# USE OF THE INTERNAL AND EXTERNAL STANDARD TECHNIQUES IN DETERMINATIONS OF AMINO ACIDS BY ION EXCHANGE CHROMATOGRAPHY

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Influence of the internal standard technique on the precision of amino acid determination was studied. The law of propagation of errors and the information theory was applied in this paper. From the results it follows that the internal standard technique did not inevitably improve the precision of the determination. In the case of a multicomponent sample, the external standard technique improved the accuracy only if calibration curve was used.

Internal and external standard techniques are widely used in chromatography. These techniques are also used in the determination of amino acids on automatic amino acid analysers by means of ion exchange chromatography.

Generally, it is assumed that internal standard techniques improve the precision of determination. On the other hand, some critical remarks have been published about usefulness of the internal standard technique[1,2]. Requirements for application of the internal standard technique are given in literature[2,3]. Use of the internal standard in amino acid determinations has, in comparison to other chromatographic methods, some specifities. Only one internal standard is used for the whole amino acid spectrum (in the case of the dual column system, one internal standard for the short column and one for the long column are used). For this reason, the internal standard is not eluted near all the peaks of interest. Similarly, the peak height (or peak area) of the internal standard is not similar to all the amino acid peaks heights (peak areas).

The use of external standard technique is attractive for chromatographs with automatic sample injectors which guarantee the precision of sample injection. These chromatographs, in connection with integrators save a considerable amount of time in calculation and presentation of results. However, some critical remarks on the use of this technique in amino acids determination have been presented[4,5].

The aim of this study was to investigate influence of the internal standard technique use on the precision of amino acids determination. The law of propagation of errors

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and the information theory were applied for these purposes. Influence of the external standard technique on the accuracy of amino acids determination was also studied.

## THEORETICAL

If the internal standard technique improves the precision of a method, the following has to be hold true[2]

$$s_{q,rel} < s_{x,rel} , \tag{1}$$

where $s_{x,rel}$ is the relative standard deviation (the coefficient of variation) of the peak of the substance to be determined and $s_{q,rel}$ is the relative standard deviation of the quotient $q_i$

$$q_i = \frac{x_i}{x_I} , \tag{2}$$

where $x_i$ is the height (area) of the peak of the substance to be determined and $x_I$ is that of the internal standard one.

From the law of error propagation, Haefelfinger[2] derived the relation

$$s_{I,rel} < 2rs_{x,rel} , \tag{3}$$

where $s_{I,rel}$ is the relative standard deviation of the internal standard peak and $r$ is the correlation coefficient for relatiosnhip $x_i$ vs $x_I$. In many cases, the value of $s_{q,rel}$ is calculated directly from the values $q_i$. It is also possible to calculate this parameter from the equation

$$s_{q,rel} = (s^2_{x,rel} + s^2_{I,rel} - 2rs_{x,rel}s_{I,rel})^{1/2} . \tag{4}$$

Analysis is a process of obtaining information on the chemical composition of matter. The information content is used as the characteristics for evaluating information properties of analytical results[6,7].

$$I_1(p, p_0) = \log_2 \frac{x_2 - x_1}{\sigma \sqrt{2\pi e}} \tag{5a}$$

$$I_1(p, p_0) = \log_2 \frac{(x_2 - x_1)\sqrt{n}}{2st_v} , \tag{5b}$$

where $x_1$ and $x_2$ are minimum and maximum limits presumed in which content of the component to be determined is involved (%), $\sigma$ is standard deviation of a random variable, $s$ is estimation of the standard deviation, $n$ is the number of parallel determinations, $t_v$ is the critical value of the Student distributor for the numbers of

degrees of freedom $v = n - 1$ and at the significance level $\alpha = 0{\cdot}038794$ (ref.[6]).

Chromatographic methods are two-dimensional analytical methods. Thus, from the signal position we determine which component of the analysed sample is present and from the signal intensity its amount. The amount of information is given as

$$M(p, p_0) = \sum_{i=1}^{k} I(p, p_0)_i , \tag{6}$$

where $k$ is the number of all components determined simultaneously.

The accuracy of the results can be inflenced by calibration. The accuracy is characterized by the difference

$$d = |\bar{x} - X| , \tag{7}$$

where $d$ is the estimated mean error of the determination, $\bar{x}$ is a mean of $n$ parallel determinations, $X$ is the true value of the component. If $d > 0$ does not differ significantly from zero, we express the information content of the results of the quantitative analysis by Eqs (*5a*) and (*5b*). On the contrary, for a significant difference, the information content is given as ref.[6]

$$I_2(p, p_0) = \log_2 \frac{(x_2 - x_1)\sqrt{n}}{2st_v} - \frac{1}{2}\left(\frac{d}{s}\right)^2 . \tag{8}$$

## EXPERIMENTAL

Evaluation of the effect of the use of the internal standard technique on the precision of determination of amino acids was carried out using a AAA-881 (Mikrotechna, Prague) amino acid analyzer with a dual column system. Samples were injected onto the column by manually operated sampling valve, a loop with capacity of 200-μl being used to load 100 nmol of the amino acid. Either tryptophan (TRY) or α-amino-β-guanidin propionic acid (AGP) were used as internal standards for the short column and L-norleucine (NLEU) for the long one.

Evaluation of the effect of the used of the external standard technique on the accuracy of amino acid analysis was carried out using an AAA-T339 (Mikrotechna, Prague) amino acid analyzer with a single column system. Samples were injected onto the column by an automatically operated sampling valve a loop with a capacity of 100-μl being used to load 25 nmol of the amino acid. The peak area was determined by means of the System I integrator (Spectra Physics, San Jose). Amino acid content in beef meat meal and *Psophocarpus tetragonolobus* (L.) DC was determined after acid hydrolysis[7]. Samples were hydrolyzed in 6M HCl in vacuum-sealed tubes at 110°C for 24 h.

The statistical calculations were carried out according to Eckschlager et al.[8].

## RESULTS AND DISCUSSION

Effect of the internal standard peak positions on precision of the amino acids determination on a short column were studied (Table I). The use of tryptophan as the internal standard did not improve the precision of the determination, as its relative

standard deviation was higher than those of the other amino acids. It was evidently caused by the dosing buffer (pH 2·2) which changed the chromatographic conditions on the top of the chromatographic column. In the second example (AGP was used as the internal standard), although $s_{x,rel}$ values were higher than for tryptophan, the use of the internal standard improved the precision of the determination. It has been found that Eq. (*4*) did not apply for $s_{x,rel} > 10\%$ (see arginine with AGP as the internal standard).

The effects of the internal standard on the precision of amino acids determination (long column) are given in Table II.

The correlation coefficient is within the limits $-1 < r < 1$. It is obvious that between the areas of the internal standard peak and the other amino acid peaks only positive correlations may exist ($r > 0$). But in fact, even negative values were calculated. This was caused probably by the effect of the matrix of the sample on the shape of chromatographic peaks an their area calculation. For $r < 0$ values, it is not surprising there is no improvement in the precision of the amino acids determination when internal standard is used. No conditions given by Eq. (*3*) are fulfilled. On the contrary, true values of the standard deviation $s^c_{q,rel}$ for $r < 0$ were calculated by using Eq. (*4*). This is in agreement with the results of Haefelfinger[2]. The agreement between both $s_{q,rel}$ and $s^c_{q,rel}$ values are affected by the number of parallel determinations.

TABLE I

Precision of determination of amino acids in beef meat meal on a short column ($n = 12$)[a]

| Amino acid | $\bar{x}$ a.u. | $s_{x,rel}$ % | $r$ — | $2rs_{x,rel}$ % | $\bar{q}$ — | $s_{q,rel}$ % | $s^c_{q,rel}$ % |
|---|---|---|---|---|---|---|---|
| | | | Internal standard: Try | | | | |
| Try | 4·89 | 4·04[b] | — | — | — | — | — |
| Lys | 8·49 | 3·63 | −0·376 | −2·729 | 1·76 | 6·25 | 6·36 |
| His | 4·38 | 2·73 | 0·234 | 1·277 | 0·90 | 4·33 | 4·31 |
| Arg | 4·05 | 6·31 | 0·157 | 1·982 | 0·83 | 6·86 | 6·94 |
| | | | Internal standard : Agp | | | | |
| Lys | 7·85 | 7·53 | 0·548 | 8·248 | 1·31 | 6·40 | 6·38 |
| His | 3·95 | 8·30 | 0·400 | 6·643 | 0·66 | 7·72 | 7·83 |
| Agp | 6·00 | 5·15[b] | — | — | — | — | — |
| Arg | 4·14 | 14·88 | 0·253 | 7·528 | 0·70 | 15·31 | 14·46 |

[a] All values related to peak area; [b] used as $s_{I,rel}$ values in Eq. (*3*).

TABLE II

Precision of determination of amino acids on a long column

| Parameter | | Amino acid | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Asp | Thr | Ser | Glu | Gly | Ala | Val | iLeu | Leu | nLeu | Tyr | Phe |
| | | | | | | | Standards ($n = 6$) | | | | | | |
| $\bar{x}$ | (a.u.) | 7·39 | 7·57 | 7·81 | 7·55 | 7·64 | 7·68 | 7·64 | 8·20 | 8·28 | 8·19 | 8·35 | 1·48 |
| $s_{x,rel}$ | (%) | 4·88 | 3·93 | 2·16 | 3·92 | 4·57 | 4·77 | 0·90 | 1·40 | 1·26 | 2·02 | 1·34 | 1·11 |
| $r$ | — | 0·355 | 0·625 | 0·721 | 0·394 | 0·252 | 0·358 | 0·492 | 0·471 | 0·773 | — | 0·236 | 0·317 |
| $2rs_{x,rel}$ | (%) | 3·467 | 4·906 | 3·119 | 3·090 | 2·301 | 3·418 | 0·887 | 1·319 | 1·945 | — | 0·632 | 0·704 |
| $q$ | — | 0·90 | 0·92 | 0·95 | 0·92 | 0·93 | 0·94 | 0·93 | 1·00 | 1·01 | — | 1·02 | 1·03 |
| $s_{q,rel}$ | (%) | 3·97 | 2·91 | 1·32 | 3·10 | 4·39 | 3·88 | 2·57 | 1·83 | 1·34 | — | 2·56 | 2·18 |
| $s^c_{q,rel}$ | (%) | 3·98 | 3·10 | 1·52 | 3·92 | 5·48 | 4·96 | 2·29 | 1·70 | 1·33 | — | 2·60 | 2·11 |
| | | | | | | | Beef meat meal ($n = 12$) | | | | | | |
| $\bar{x}$ | (a.u.) | 9·14 | 4·21 | 5·93 | 13·68 | 7·48 | 7·32 | 4·85 | 4·19 | 8·84 | 8·52 | 3·26 | 3·83 |
| $s_{x,rel}$ | (%) | 6·90 | 6·86 | 7·36 | 3·54 | 4·42 | 4·73 | 7·00 | 3·79 | 4·51 | 2·49 | 4·46 | 4·49 |
| $r$ | — | 0·615 | 0·058 | 0·555 | 0·855 | −0·842 | 0·235 | −0·450 | 0·270 | 0·675 | — | −0·423 | 0·087 |
| $2rs_{x,rel}$ | (%) | 8·488 | 0·685 | 8·174 | 6·049 | 7·446 | 2·226 | −6·301 | 2·048 | 6·089 | — | −3·770 | 0·782 |
| $q$ | — | 1·07 | 0·49 | 0·70 | 1·61 | 0·88 | 0·86 | 0·57 | 0·49 | 1·04 | — | 0·38 | 0·45 |
| $s_{q,rel}$ | (%) | 5·59 | 7·23 | 6·22 | 1·97 | 2·70 | 4·67 | 6·34 | 3·93 | 3·42 | — | 5·79 | 4·81 |
| $s^c_{q,rel}$ | (%) | 5·72 | 7·16 | 6·32 | 1·91 | 2·69 | 4·80 | 6·29 | 3·94 | 3·37 | — | 5·95 | 4·95 |

From the results given in Table II it follows that in the case of using the internal standard technique, for 7 amino acids from the 11 determined, improvements of the precision were achieved. From the law of error propagation it is not possible to draw an explicit conclusion on the acceptibility of the use the internal standard technique for the whole tested set.

For this purpose, the values $x_1 = 0$, $x_2 = 14$ a.u. and with quotients $q_1 = 0$, $q_2 = 1{\cdot}75$ for the calculation of information content $I(p, p_0)$ were applied (Tables III and IV). Information content depends not only on the value of the standard deviation $s$ but also on the number of parallel determinations $n$. The following two cases considered:

*a*) standard deviation is computed from the all parallel determinations;

*b*) less parallel determinations are carried out than the number of those from which $s$ was determined in another series. In this paper, both methods for $I(p, p_0)$ calculation were used (Table III). For interpretation of the results it is necessary to determine the least information content at which resolution of chromatographic peaks is possible using the given method. It is given by the ratio $(x_2 - x_1)/s$ and must always hold $x_2 - x_1 > 6s$, since for intervals $\langle x_1, x_2 \rangle$ narrower than $6s$ Eq. (*5*) is not applicable. Under the above mentioned condition, the following values of

TABLE III

Information content (In bit) of determination of amino acids in beef meat meal on a short column ($n = 12$)

| Amino acid | $I_1(p,p_0)_x{}^a$ | $I_1(p,p_0)_q{}^a$ | $D^a$ | $I_1(p,p_0)_x{}^b$ | $I_1(p,p_0)_q{}^b$ | $D^b$ |
|---|---|---|---|---|---|---|
| | | | Internal standard: Try | | | |
| Try | 5·712 | — | — | 4·420 | — | — |
| Lys | 5·070 | 3·177 | 1·893 | 3·778 | 1·885 | 1·893 |
| His | 6·436 | 5·059 | 1·377 | 5·144 | 3·767 | 1·377 |
| Arg | 5·341 | 4·510 | 0·831 | 4·049 | 2·218 | 0·831 |
| $M(p, p_0)$ | 16·847 | 12·746 | — | 12·971 | 8·870 | — |
| | | | Internal standard: Agp | | | |
| Lys | 4·132 | 3·951 | 0·181 | 2·840 | 2·658 | 0·181 |
| His | 4·979 | 4·668 | 0·311 | 3·687 | 3·376 | 0·311 |
| Agp | 5·068 | — | — | 3·775 | — | — |
| Arg | 4·071 | 3·595 | 0·476 | 2·779 | 2·302 | 0·476 |
| $M(p, p_0)$ | 13·182 | 12·214 | — | 9·306 | 8·336 | — |

[a] Calculated for $n = 12$; [b] calculated for $n = 2$ (the standard deviation was estimated for $n = 12$).

**Table IV**

**Information content (in bit) of determination of amino acids on a long column**

| | Amino acid | | | | | | | | | | | | $M(p, p_0)$ bit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Asp | Thr | Ser | Glu | Gly | Ala | Val | iLeu | Leu | nLeu | Tyr | Phe | |
| | | | | | | Standards ($n = 6$) | | | | | | | |
| $I_1(p, p_0)_x$ | 4·646 | 4·734 | 5·582 | 4·925 | 4·505 | 4·432 | 6·841 | 6·112 | 6·238 | 5·362 | 5·966 | 6·475 | 60·456 |
| $I_1(p, p_0)_q$ | 4·749 | 5·128 | 5·943 | 5·012 | 4·531 | 4·545 | 5·190 | 5·544 | 5·923 | — | 5·278 | 5·416 | 57·259 |
| $D$ | −0·013 | −0·394 | −0·361 | −0·087 | −0·026 | −0·113 | 1·651 | 0·569 | 0·314 | — | 0·688 | 1·059 | — |
| | | | | | | Beef meat meal ($n = 12$) | | | | | | | |
| $I_1(p, p_0)_x$ | 4·037 | 5·165 | 4·568 | 4·411 | 4·967 | 4·004 | 4·931 | 6·025 | 4·699 | 5·608 | 6·157 | 5·912 | 55·776 |
| $I_1(p, p_0)_x$ | 4·436 | 5·180 | 4·902 | 5·356 | 5·772 | 5·013 | 5·166 | 6·067 | 5·189 | — | 5·871 | 5·904 | 58·856 |
| $D$ | 0·399 | 0·015 | 0·334 | 0·945 | 0·805 | 0·109 | 0·235 | 0·042 | 0·490 | — | −0·286 | −0·008 | — |

minimal information content were calculated: for $n = 2\ I(p, p_0) = 1{\cdot}038$ bits; $n = 6 I(p, p_0) = 1{\cdot}648$ bits; $n = 12 I(p, p_0) = 2{\cdot}331$ bits. Only for lysin and histidine (Table III, with TRY as internal standard) were differences $D = I(p, p_0)_x - I(p, p_0)_q$ higher than the minimal information content for $n = 2$. From this point of view it is possible to evaluate the use of the interval standard technique as insignificant.

Further, the influence of the external standard technique on the accuracy of amino acids determination was studied. It is well known that the highest accuracy of results is obtained if the peak area (height) of the standard and the compounds determined are the same. The proportion of the amino acid peaks depends on the type of analyzed material. For this reason each type of material should have its own external standard. It is difficult to meet this requirement in practice. Hence, for different dilutions of the sample various amino acid contents were calculated (Table IV). It is suitable to use a calibration curve for each component to estimate its content. The advantage of the calibration curve is the possibility to use it for non-linear dependences, too. On the other hand, this way of calculation is more time consuming in comparison to the external standard technique.

The values $x_1 = 0$ and $x_2 = 50$ g/kg were used for the calculation of the information content in Table V. Due to poor knowledge of the true amino acids content

TABLE V

Information content of determination of amino acids in *Psophocarpus tetragonolobus* (L.) DC using the external standard technique

| Amino acid | $\bar{x}^a$ g/kg | $I(p, p_0)^a$ bit | $\bar{x}^b$ g/kg | $I(p, p_0)^b$ bit | $\bar{x}^c$ g/kg | $I(p, p_0)^c$ bit |
|---|---|---|---|---|---|---|
| Asp | 43·5 | 0·033 | 43·2 | 0·306 | 37·5 | 2·458 |
| Thr | 15·9 | 4·211 | 15·3 | 4·600 | 15·4 | 4·604 |
| Ser | 21·2 | −0·402 | 20·2 | 3·097 | 19·0 | 4·412 |
| Gly | 17·6 | −21·086 | 16·3 | 3·266 | 15·7 | 5·813 |
| Ala | 16·7 | 2·480 | 15·2 | 4·690 | 15·5 | 4·742 |
| Val | 19·7 | −314·896 | 17·3 | 5·979 | 17·5 | 7·412 |
| iLeu | 16·6 | −1·312 | 15·4 | 5·402 | 15·3 | 5·412 |
| Leu | 33·7 | −8·952 | 32·2 | 4·925 | 31·8 | 5·412 |
| Tyr | 18·9 | −5·480 | 18·1 | −2·774 | 18·5 | 7·412 |
| Phe | 18·0 | −3·625 | 17·7 | 4·014 | 17·1 | 5·090 |
| His | 11·0 | 4·980 | 10·8 | 2·433 | 11·3 | 6·412 |
| Lys | 28·3 | 4·776 | 25·7 | −11·310 | 27·7 | 5·412 |
| Arg | 26·7 | 2·773 | 25·0 | 3·598 | 25·1 | 3·605 |
| $M(p, p_0)$ | — | −336·500 | — | 28·226 | — | 68·196 |

[a] Dilution 1 : 250; [b] dilution 1 : 500; [c] calculated from calibration curve.

in the sample, values calculated from a calibration curve were used as $X$ in Eq. (7). It is apparent that the information content of results decreases with the increasing value of $d$. This fact is also manifested in the amount of information $M(p, p_0)$ values.

In conclusion, it may be remarked that the internal standard technique will not improve the precision of a determination in many cases. In the case of a multicomponent sample the external standard technique will improve the accuracy only if a calibration curve is used.

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## SYMBOLS

| | |
|---|---|
| $D$ | difference of $I(p, p_0)_x - I(p, p_0)_q$ |
| $d$ | estimate of the mean error of the determination (Eq. (7)) |
| $I(p, p_0)$ | information content (Eqs (5*a*) and (5*b*)) |
| $M(p, p_0)$ | amount of information (Eq. (6)) |
| $n$ | number of parallel determinations |
| $q$ | ratio of peak heights or areas (Eq. (2)) |
| $\bar{q}$ | mean of peak hieghts or areas |
| $r$ | correlation coefficient (for relationship $x_i$ vs $x_I$) |
| $s$ | estimate of the standard deviation |
| $t_\nu$ | percentage value of the Student distribution for $\alpha = 0{\cdot}038794$ and $\nu = n - 1$ degrees of freedom |
| $X$ | true content of the component in an analyzed sample |
| $x$ | value of the result of analysis |
| $\bar{x}$ | mean of parallel determinations |
| a.u. | arbitrary units |
| $\alpha$ | probability of a type I error (the significance level) |
| $\sigma$ | standard deviation of a random variable |

Subscripts

| | |
|---|---|
| I | internal standard |
| i | index of the component |
| q, x | concerning to symbols $q$, $x$ |
| rel | relative |
| v | number of degrees of freedom |

Superscript

| | |
|---|---|
| c | calculated according to Eq. (*4*) |