Comparison of Two Quantitation Methods in HPLC: Standardless Versus Calibration with External Standards. Application to the Analysis of Amino Acids in Fruit Juices

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Abstract

A distinctive method of high-performance liquid calibration peak-area quantitation, called the standardless or absolute method, allows the absolute number of moles of the main amino acids found in twenty-six commercial fruit juices to be determined. The theory of the standardless model is briefly illustrated. The results found with this alternative method are compared with the reference procedure (calibration with external standards) by using difference plots and regression analysis. The presence of both systematic, constant, and proportional errors between the two methods is disclosed and discussed.

Introduction

There is an ongoing interest in the development of an accurate method of analysis and measurement for routine and research purposes. Quantitative results in high-performance liquid chromatography (HPLC) are usually based on calibration with standards and peak-area measurement. A distinctive method to determine the absolute number of moles of a compound from peak-area measurements using HPLC with a UV–vis detector has been proposed (1,2). The model requires the knowledge of (*i*) cell thickness, (*ii*) molar absorptivity of the analyte, (*iii*) flow-rate, and (*iv*) absorbance. The alternative method of peak-area quantitation may replace the often tedious and sometimes expensive calibration procedure. The main advantage of the standardless method of analysis is the possibility of obtaining a quantitation of the analyte from a single measurement if the system has been conveniently calibrated.

The validation of a new analytical method requires a comparison with a formerly used standard method (3). The comparison is done by analyzing a set of samples over the concentration range to be validated, and the main interest is the identification of systematic errors (constant or proportional). A statistical test is applied to compare the results obtained with the two methods. Linear regression and paired *t*-test are commonly used to estimate the bias of the new method (4). If no bias exists, the two methods differ only by random error; therefore, each sample yields an identical result with both analytical methods, and the regression line has a zero intercept ($\alpha = 0$) and a slope (β) of 1. Least-squares analysis can estimate proportional error and should be considered a prerequisite to the Student's *t*-test analysis (5). Moreover, visual examination of results is always recommended to detect a trend in the data (6).

In this study, the main amino acids of 26 fruit juices were analyzed by HPLC, then the peaks-area was quantitated by conventional external standard calibration and values compared with those obtained using the alternative absolute method of quantitation.

Experimental

Sample analysis

Nine amino acids in 26 commercial fruit juices (apricot, peach, apple, pear, orange, pineapple, grapefruit, and blueberry) were analysed by reversed phase-HPLC–UV (LC-1500 HPLC system, JASCO, Tokyo, Japan) (Figure 1) as previously described in the literature (7).

Peak quantitation

Peaks-area quantification was based on (*i*) the absolute analysis (i.e., standardless method) and (*ii*) the external standard calibration using linear regression analysis (8) (Statistica 6.0, StatSoft, Tulsa, OK; Analyse-it 1.50, Leeds, UK). The calibration plots, relating the signal (peak area) to the concentration, yielded the linear equation $y = \alpha + \beta x$, where y is the signal (μAU), α the intercept (signal), β the slope (signal/concentration), and x the amino acid concentration (range 0–1.25mM, measured in duplicate, except for asparagine). In order to determine the absolute number of moles, the flow rate, the cell thickness, the correction factor, and the molar absorp-

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tivity at 263 nm of each amino acid were measured as described in the literature (1). As concentration values are often used in food science, the moles of each amino acid were converted to mg/L.

Statistical analysis

A preliminary visual examination of results was assessed by the residual analysis and by plotting the difference between the methods versus their mean (6). Data of the two methods of measurement, absolute method and external calibration, were compared at 5% level by Deming's regression, which considers the errors in both variables and minimizes the distance of the data points orthogonal to the regression line (9). The comparison between the two methods of quantitation tested the deviations of the intercept ($\alpha = 0$) and the slope ($\beta = 1$).

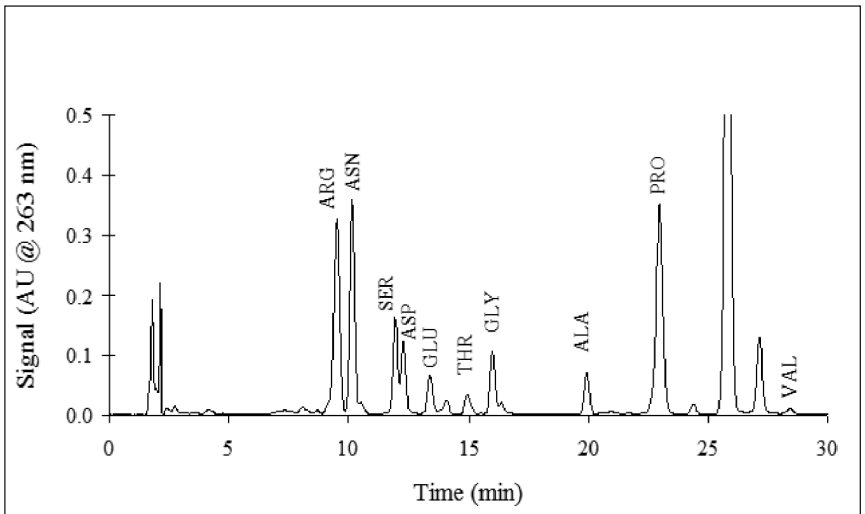

Figure 1. Chromatogram of a fruit juice. For abbreviation identification see Table II.

Results and Discussion

The absolute method of quantitation yielded the final equation:

$$\hat{A}\,F = 10^3\,\varepsilon\,b\,N = 10^3\,\varepsilon\,c\,V_{loop}$$

where $\hat{A}$ (min) is the area of the chromatographic peak of the analyte; F (1.011 cm^3/min) the flow rate of the mobile phase; ε (l/mol/cm) the molar absorptivity of the analyte at the wavelength used; b (0.954 cm) the cell tickness; c (mol cm^{-3}) the concentration of the analyte; N (mol) the number of moles eluted; V_{loop} (21.8 10^{-6} cm^3) the loop volume; and 10^3 is a numerical factor necessary because the values of ε found in the literature are frequently reported in l/mol/cm, and if F is expressed in cm^3/min, the liters must be converted into cm^3.

The estimated value of cell thickness, flow rate and loop volume was in good agreement with the expected values of 1 cm, 1 cm^3/min, and 20-µL, respectively. Amino acids showed a similar molar absorptivity and sensitivity (slope) as a consequence of the derivatization procedure (Table I). For each amino acid, the linearity (r) of calibration was significant, the lack of linear fit was rejected at 5% level (i.e., the linear model fully described the data), and the distribution of residuals showed a lack of trend (data not shown).

The difference or bias plot was used to compare the results of the two methods by plotting the differences (method A – method B) as a function of the measurements

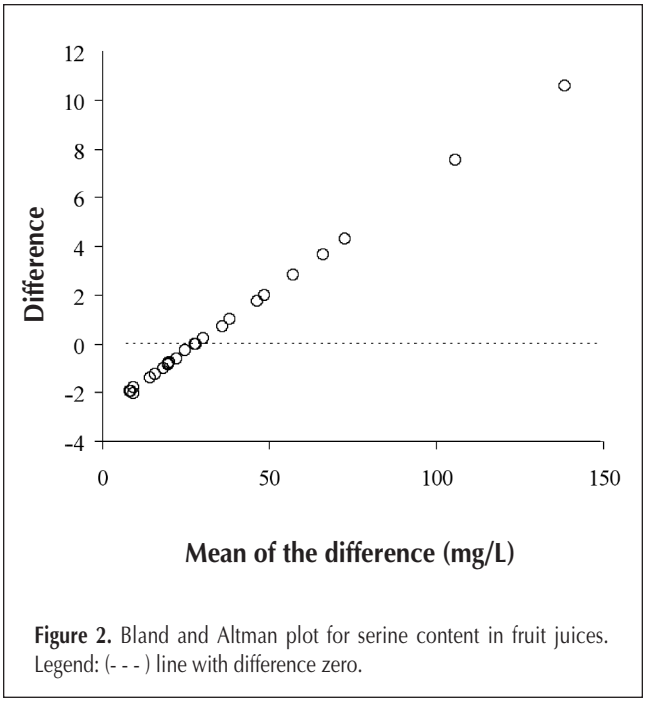

Figure 2. Bland and Altman plot for serine content in fruit juices. Legend: (- - -) line with difference zero.

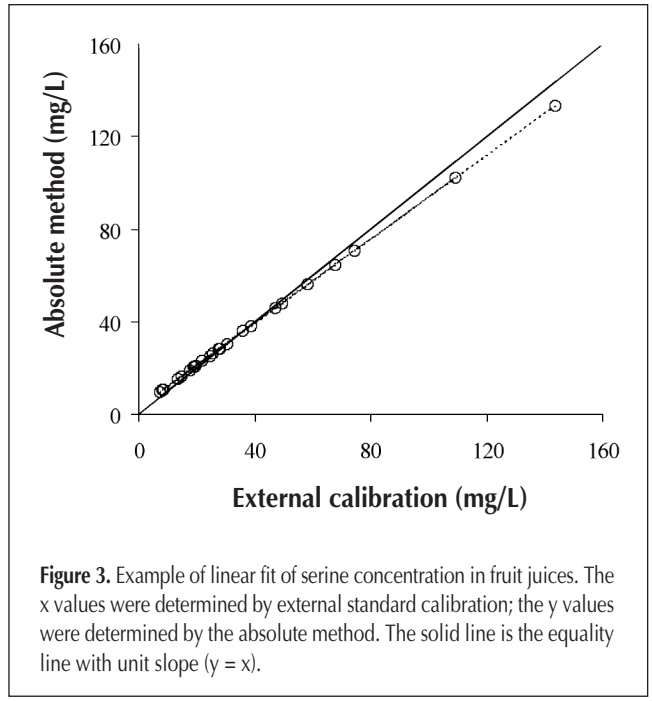

Figure 3. Example of linear fit of serine concentration in fruit juices. The x values were determined by external standard calibration; the y values were determined by the absolute method. The solid line is the equality line with unit slope (y = x).

Table I. Amino Acids, Code, Molar Absorptivity (ε), and Calibration Parameters with External Standards

Amino acid	code	ε (*l* mol^{-1}/cm)	Calibration parameters: Intercept ± SE* (Â)	Slope ± SE* (Â mM^{-1})	Model SE	Lack of fit†
Arginine	ARG	17600	0.4 ± 0.3	3.0 ± 0.4	0.04	0.0883
Asparagine	ASN	19100	–1 ± 2	4.0 ± 0.3	1	n.d.‡
Serine	SER	19300	0.0 ± 0.1	3.0 ± 0.1	0.1	0.0984
Aspartic acid	ASP	18400	–0.02 ± 0.04	3.2 ± 0.1	0.1	0.0698
Glutamic acid	GLU	18900	0.01 ± 0.02	2.95 ± 0.04	0.03	0.3621
Threonine	THR	18600	0.01 ± 0.02	3.03 ± 0.03	0.03	0.3489
Glycine	GLY	19600	0.02 ± 0.03	3.2 ± 0.1	0.1	0.2710
Alanine	ALA	19500	0.00 ± 0.03	3.19 ± 0.04	0.04	0.2154
Proline	PRO	18200	0.0 ± 0.1	3.14 ± 0.01	0.1	0.5355
Valine	VAL	19500	0.00 ± 0.03	3.20 ± 0.04	0.04	0.1241

* SE, standard error (× 10^{-6});
† *p*-level [F_{crit} (α = 0.05; df_{LOF} = 4; df_{PE} = 4) = 6.39];
‡ n.d., not determined.

Table II. Content of Amino Acids in 26 Fruit Juices Based on Quantitation with Absolute Method and External Calibration, Bias, and Method Comparison by Deming Regression Analysis

Amino acid	Fruit juice composition* (mg/L): Absolute method	External calibration	Bias‡	Deming regression†: Intercept (α)	Slope (β)
ARG	138 ± 241	137 ± 249	1 ± 6	2.7 ± 0.1	0.97 ± 0.01
ASN	718 ± 531	626 ± 636	–90 ± 100	n.d.§	n.d.§
SER	36 ± 30	37 ± 33	1 ± 3	2.62 ± 0.03	0.910 ± 0.001
ASP	416 ± 61	42 ± 61	1.1 ± 0.6	–0.7 ± 0.1	0.990 ± 0.001
GLU	20 ± 17	22 ± 19	2 ± 2	0.6 ± 0.1	0.900 ± 0.004
THR	256 ± 9	26 ± 10	1.1 ± 0.6	0.4 ± 0.2	0.940 ± 0.007
GLY	189 ± 18	19 ± 19	1 ± 1	0.4 ± 0.1	0.930 ± 0.002
ALA	20 ± 16	21 ± 17	1 ± 0.9	0.2 ± 0.1	0.940 ± 0.002
PRO	100 ± 134	101 ± 137	1 ± 2	0.8 ± 0.2	0.980 ± 0.001
VAL	11 ± 9	12 ± 9	0.8 ± 0.4	–0.3 ± 0.1	0.960 ± 0.009

* mean ± SD of the composition of twenty-six different fruit juices.
† Value ± 95% confidence limits ($t_{0.05;\ df\ 24}$ = 2.064).
‡ (absolute method – external calibration).
§ not determined (due to missing the lack of fit test).

average [(method A + method B)/2]. Each amino acid showed a strong correlation ($r \geq 0.96$) between the differences and the means of difference; thus, a trend in the data was revealed (Figure 2). The result implied the presence of a systematic proportional difference between the two methods of measurement. Torsi et al. (2) have disclosed a systematic error up to 20%, which is dependent on the type of detector used. No mention was made regarding the type of systematic error found, that is, proportional or constant.

The two methods of quantitation showed a similar variance, and the comparison by Deming's regression showed the presence of both systematic constant ($\alpha \neq 0$) and proportional errors ($\beta \neq 1$). The proportional error was below 10%, whereas the absolute constant error was maximum for arginine, being 2.7 mg/L (Table II). The absolute method measured higher than the reference in the lower range of values and lower than the reference in the upper range (Figure 3). Under this condition, the paired *t*-test is unsuitable for testing the relationship between the two methods. In fact, the *t*-test allows the evaluation of random and constant errors, but only when the proportional error is absent.

Conclusion

Despite the high correlation between the two methods, these are not in perfect agreement. Further investigation is needed to understand the origin of these analytical errors.

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