
	c_{added} (mM)	0.120, 0.240, 0.360	0.120, 0.240, 0.360	0.120, 0.240, 0.360
	c_{found} (mM)	0.118, 0.227, 0.360	0.117, 0.244, 0.358	0.122, 0.240, 0.360
	Recovery (%)	98.3, 94.6, 100.0	97.2, 101.8, 99.5	101.5, 100.0, 100.0
	$\bar{\Re}$[b]	97.6	99.5	100.5
	P-value[c]	0.336	0.782	0.420
Liver	$c_{x,EC}$ (mM)	0.107	0.062	0.287
	$c_{x,AC}$ (mM)	0.258	0.166	0.772
	c_{added} (mM)	0.120, 0.240, 0.360	0.120, 0.240, 0.360	0.120, 0.240, 0.360
	c_{found} (mM)	0.120, 0.230, 0.351	0.119, 0.238, 0.350	0.118, 0.245, 0.350
	Recovery (%)	100.0, 95.9, 97.6	99.2, 99.3, 97.2	98.3, 102.2, 97.2
	$\bar{\Re}$[b]	97.8	98.6	99.2
	P-value[c]	0.268	0.224	0.656

[a] Analyte sample concentration was obtained from the estimated test portion concentration (from Eqs. (2) and (4)) and the appropriate dilution factor.
[b] Average recovery.
[c] *P*-value of the recovery test. If $P > 0.20$, recovery is not significantly different from 100%.

that the amount of sample used for analysis is always the same. This is because the slope of the AC curve (b_{AC}) to correct the proportional error introduced by the matrix, may change when different matrix amounts are used and consequently Eq. (5) would not be valid ($b_{MCC} \neq b_{AC}$). For each type of matrix and analyte, characteristic values for the coefficients of Eq. (7) can be established (from the AC and YC of each matrix) and this function can be used to calculate the analyte concentration in any sample without matrix systematic errors.

Fig. 1 shows the relationship among parameters of the different calibrations. MCC curve is the line that would be obtained if an amino acid free matrix were available and a MMC were performed. Slopes of both AC (b_{AC}) and MCC (b_{MCC}) curves are identical but different from the slope of the EC curve (b_{EC}). The intercept of MCC curve (a_{MCC}) is the same as the one of YC curve (a_{YC}).

In our study, both method blank (a_{EC}), and total Youden blank (a_{YC}) are zero for all amino acids and matrices, and consequently Eq. (7) would be rewritten as:

$$y = a_{MCC} + b_{MCC} \cdot x = b_{AC} \cdot c_{x,MCC} \quad (8)$$

3.3.2. Validation of MCC using a recovery test

A recovery study was performed to validate the matrix-corrected calibration. Four aliquots of plasma, muscle and liver were used and three of them were spiked with known amounts of standard. As we know the analytical signal of sample spiked or not with the standard we can calculate the analytical signal due the spiked standard [28]:

$$y_{anal(added)} = y_{anal(sample+added)} - y_{anal(sample)} \quad (9)$$

where $y_{anal(added)}$ is the signal due to the added standard to each sample, $y_{anal(sample+added)}$ the total signal from the spiked samples and $y_{anal(sample)}$ is the net signal from the non-spiked sample.

The signal corresponding to the added standard, $y_{anal(added)}$, is substituted for y_{MCC} in Eq. (8) to obtain the found concentration, $c_{x,MCC}$ (or c_{found}) (Table 3). Recovery, $\Re$, is calculated from:

$$\Re = \frac{c_{found}}{c_{added}} \times 100\% \quad (10)$$

Recovery was calculated at three different levels of addition and a recovery test performed where the average recovery for each amino acid is compared to 100 [26]. Table 3 shows that recoveries are close to 100% in plasma, muscle and liver ($P > 0.20$). Therefore, the MCC-based method of correction of the matrix effect has been validated.

Establishment of a MCC function from AC and YC for each type of matrix and analyte overcomes the inconvenience of using the usual method of standard addition where AC and YC curves are necessary for each individual sample.

Advances in the knowledge of amino acid metabolism in farm animals are very dependent on the accurate and precise determination of amino acids in body fluids and tissues. Current development in the chromatography of amino acids, although improving the speed and sensitivity of the methods

have done little to improve their precision and accuracy. We have demonstrated the suitability of the Pico-Tag method for routine analyses of free physiological amino acids in plasma, muscle and liver, provided that the matrix effect is considered and corrected. The no consideration of the matrix effect when analyzing free amino acids in physiological fluids may lead to inaccurate results and this is of paramount importance when quantitative analysis is necessary.

4. Conclusions

Establishment of a Youden calibration and an standard-added calibration together with the external calibration is necessary to take into account the constant and proportional errors introduced by plasma, muscle and liver matrices when free amino acids are determined using the Pico-Tag method. A new function, matrix-corrected calibration, is proposed to correct for constant and/or proportional errors introduced by a complex matrix when it is not possible to use an analyte free matrix for standard-added calibration.

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