Determination of tryptophan in feed samples by cyclic voltammetry and multivariate calibration methods

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A voltammetric method for the determination of tryptophan (Trp) in feed samples using multivariate calibration techniques is proposed. The multivariate data consisted of the oxidation wave of cyclic voltammograms registered from 400 to 925 mV using a graphite–methacrylate composite electrode, at which Trp was oxidized around a potential of 900 mV. The interference from other oxidizable amino acids as well as the feed sample matrix was circumvented by using multivariate calibration methods. Principal component regression (PCR) and partial least squares regression (PLS) and non-linear PLS (NL-PLS) were checked for quantifying Trp in these samples. The results obtained using NL-PLS were in agreement with those obtained with the standard method for the determination of amino acids, with an overall prediction error of 7.9%.

Introduction

Amino acids are often added to feed samples as food fortifiers during their elaboration process in order to correct for possible dietary deficiencies. Among these amino-acids, tryptophan (Trp) is especially relevant since it is an essential amino acid scarcely present in vegetable products. Hence, analytical methods for controlling the raw materials of the feed samples are needed.

As with other amino acids, Trp is usually determined by liquid chromatography with pre- or post-column derivatization with general reagents for the amino group such as ninhydrin, o-phthaldialdehyde and phenylisothiocyanate. However, the UV absorption and the native fluorescence of Trp are features which enable its direct spectrophotometric and fluorometric detection. Other separation techniques such as capillary electrophoresis, gas chromatography and thin-layer chromatography have also been used for determining Trp.

Non-chromatographic applications have also been reported for the determination of Trp, including a flow-through spectrophotometric cyclodextrin optosensor system, and fluorometric and chemiluminometric methods. In some cases, the detection is not sufficiently selective for this analyte so derivative spectroscopy and multivariate calibration techniques can be used to overcome this lack of selectivity. Since Trp can be easily oxidised electrochemically, detection was used. Enzymatic methods for determining Trp commonly involve amperometric biosensors with L-tryptophan-2-monooxygenase.

Multivariate calibration methods have usually been used in the analysis of spectroscopic data, but they have rarely been used for studying voltammetric data. The chemometric approach could be especially suitable for supplying the wide demand for Trp determinations in many fields, such as biochemistry, clinical chemistry, nutrition, agrochemistry and pharmaceutical products.

This paper describes a cyclic voltammetric method for determining Trp in extracts of feed samples using multivariate calibration. The cyclic voltammograms were registered from 400 to 1100 mV by using a rigid-composite working electrode. With this electrode, Trp was oxidised at potentials of around 900 mV. This oxidation was not selective and, thus, other amino acids and the feed sample matrix interfered with the analysis when classical univariate calibration methods were used. Here, multivariate calibration techniques, e.g., principal component regression (PCR), partial least squares regression (PLS) and non-linear PLS (NL-PLS) have been successfully applied to the resolution of overlapped voltammetric signals to determine Trp in this kind of samples. The range of potentials recommended for the analysis was from 400 to 925 mV.

In this study, a graphite–methacrylate rigid composite has been chosen for the fabrication of the working electrode. Rigid composites are the result of the dispersion of a powdered conducting material (e.g. graphite) in the bulk of a polymer. This type of electrode has some interesting properties such as microelectrode array surface, high signal-to-noise ratio, high mechanical and chemical stability, easily reproducible and renewable surface. In some applications, additionally, enzymes, antibodies, cofactors, redox mediators, etc. can be incorporated to modify the response or the selectivity of the electrode towards different analytes.

Experimental

Reagents

All reagents were of analytical grade. Solutions were prepared using Millipore water. The background electrolyte solution was prepared from potassium chloride (Merck, Darmstadt, Germany) and potassium monohydrogen phosphate (Merck) at pH 7.5 [pH was adjusted with a 37% (w/w) hydrochloric acid solution from Merck]. The working electrode was prepared with Sealer-Healer 1540 commercial methacrylate monomer (QHSealer-Healer, UK), benzoyl peroxide (Fluka, Buchs, Switzerland) and graphite powder (Merck) as outlined in ref. 39. Commercial feed samples were supplied by Cooperativa Agropecuaria de Guissona, Lleida, Spain.
Apparatus and measurement procedure

Cyclic voltammograms were registered with a PGSTAT 20 Autolab potentiostat from Eco Chemie, Utrecht, Netherlands in the range 400 to 1100 mV, in steps of 25 mV and a scan rate of 25 mV s$^{-1}$. This scan rate enabled the analytical response of Trp to be sufficiently differentiated from the background signal. A double-junction reference electrode Ag/AgCl Orion (Cambridge, MA, USA) 90-02-00, with the external chamber filled with 0.1 M KCl solution, and a platinum auxiliary electrode were used. The working electrode was home-made and consisted of a PVC electrode body (18 mm length and 6 mm id) filled with a graphite–methacrylate rigid composite.39

Voltammetric measurements were carried out by adding 1 ml of the solution of feed sample extract to 25 ml of the background electrolyte solution (0.1 M potassium chloride–0.1 M phosphate solution, pH 7.5). In order to ensure reproducibility of the voltammetric data, cyclic voltammograms used for multivariate calibration were registered in steady-state conditions, which were achieved after 10 to 15 successive scans.

Feed sample treatment

Trp, added as a fortifier to the feed samples, was recovered by dissolving it in acidic aqueous solution. For this purpose, amounts of 5 g of feed sample were leached with 50 ml of 0.1 M HCl solution for 30 min in conical flasks with magnetic stirring. The resulting extract solutions were subsequently filtered through a nylon membrane of 0.45 μm of pore size and refrigerated. Hydrochloric acid facilitated the dissolution of Trp, added as a fortifier to the feed samples, was recovered by dissolving it in acidic aqueous solution for this purpose. Amounts of 5 g of feed sample were leached with 50 ml of 0.1 M HCl solution for 30 min in conical flasks with magnetic stirring. The resulting extract solutions were subsequently filtered through a nylon membrane of 0.45 μm of pore size and refrigerated. Hydrochloric acid facilitated the dissolution of Trp, added as a fortifier to the feed samples, was recovered by dissolving it in acidic aqueous solution. For this purpose, amounts of 5 g of feed sample were leached with 50 ml of 0.1 M HCl solution for 30 min in conical flasks with magnetic stirring. The resulting extract solutions were subsequently filtered through a nylon membrane of 0.45 μm of pore size and refrigerated. Hydrochloric acid facilitated the dissolution of Trp, added as a fortifier to the feed samples, was recovered by dissolving it in acidic aqueous solution. For this purpose, amounts of 5 g of feed sample were leached with 50 ml of 0.1 M HCl solution for 30 min in conical flasks with magnetic stirring. The resulting extract solutions were subsequently filtered through a nylon membrane of 0.45 μm of pore size and refrigerated. Hydrochloric acid facilitated the dissolution of Trp, added as a fortifier to the feed samples, was recovered by dissolving it in acidic aqueous solution. For this purpose, amounts of 5 g of feed sample were leached with 50 ml of 0.1 M HCl solution for 30 min in conical flasks with magnetic stirring.

Data sets under study

In first-order calibration, standards to be used in the calibration step must have the same characteristics and composition as the unknown samples in order to implicitly model any possible interferences and background contributions present in the samples. Therefore, in the analysis of free (non-proteic) Trp in the feed samples, the standards must be other extract solutions previously analysed using an independent method (e.g. by liquid chromatography, as described below).

In this study, 17 extracts of feed samples were analysed. Each particular sample was predicted using the 16 remaining samples as standards in order to ensure similarity between standards and unknown samples. The dimension of the corresponding raw data matrices was the number of potentials of each cyclic voltammogram NP (NP = 58 values of current intensities taken at regular intervals of 25 mV from 400 to 1100 mV) by the number of samples NS (NS = 16 and 1, for the calibration and prediction sets, respectively).

Standard method for the determination of Trp

The free (non-proteic) Trp in the feed samples was determined using the standard method for amino acid analysis, which permitted the quantification of the other amino acids also present in the extract (see Table 1). The standard method consisted of a separation of common amino acids by cation exchange liquid chromatography and post-column derivatization with ninhydrin.40

Multivariate calibration methods

For calculations, MATLAB for Windows (Version 4.1) was used. Multivariate calibration methods were from PLS_Toolbox of Wise and Gallagher.41 Multivariate calibration methods have extensively been described elsewhere.29,30,42

The optimum number of latent variables for each multivariate calibration method was estimated by leave-one-out cross-validation from a data set including the 17 feed sample extracts. Therefore, the number of latent variables chosen was that which minimised the PRESS value calculated as follows:

$$\text{PRESS}(k) = \sum_{i=1}^{\text{Samples}} (C_{\text{true}} - C_{\text{calc}(k)})^2$$

where $C_{\text{true}}$ is the real concentration of analyte in the sample $i$ and $C_{\text{calc}}$ is the concentration predicted by multivariate calibration methods; $k$ refers to the number of latent variables used in the model.

The prediction error in Tables 2 and 3 for the calibration and prediction steps was calculated using the expression:

$$\text{Error(%) = } \sum_{i=1}^{\text{Samples}} \left( \frac{(C_{\text{true}} - C_{\text{calc}})^2}{ \sum_{i=1}^{\text{Samples}} (C_{\text{calc}})^2 } \right) \times 100$$

### Table 1  Composition of free amino acids in the feed samples obtained using the standard method of amino acid analysis

<table>
<thead>
<tr>
<th>Feed sample</th>
<th>Trp</th>
<th>Arg</th>
<th>Asp</th>
<th>Cys</th>
<th>Glu</th>
<th>Met</th>
<th>Lys</th>
<th>Tyr</th>
<th>Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>0.249</td>
<td>0.100</td>
<td>0.058</td>
<td>0.131</td>
<td>0.045</td>
<td>0.137</td>
<td>0.133</td>
<td>0.182</td>
<td>0.025</td>
</tr>
<tr>
<td>S2</td>
<td>0.155</td>
<td>0.103</td>
<td>0.053</td>
<td>0.124</td>
<td>0.043</td>
<td>0.139</td>
<td>0.144</td>
<td>0.255</td>
<td>0.033</td>
</tr>
<tr>
<td>S3</td>
<td>0.244</td>
<td>0.101</td>
<td>0.058</td>
<td>0.190</td>
<td>0.044</td>
<td>0.140</td>
<td>0.138</td>
<td>0.181</td>
<td>0.035</td>
</tr>
<tr>
<td>S4</td>
<td>0.167</td>
<td>0.104</td>
<td>0.052</td>
<td>0.192</td>
<td>0.042</td>
<td>0.138</td>
<td>0.145</td>
<td>0.275</td>
<td>0.032</td>
</tr>
<tr>
<td>S5</td>
<td>0.247</td>
<td>0.121</td>
<td>0.068</td>
<td>0.128</td>
<td>0.057</td>
<td>0.105</td>
<td>0.132</td>
<td>0.268</td>
<td>0.024</td>
</tr>
<tr>
<td>S6</td>
<td>0.163</td>
<td>0.101</td>
<td>0.055</td>
<td>0.191</td>
<td>0.050</td>
<td>0.137</td>
<td>0.139</td>
<td>0.091</td>
<td>0.034</td>
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<tr>
<td>S7</td>
<td>0.167</td>
<td>0.120</td>
<td>0.066</td>
<td>0.069</td>
<td>0.060</td>
<td>0.221</td>
<td>0.120</td>
<td>0.181</td>
<td>0.036</td>
</tr>
<tr>
<td>S8</td>
<td>0.126</td>
<td>0.130</td>
<td>0.067</td>
<td>0.187</td>
<td>0.055</td>
<td>0.136</td>
<td>0.135</td>
<td>0.089</td>
<td>0.035</td>
</tr>
<tr>
<td>S9</td>
<td>0.184</td>
<td>0.087</td>
<td>0.060</td>
<td>0.191</td>
<td>0.030</td>
<td>0.139</td>
<td>0.131</td>
<td>0.272</td>
<td>0.033</td>
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<tr>
<td>S10</td>
<td>0.245</td>
<td>0.129</td>
<td>0.066</td>
<td>0.069</td>
<td>0.057</td>
<td>0.117</td>
<td>0.118</td>
<td>0.092</td>
<td>0.022</td>
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<tr>
<td>S11</td>
<td>0.167</td>
<td>0.085</td>
<td>0.059</td>
<td>0.070</td>
<td>0.029</td>
<td>0.116</td>
<td>0.215</td>
<td>0.181</td>
<td>0.035</td>
</tr>
<tr>
<td>S12</td>
<td>0.084</td>
<td>0.120</td>
<td>0.068</td>
<td>0.106</td>
<td>0.056</td>
<td>0.220</td>
<td>0.123</td>
<td>0.220</td>
<td>0.030</td>
</tr>
<tr>
<td>S13</td>
<td>0.166</td>
<td>0.099</td>
<td>0.065</td>
<td>0.128</td>
<td>0.058</td>
<td>0.100</td>
<td>0.119</td>
<td>0.214</td>
<td>0.033</td>
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<tr>
<td>S14</td>
<td>0.165</td>
<td>0.088</td>
<td>0.067</td>
<td>0.155</td>
<td>0.058</td>
<td>0.118</td>
<td>0.117</td>
<td>0.145</td>
<td>0.025</td>
</tr>
<tr>
<td>S15</td>
<td>0.167</td>
<td>0.098</td>
<td>0.053</td>
<td>0.147</td>
<td>0.043</td>
<td>0.116</td>
<td>0.144</td>
<td>0.170</td>
<td>0.021</td>
</tr>
<tr>
<td>S16</td>
<td>0.134</td>
<td>0.122</td>
<td>0.066</td>
<td>0.154</td>
<td>0.032</td>
<td>0.120</td>
<td>0.216</td>
<td>0.204</td>
<td>0.025</td>
</tr>
<tr>
<td>S17</td>
<td>0.136</td>
<td>0.105</td>
<td>0.060</td>
<td>0.101</td>
<td>0.032</td>
<td>0.120</td>
<td>0.216</td>
<td>0.204</td>
<td>0.025</td>
</tr>
</tbody>
</table>

*Percentage as g of free (non-proteic) amino acid in 100 g of sample.*
Results and discussion

The amino acid composition of the feed samples is summarised in Table 1. In addition to Trp, the extract solutions contained variable amounts of other amino acids. Some of them (e.g., Lys and Met) were also essential amino acids used as fortifiers, whereas others came from raw products for preparation of the feed samples. The contents ranged between 0.21 and 0.12% for Lys, 0.22 and 0.10% for Met, and 0.13 and 0.09% for Arg. Other amino acids such as Val, Gly, Glu, Gln and Asp were present at much lower concentrations (ranging from 0.01 to 0.06%).

Cyclic voltammetric measurements

Graphite composites have often been used in our working group to prepare electrodes as they present some other attractive features such as surfaces with microelectrode array properties, high signal-to-noise ratio, high mechanical and chemical stability and reproducible and renewable surfaces.

The reproducibility in the fabrication of the composites was extensively studied elsewhere, where RSD of the response for a series of electrodes were about 1–2%. The surface of the electrode is renewable by polishing with abrasive papers and, the reproducibility of this process, estimated as RSD of response, ranged from 1 to 4%, depending on the complexity of the composite and the electrochemistry.

When the steady-state of the diffusion–oxidation processes was achieved, the shape of the cyclic voltammograms did not vary from scan to scan. Under these conditions, reproducible measurements were possible. Experimentally, the reproducibility was studied by registering the cyclic voltammograms for a series of six 1 \( \times \) \( 10^{-4} \) M Trp solutions. The RSD at 900 mV was about 2.5%.

Fig. 1 shows the cyclic voltammograms of 1 \( \times \) \( 10^{-4} \) M Trp solution, a feed sample extract solution and the background electrolyte solution registered in the range 400 to 1100 mV obtained with a graphite–methacrylate working electrode as explained in the experimental section. Altering the composition of the graphite electrode with polymeric materials shifted the oxidation processes to higher potentials.38 The widening of the oxidation range may help to differentiate Trp from the other oxidizable amino acids (Cys and Tyr).

The presence of Cys and Tyr in these samples was especially remarkable since these amino acids were oxidized at similar potentials to Trp. Additionally, the feed sample matrix could interfere with the voltammetric measurements at potential values over 1000 mV. As a result, the response for Trp was overlapped with the contributions of the oxidizable amino acids and the feed sample matrix and consequently no selective potential for Trp was found. However, this lack of selectivity for Trp was circumvented with the aid of multivariate calibration techniques since the Trp response was characteristic and sufficiently distinguishable from the other contributions.

Application of multivariate calibration methods

The principal component analysis of the extracts of feed samples showed that PC-1 explained the variance related to the maximum current intensity of voltammograms and, in principle, there was no correlation with the Trp concentration. Information on the Trp contents seemed to appear in further factors. The interpretation of the score and loading plots was rather difficult which suggested the complexity of the voltammetric data and the high weight of the sample matrix in the model.

The shift in potential of peaks associated with the change of the analyte concentration has been described in voltammetric techniques. Moreover, non-additive signals can also be found. These phenomena have been attributed to the influence of high analyte concentrations or other electroactive compounds on the reversibility of the electrochemical process. However, data with moderate shifts can be correctly modelled. However, when these effects are marked, significant drawback that might be encountered in the analysis of the voltammetric data is the lack of bilinearity (i.e. the shape of the voltammogram of the analyte was not unique). Apparently, this type of system should be modelled by including more than one contribution per analyte, as the rank of each compound is two or more. However, the resolution and quantification of the analyte

### Table 3

<table>
<thead>
<tr>
<th>Method</th>
<th>Overall prediction error (%)</th>
</tr>
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<tbody>
<tr>
<td>PCR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.3</td>
</tr>
<tr>
<td>PLS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.0</td>
</tr>
<tr>
<td>NL-PLS&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Using a working range from 400 to 925 mV and 5 factors. <sup>b</sup> The degree of polynomial was two.

![Cyclic voltammograms of a 1 x 10^{-4} M Trp solution and an extract of a feed sample containing 0.155% Trp (6.4 x 10^{-6} M Trp) in 0.1 M KCl and 0.1 M phosphate (pH = 7.5) background electrolyte solution. Trp molarities correspond to concentrations in the measurement cell.](image)

### Table 2

<table>
<thead>
<tr>
<th>Method</th>
<th>Number of latent variables</th>
<th>Cumulative explained Y variance (current intensity) (%)</th>
<th>Cumulative explained X variance (concentration) (%)</th>
<th>Overall prediction error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
<td>99.995</td>
<td>99.562</td>
<td>6.6</td>
</tr>
<tr>
<td>PLS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
<td>99.999</td>
<td>99.777</td>
<td>4.7</td>
</tr>
<tr>
<td>NL-PLS&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5</td>
<td>99.992</td>
<td>99.812</td>
<td>4.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Using a working range from 400 to 925 mV. <sup>b</sup> The degree of polynomial was two.
Calibration models built for PCT, PLS and NL-PLS methods using the optimum number of latent variables were examined and validated using different tools. Firstly, as shown in Table 2, these models were able to explain a large amount of variance of both concentration and response matrices. Secondly, the error in the prediction of the same samples utilised as standards for building the model enabled the checking of the ability of the model to make predictions. Thus, using eqn. (2), prediction errors were 6.6–4.7% and 4.3% for PCR, PLS and NL-PLS, respectively (see Table 2).

Once calibration models were examined, they were used to predict Trp in other samples which were not used in the calibration set. As explained in the experimental section, the general strategy was based on the prediction of each particular sample using the 16 remaining samples as standards. In this way, Trp concentrations calculated with PCR, PLS and NL-PLS were statistically compared with those given by the standard method using a $t$-test of paired measurements. At $\alpha = 0.05$ ($t_{\text{crit}} = 2.120$, for 16 degrees of freedom), $t_{\text{calc}}$ values were 0.906, 0.991 and 0.980, respectively, for PCR, PLS and NL-PLS, so that there were no significant differences between the Trp contents from the standard chromatographic method and the multivariate calibration methods. Moreover, the $\alpha_{\text{actual}}$ values estimated for PCR, PLS, and NL-PLS were 0.503, 0.346, and 0.621, respectively.

The accuracy in the quantification of Trp in the feed samples, expressed as percentage of the prediction error [see eqn. (2)], is shown in Table 3 for PCR, PLS and NL-PLS. In all cases, the overall prediction error was lower that 11%. Apparently, the PLS method, and particularly NL-PLS using a polynomial of second degree seemed to provide a better performance than PCR. However, this slightly higher accuracy of NL-PLS was not statistically significant when compared with PCR and PLS.

As an example, Fig. 3 plots the results of the determination of Trp in the feed samples using the method proposed against those obtained by the standard procedure. In this case, the chemometric method chosen was NL-PLS with 5 factors and using the oxidation wave in a working range of potentials from 400 to 925 mV. As may be seen in Fig. 3, both methods are in satisfactory agreement, with an overall quantification error of 7.9%.

**Conclusions**

This paper presents a study of the possibilities of using a voltammetric technique for the determination of Trp in extracts of feed samples. Multivariate calibration may constitute an attractive alternative for fast analysis of Trp in samples of a similar nature and composition. Cyclic voltammograms can be used as multivariate data. Although all methods tested gave
reasonably good predictions, the accuracy was slightly better for NL-PLS. Hence, Trp can be quantified in these complex samples, in the presence of interference from other oxidizable amino acids and the feed sample matrix, with an overall prediction error of 7.9% using NL-PLS. Moreover, no significant differences in the Trp concentrations have statistically been found when comparing the standard and proposed methods.

Hence, the method proposed here can serve for the analysis of industrial feed, where speed in obtaining the results is crucial, and may become an attractive alternative to chromatographic techniques, which are more tedious and time-consuming. Moreover, the electrochemistry can be improved by using potential step methods to enhance the peak resolution. In addition, the electrode can be modified with electrocatalysts to increase the selectivity of Trp towards other oxidizable amino acids and the sample matrix. Further work will be done in these directions.

References

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