Modified tubular electrode in a multi-commutated flow system

Determination of acetaminophen in blood serum and pharmaceutical formulations

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Abstract

This work describes the construction of a Nafion coated glassy carbon tubular electrode and its use, coupled to a multi-commutated flow system, for the voltammetric determination of acetaminophen in serum and pharmaceutical formulations. The modification of the electrode enhanced the analytical signal intensity and, simultaneously, prevented the electrode surface fouling. The multi-commutated system conferred high versatility to the manifold, allowing it to be easily adjusted to each determination without the need to introduce any physical reconfiguration. The on line enzymatic hydrolysis of acetaminophen, giving rise to 4-aminophenol, allowed the problem of interferences resulting from the oxidation of the matrix serum constituents to be overcome.

The method presented a linear range up to $5.0 \times 10^{-4}$ mol l$^{-1}$ with a detection limit of $1.7 \times 10^{-5}$ mol l$^{-1}$, a sampling rate of 24 determinations per hour and a repeatability (expressed in relative standard deviation) always lower than 3%. The method was applied to serum samples and pharmaceutical formulations and no statistically significant difference between the results obtained by the proposed method and by the reference methods was found, for a confidence level of 95%.

Keywords: Acetaminophen; 4-Aminophenol; Modified tubular electrode; Nafion; Voltammetry; Multi-commutation; Flow analysis

1. Introduction

Acetaminophen ($N$-acetyl-$p$-aminophenol), also known as paracetamol, is a drug with antipyretic and analgesic action, frequently used in therapy and which has low toxicity when used at the recommended doses [1]. Nevertheless, the ease with which the general public can access this drug makes it frequently associated with overdoses, in this situation causing serious or even fatal hepatic damage [2]. The treatment of a supposed overdose of acetaminophen demands a rapid and reliable determination of its concentration in blood serum.

This work describes for the first time, to our knowledge, the use of a tubular detector with Nafion film coated glassy carbon electrodes, applied to the determination of acetaminophen in blood serum and pharmaceutical formulations. Due to the presence in blood serum of other compounds which are oxidised in the same potential range of acetaminophen, this was selectively hydrolysed on line, through the action of the enzyme ary acylamidase [3], giving rise to 4-aminophenol and acetate, with 4-aminophenol being determined voltametrically at an oxidation potential at which the occurrence of the referred interferences is no longer observed. The tubular electrode was modified with a Nafion film, whose electrocatalytic effect to the oxidation reaction of 4-aminophenol was already demonstrated [4].

The tubular detector was coupled to a multi-commutated flow system [5] which offers superior versatility to the manifold since this can be easily adjusted to each determination without the need for physical reconfiguration, sufficing just to reprogram the functioning of the commutation devices (solenoid valves).
2. Experimental

2.1. Reagents and solutions

Reagents of p.a. quality or similar were used without having been subject to any additional purification. In the preparation of the solutions, water purified by the Millipore Milli Q system (conductivity < 0.1 \(\mu\)S cm\(^{-1}\)) was used.

As carrier solutions, a glycine and sodium hydroxide 0.2/0.2 mol l\(^{-1}\) buffer solution (pH 8.6) and a citric acid and di-sodium hydrogen phosphate 0.5/1.0 mol l\(^{-1}\) buffer solution (pH 5.5) [6] were used, the later also functioning as supporting electrolyte.

Acetaminophen (Sigma) standard solutions, with concentrations between \(5.0 \times 10^{-5}\) and \(5.0 \times 10^{-4}\) mol l\(^{-1}\), were prepared daily by diluting a stock solution of acetaminophen in the glycine and sodium hydroxide buffer solution.

For the enzymatic reactor, the enzyme aryl acylamidase (E.C. 3.5.1.13) from *Pseudomonas fluorescens* ATCC 39005 (Sigma) was used, in the lyophilised form and with the specific activity of 6.0 U/mg protein, being immobilised 43 U in 0.5 g of aminopropyl glass beads (Sigma) of average pore size 700˚A, activated with a 2.5% (v/v) glutaraldehyde solution [7]. The beads were packed in a homemade PVC column of 4 mm inner diameter and 50 mm length, and cotton fibre was placed in its extremities in such a way as to retain the beads in its interior. When not in use, the enzymatic reactor was stored in sodium dihydrogen phosphate/di-sodium hydrogen phosphate 0.1 mol l\(^{-1}\) buffer solution (pH 7.0), at 4\(^{\circ}\)C.

For the electrode coating, a 2% (v/v) Nafion solution in ethanol was used, prepared from a Nafion perfluorinated ion-exchange resin 5% (m/v) solution in a mixture of lower aliphatic alcohols and water (Aldrich).

The blood serum samples, without being subjected to additional treatments, were diluted 100 times in glycine and sodium hydroxide buffer solution. Samples of pharmaceutical formulations containing acetaminophen, available commercially in Portugal, were also analysed. For each sample of tablets and effervescent tablets, 20 U were weighed and the average weight calculated. These 20 U were pulverised and mixed, and a stock solution of each sample (with \(2.0 \times 10^{-3}\) mol l\(^{-1}\) in acetaminophen) was prepared by weighing the corresponding quantity of powder and dissolving it in glycine and sodium hydroxide buffer solution. The working solutions of \(2.0 \times 10^{-4}\) mol l\(^{-1}\) were obtained by dilution of the respective stock solution in glycine and sodium hydroxide buffer solution.

The syrups were diluted in glycine and sodium hydroxide buffer solution, presenting a final acetaminophen concentration of \(7.5 \times 10^{-5}\) mol l\(^{-1}\).

To evaluate the quality of the results supplied by the proposed method, the serum samples and pharmaceutical formulations were analysed by the reference methods (Glynn and Kendal method) [8] and (BP 1998) [9], respectively.

2.2. Equipment

In the developed multi-commutated flow system (Fig. 1A) the solutions and samples were aspirated through an automatic syringe with a 10ml total capacity (Crisom model Micro BU 2031). To control the direction of the solutions and samples inside the manifold, three-way solenoid valves (161 T031, NResearch) were used. A homemade power driver, based on an integrated ULN 2003 circuit, was used to operate the solenoid valves. Control of the analytical system was carried out through an interface card (PC-LABCard model PCL-711B, Advantech) and a microcomputer. The software was developed in Quick-
Basic version 4.5 (Microsoft) and permitted control of the automatic burette with the syringe and solenoid valves. Connections between the various components of the flow system were made with Teflon tubes (Omnifit) of 0.8 mm inner diameter. Voltammetric measurements were carried out in an Autolab electrochemical system (Eco Chemie model PGSTAT 10) and data acquisition was accomplished through GPES software (Version 4.6).

2.3. Construction of the tubular detector

The voltammetric detector was constructed to permit its application in multi-commutated flow systems with the propulsion device placed after the detector (aspiration of solutions), thereby creating an internal pressure lower than atmospheric pressure. This positioning of the propulsion device simplifies the manifold, given that the solutions are aspirated by a single channel, thereby reducing their consumption.

On the basis of its construction was a tubular detector recently described [10] but adjustments were introduced which led to the sealed fixation of the electrodes to the detector, making the aspiration of the solutions through its interior viable without the entry of air bubbles. The tubular-configuration detector (Fig. 1C) with a 35 μl dead volume and 15 μl inner volume, was constituted by a central Perspex support, which encased the working and auxiliary electrodes, both of glassy carbon, constructed from a glassy carbon rod of 7 mm diameter. The resulting cylinders were of 2 mm thickness and were perforated in the centre with a 0.8 mm diameter orifice (a dimension similar to the tubing in the flow system), being firmly fixed to the support by two rubber disks also perforated in the centre. As reference electrode, a Metrohm electrode (Ag/AgCl–KCl 3.0 mol l−1, model 6.0727.000) was used, fixed by a threaded screw and superficially touching the central channel which linked the working electrode to the auxiliary electrode. The electric contact with the working and auxiliary electrodes was established through two metallic contacts threaded into the Perspex support.

For cleaning and coating the working electrode, this was withdrawn from the detector, being initially subjected to a mechanical polishing, using a cotton thread soaked in alumina aqueous slurry of 0.075 μm. This was then cleaned through ultrasound, initially in an aqueous solution of nitric acid and thereafter in ethanol, for 1 min. Finally, it was washed with water.

The electrode coating, through the droplet evaporation method, consisted of depositing 30 μl of the 2% (v/v) Nafion solution directly into the central orifice of the electrode. It was kept at room temperature until the solvent was completely evaporated (about 30 min), being washed with water before encapsement in the voltammetric detector.

Between determinations, the surface of the modified electrode was renewed on line, by cyclic scanning between −0.1 and +2.0 V (two cycles), with a step potential of 0.024 V and a scan rate of 0.200 V s−1, making the citric acid and di-sodium hydrogen phosphate buffer solution flow simultaneously.

2.4. Automatic procedure

The multi-commutated flow system (Fig. 1A) comprised three 3-way solenoid valves (V1, V2 and V3). Valves V2 and V3 were simultaneously operated in order to define two parallel analytical pathways, in which one was intended to carry out the exchange of samples and standard solutions, avoiding their passage through the detector, thereby reducing the passivation of the working electrode surface, and the other included the tubular detector (D). The automatic burette (Bu) with the syringe was placed at the end of the analytical manifold, after the detector, and was used to aspirate all solutions. This positioning of the propulsion device simplified the configuration of the manifold and allowed a reduction in the consumption of samples and carrier solutions.

The analytical cycle (Fig. 1B) was initiated with V1 and V3 in position 2 and V2 in position 1, enabling the passage of a standard solution or sample in the enzymatic reactor, being rejected to waste without passing in the detector. After 60 s, V1 and V3 moved to position 1 and V2 to position 2, making it possible for the buffer solution to enter to renew the surface of the working electrode (50 s). Subsequently, V1 moved to position 2, permitting the aspiration of the standard solution or sample, already subjected to the enzymatic hydrolysis of acetaminophen, directing it to the detector. Finally, V1 returned to position 1 for 15 s, directing the sample to the detector and allowing the detection of 4-aminophenol.

3. Results and discussion

3.1. Voltammetric studies

By continuously passing a 2.0 × 10−3 mol l−1 4-aminophenol standard solution in the multi-commutated flow system at a flow rate of about 0.5 ml min−1 and using the bare electrode, the relationship between the potential scan rate (cyclic voltammetry) and the anodic peak current intensity (Ip) was studied. It was observed that the Ip varied linearly with the square root of the scan rate, from 0.010 to 0.200 V s−1.

The effect of pH (from 2 to 8) on the Ip was also evaluated. It was shown that the Ip was higher for pH values between 5 and 7, with a pH value of 5.5 being selected for subsequent analyses.

Frequency (f) values between 10 and 50 Hz were tested, maintaining as fixed the step potential (∆Ep) and pulse amplitude (α) (at 2 and 60 mV, respectively). It was observed that Ip decreased with the increase of f over the full interval of tested values. The value of 25 Hz was chosen, at which the best relationship between Ip and scan rate was obtained. By fixing this f value, ∆Ep was made to vary between 2 and 10 mV, and the Ip was highest for 4 mV. Finally, α was made to vary between 10 and 110 mV. It was observed that Ip increased almost linearly with the increase of α up to 80 mV, beyond which it remained almost constant. Therefore, the values of f = 25 Hz, ∆Ep = 4 mV and α = 80 mV were selected for subsequent analyses.
3.2. Characterization of the modified electrode

A study of the voltammetric response of 4-aminophenol obtained with the modified electrode was carried out. By performing 10 consecutive determinations of a 5.0 \times 10^{-5} \text{ mol} \cdot \text{l}^{-1} 4-aminophenol standard solution, it was shown that the coated electrode allowed the attainment of an \( I_p \) value with good repeatability (relative standard deviation (R.S.D.) of 3.0%) and higher \( I_p \) increase of about 35% than that obtained with the bare electrode, whose repeatability was much lower (R.S.D. of 16.5%). The same was evidenced in relation to the \( E_p \) (R.S.D. of 1.1% for the coated electrode and 19.2% for the bare electrode), making it possible to conclude that the electrode coating with Nafion film allowed the enhancement of signal intensity and repeatability of the determinations, by preventing the passivation of the electrode surface.

The effect of the Nafion solution concentration and volume deposited in the working electrode (that condition the thickness of the film) on \( I_p \) and \( E_p \) was evaluated, with Nafion solutions being prepared with concentrations between 0.5% and 3% (v/v) and depositing a volume of 30 \( \mu \text{l} \) in the electrode. It was shown that the 2% (v/v) concentration gave rise to the highest \( I_p \) value. With that concentration of Nafion solution, the volume deposited in the electrode was made to vary, with the highest \( I_p \) being observed for the 30 \( \mu \text{l} \) volume.

When the film was prepared with Nafion concentrations of 0.5% and 1% (v/v) and volumes of 10 and 20 \( \mu \text{l} \), the quantity of active sites was insufficient to show an increase in \( I_p \). However, for the 3% (v/v) concentration and volumes greater than 30 \( \mu \text{l} \), the process of diffusion of the analyte on the film declined and, for this reason, the \( I_p \) decreased. In relation to \( E_p \) it was verified that, for the 0.5% (v/v) concentration and 10 \( \mu \text{l} \) volume, the displacement in the \( E_p \) values for successive measurements was observed. Therefore, the thickness of the film was insufficient to protect the electrode from passivation but for higher concentrations and volumes, the \( E_p \) maintained itself constant, even after 20 determinations. For the subsequent analyses, the working electrode was modified with a 2% (v/v) Nafion solution, with a 30 \( \mu \text{l} \) volume being applied. The modified electrode presented a response time lower than 5 s, given that the voltammetric scan was carried out after an equilibration time of 5 s and the running of a second scan after the first did not give rise to a higher \( I_p \) value.

The stability of the coated electrode was evaluated, by carrying out consecutive determinations of a 5.0 \times 10^{-5} \text{ mol} \cdot \text{l}^{-1} 4-aminophenol standard solution. After 100 determinations, the \( I_p \) and \( E_p \) values showed good repeatability with a R.S.D. of 4.4% and 1.1%, respectively, concluding that the modified electrode exhibited high stability. The same modified electrode could be used to carry out up to 500 determinations (equivalent to 2 weeks of work) without loss of reproducibility. The influence of flow rate on the stability of the modified electrode was also evaluated, showing that flow rates lower than 1.5 \text{ ml} \cdot \text{min}^{-1} did not affect the reproducibility of \( I_p \) values for successive measurements.

The reproducibility of the modification process was studied, that is, if the modification of an electrode on different days always gave rise to the same \( I_p \) and \( E_p \) values. According to the results, it was concluded that the process of modifying the electrode was reproducible, since, for the same electrode on different days, similar values were obtained for \( I_p \) (with R.S.D. lower than 5%) and \( E_p \) (with R.S.D. of 2%).

Bearing in mind the concentration interval of acetaminophen in blood serum [11], standard solutions of 4-aminophenol were prepared with concentrations between 2.5 \times 10^{-5} and 5.0 \times 10^{-3} \text{ mol} \cdot \text{l}^{-1} and the \( I_p \) was measured. A linear relationship was observed between 4-aminophenol concentration and \( I_p \) up to 5.0 \times 10^{-5} \text{ mol} \cdot \text{l}^{-1}, with the electrode being saturated beyond this value.

3.3. Enzymatic reactor

Literature data suggests that the enzymatic hydrolysis of acetaminophen is preferable to acidic hydrolysis because it does not require drastic temperature and pH conditions [12]. Additionally, the use of solutions at high temperatures in flow systems is difficult to implement and increases the complexity of the manifolds, so enzymatic hydrolysis was used to convert acetaminophen into 4-aminophenol. To get a good return on the use of the enzyme, this was immobilised in a column which was inserted into the flow system.

Aryl acylamidase is active in the pH range between 7 and 12 with 8.6 being the optimum pH [13]. In this way, a glycine and sodium hydroxide 0.2/0.2 \text{ mol} \cdot \text{l}^{-1} buffer solution (pH 8.6) was used to prepare the solutions. In relation to the temperature, the enzyme functions between 25 and 55 °C, showing maximum activity at 45 °C [13]. However, the analyses were carried out at room temperature with a view to making the flow manifold simpler and suited to the intended determinations.

Different quantities of enzyme immobilised in the column were tested, in order to establish the minimum quantity of enzyme capable of hydrolysing all the acetaminophen in a 160 \( \mu \text{l} \) volume of standard solution with a maximum concentration of 5.0 \times 10^{-3} \text{ mol} \cdot \text{l}^{-1} (upper limit of the linear range). It was shown that 43 \text{ U} were needed, which corresponded to 360.0 \text{ mg} of enzyme immobilised in the column.

The enzymatic column maintained good functioning characteristics over approximately 2 months, a period during which the only peak observed related to 4-aminophenol. Subsequently, the enzyme began to lose activity, as evidenced by the appearance of two peaks, one referring to 4-aminophenol and the other referring to acetaminophen, resulting from an incomplete hydrolysis.

3.4. Interference studies

The effect of the constituents of blood serum (albumin, glucose and N-acetylcysteine) and pharmaceutical excipients (sucrose, sorbitol, sodium benzoate, glycerol and citric acid) which are present in the analysed pharmaceutical formulations was studied. In these studies, various solutions were analysed containing 2.5 \times 10^{-4} \text{ mol} \cdot \text{l}^{-1} of acetaminophen and the foreign compound at a higher concentration (maximum 100:1). The interfering concentration of each compound was considered as being that which caused a variation in the response greater than or equal to ±5% in relation to the response obtained in its
absence. It was possible to conclude that none of the studied compounds interfered in the determination of acetaminophen, at the concentrations in which they are present in the samples.

3.5. Optimization of multi-commutated flow system parameters

In the developed multi-commutated flow system several factors had to be optimised. Consequently, the Taguchi Parameter Design (TPD) was selected as the optimisation methodology, since this provides the necessary information with the minimal experimentation. TPD discriminates between control factors, uncontrollable factors and experimental noise, treating them separately by means of special design matrices (orthogonal arrays) in which the columns (corresponding to factors and their interactions) and rows (corresponding to trials) are arranged in a convenient and fixed way [14], indicating the combination of factor levels in each experiment and enabling the simultaneous evaluation of several parameters with the minimum number of trials. The results obtained in these experiments were statistically analysed in order to find the lesser variability and to adjust each variable to its optimum level [15–18]. The main advantage of the Taguchi parameter design is that it introduces noise factors in the experimentation, causing an uncontrolled variation in such a way that the obtained optimum becomes insensitive to noise, thus with a higher reproducibility.

The first step of implementing the TPD was to identify the output variable to optimise. In the multi-commutated flow system, the desired response was the maximum Iₚ value. The second step was to identify factors and/or interactions of interest. The flow rate (FR) for the aspiration of solutions, the aspirated sample volume (V) and the reactor length (R) from valve V₂ to the detector were selected as control factors. The factor’s levels were chosen bearing in mind preliminary analyses carried out and the physical limitations of the system. In relation to the flow rate, it was shown that the use of high flow rates led to a compaction of the beads inside the enzymatic reactor, which resulted in hindering and even impeding the flow passage. As for the aspirated sample volume, taking into account the fact that serum is difficult to obtain, it should be the lowest value which originated reproducible results. Considering the reactor, since it was not intended to increase the dispersion of the samples after the passage through the enzymatic reactor, it would have to possess a minimum length, sufficient just to link the V₂ valve to the detector. On the other hand, taking into account the composition of the matrix in the blood serum and pharmaceutical formulations, albumin concentration was selected as noise factor, since its value could vary among serum samples, altering its viscosity and consequently, affecting the response. As with the controllable factors previously referred, the noise factor was studied at three levels, which covered the predicted concentration range in blood (Table 1). The third step was to select the appropriate orthogonal array and to assign factors and/or interactions to columns. The selected orthogonal array must have a number of columns equal to or higher than the number of degrees of freedom of the system, so an L₉(3⁴) was used. Since only three controllable factors were selected for optimisation, the fourth column of the orthogonal array was used to study interactions between factors (I). The experiments were performed in duplicate, to calculate the residual error, so that the total number of experiments undertaken was 54 (9 experiments × 3 noise levels × 2 replicas). Measurements were carried out with solutions containing 2.5 × 10⁻⁴ mol l⁻¹ of acetaminophen and an albumin concentration correspondent to each one of the three levels of noise factor. Table 2 indicates the matrix of the design and the results obtained. The fourth step was to carry out the experiments, analyse the data and determine the optimum levels. The results were analysed statistically to adjust each variable to its optimum level and with the least variability possible. All calculations were carried out through the ANOVA-TM v2.5 computer programme. The levels combination that enabled the highest analytical signal to be obtained (highest Iₚ) was FR₁–V₁–R₁–I₁. The response was higher for FR₁ because in this case, the contact between acetaminophen and the enzyme was favoured.

Table 1.

<table>
<thead>
<tr>
<th>Notation</th>
<th>Factor</th>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR</td>
<td>Flow rate (ml min⁻¹)</td>
<td>0.48</td>
<td>0.96</td>
<td>1.44</td>
</tr>
<tr>
<td>V</td>
<td>Sample volume (µl)</td>
<td>80</td>
<td>120</td>
<td>160</td>
</tr>
<tr>
<td>R</td>
<td>Reactor length (cm)</td>
<td>4</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>I</td>
<td>Interactions between factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>Albumin concentration (g l⁻¹)</td>
<td>3.5</td>
<td>4.4</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Table 2. Experimental L₉(3⁴) orthogonal array with a three-level noise factor

<table>
<thead>
<tr>
<th>Trial</th>
<th>Control factors and levels</th>
<th>Iₚ × 10⁻⁷ (A)</th>
<th>S/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FR  V  R  I</td>
<td>N₁  N₂  N₃</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1   1   1   1</td>
<td>10.70 9.75</td>
<td>10.62 9.92</td>
</tr>
<tr>
<td>2</td>
<td>1   2   2   2</td>
<td>9.64 9.47</td>
<td>11.50 11.01</td>
</tr>
<tr>
<td>3</td>
<td>1   3   3   3</td>
<td>11.51 12.73</td>
<td>12.20 11.59</td>
</tr>
<tr>
<td>4</td>
<td>2   1   2   3</td>
<td>7.57 7.64</td>
<td>7.02 7.53</td>
</tr>
<tr>
<td>5</td>
<td>2   2   3   1</td>
<td>7.86 7.59</td>
<td>10.05 10.77</td>
</tr>
<tr>
<td>6</td>
<td>2   3   1   2</td>
<td>9.70 9.81</td>
<td>10.01 11.12</td>
</tr>
<tr>
<td>7</td>
<td>1   3   2   2</td>
<td>7.61 7.44</td>
<td>7.61 6.87</td>
</tr>
<tr>
<td>8</td>
<td>2   1   3   3</td>
<td>7.54 7.84</td>
<td>7.19 7.86</td>
</tr>
<tr>
<td>9</td>
<td>3   2   2   1</td>
<td>8.80 8.43</td>
<td>9.05 9.86</td>
</tr>
</tbody>
</table>

Anodic peak current intensity (Iₚ, × 10⁻⁷ A) was determined for each trial and noise level.
the variance ratio (F) can be inferred that all controllable factors considered in the experimental design (FR, V and R) caused a statistically significant effect at the 95% confidence level (calculated F was greater than critical F). The factor which exerted the greatest influence on the response was a contribution of 57.0% for the total variance of the results was the flow rate, followed by the sample volume (16.8%). The contribution of the residual error for the variability of the response (12.3%) indicated the good application of the experimental design used. As a rule, if such a contribution is smaller than 15%, the variance of the experimental data can be said to be explained by the effect of factors and interactions.

The effect of controllable factors on the S/N ratio was also evaluated. It was demonstrated that irrespective of the uncontrollable noise variation, the highest Ip, with V3 being that which gave rise to the higher signal. R1 produced the highest response because that length was that which was better suited to the 15 s of aspiration of the pH 5.5 buffer solution necessary to obtain the result.

The results of the analysis of variance (ANOVA) for the regular analysis are indicated in Table 3. From the calculated values of the variance ratio (F), it can be inferred that all controllable factors considered in the experimental design (FR, V and R) caused a statistically significant effect at the 95% confidence level (calculated F was greater than critical F). The factor which exerted the greatest influence on the response was a contribution of 57.0% for the total variance of the results was the flow rate, followed by the sample volume (16.8%). The contribution of the residual error for the variability of the response (12.3%) indicated the good application of the experimental design used. As a rule, if such a contribution is smaller than 15%, the variance of the experimental data can be said to be explained by the effect of factors and interactions.

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Table 3: Pooled ANOVA for the regular analysis

<table>
<thead>
<tr>
<th>Variation source</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Variance ratio (F)*</th>
<th>Pooled sum of squares</th>
<th>Contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate, FR</td>
<td>2</td>
<td>74.37</td>
<td>37.19</td>
<td>123.47</td>
<td>N 73.77</td>
</tr>
<tr>
<td>Sample volume, V</td>
<td>2</td>
<td>22.41</td>
<td>11.20</td>
<td>21.84</td>
<td>16.8</td>
</tr>
<tr>
<td>Reactor length, R</td>
<td>2</td>
<td>5.48</td>
<td>2.74</td>
<td>9.10</td>
<td>N 4.88</td>
</tr>
<tr>
<td>Interactions between control factors</td>
<td>2</td>
<td>1.50</td>
<td>0.75</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Noise, N</td>
<td>2</td>
<td>4.20</td>
<td>2.10</td>
<td>6.98</td>
<td>N 3.60</td>
</tr>
<tr>
<td>Interactions between control factors and noise</td>
<td>16</td>
<td>14.25</td>
<td>0.89</td>
<td>2.96</td>
<td>N 9.43</td>
</tr>
<tr>
<td>Residual</td>
<td>25</td>
<td>7.24</td>
<td>0.27</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>129.45</td>
<td>2.44</td>
<td>129.45</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Critical variance ratio for a 95% confidence level is 3.35 (2 d.f.).
* Contribution is defined as 100 × (pooled sum of squares/total sum of squares).

With the parameters of the flow system optimised, standard solutions of acetaminophen with concentrations between 5.0 × 10⁻³ and 5.0 × 10⁻⁴ mol l⁻¹ were aspirated to the system. A linear relationship was verified between the concentration of acetaminophen and Ip, in the referred interval with a detection limit, calculated from the regression equation (with yb = a and yb = x) [19], of 1.7 × 10⁻⁴ mol l⁻¹.

The repeatability of the measurements, expressed as R.S.D. of the Ip, was evaluated through successive determinations of a 1.0 × 10⁻⁴ mol l⁻¹ 4-acetaminophen standard solution, with a R.S.D. value of 2.4% (n = 10) being obtained.

Under the established conditions, each determination took about 2.5 min including the surface renewal step, which corresponds to 24 determinations per hour.

3.6. Sample analysis

To evaluate the applicability of the proposed method, several blood serum samples were analysed, as well as some pharmaceutical formulations existing on the Portuguese market. Table 4 shows the results obtained in the determination of acetaminophen in these samples. The agreement between the results obtained by the proposed method and by the reference methods was evaluated through the Student t-test for paired samples, in which the calculated t value (−0.31 for
The developed method shows itself as an advantageous alternative to the reference methods in that it enables the attainment of similar results while being additionally more rapid, less laborious and above all, makes it possible to analyse samples so diverse as blood serum and pharmaceutical formulations, using the same flow system. The possible chemical interferences caused by the constituents of the serum matrix were avoided using an enzymatic reactor in which acetaminophen present in the samples was hydrolysed on line.

Although chemically modified electrodes are currently widely used due to the advantages they offer, the use of tubular electrodes with modified surface is, to our knowledge, described now for the first time. This work describes the development of a tubular detector with modified electrodes and demonstrates the significant advantages of its application in the analysis of complex samples, namely blood serum and pharmaceutical formulations. The Nafion film allowed an increase in analytical signal intensity and simultaneously, protected the surface of the working electrode, avoiding its fouling by species present in the sample matrixes, conferring greater stability to the electrode and higher reproducibility to the determinations. Due to the tubular configuration of the detector, it was possible to apply it to a multi-commutated flow system, thereby associating the advantages of the multi-commutated flow systems, namely their greater versatility, which enables the...
flow manifold to be easily altered and adjusted to each determination simply by reprogramming the routine that defines the analytical cycle, without the need to introduce any physical reconfiguration in the system. The developed detector can be applied in flow systems where solutions are aspirated as well as flow systems in which solutions are propelled, thereby making it feasible to combine it with other flow system modalities, such as flow injection analysis or sequential injection analysis.

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