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Analytica Chimica Acta 462 (2002) 293–304

ANALYTICA CHIMICA ACTA

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On-line simultaneous monitoring of glucose and acetate with FIA during high cell density fermentation of recombinant *E. coli*

I. Rocha, E.C. Ferreira*

Centro de Engenharia Biológica, Universidade do Minho, 4710-057 Braga, Portugal

Received 9 November 2001; received in revised form 21 March 2002; accepted 22 April 2002

Abstract

A two-channel flow injection analysis (FIA) system was developed for the simultaneous on-line monitoring of acetate and glucose during high cell density fed-batch fermentations of recombinant *Escherichia coli*. Acetate measurement was performed with a modified and optimised version of an existing method, based on acetate diffusion through a gas-diffusion chamber into a stream containing an acid–base indicator. The subsequent decrease in the absorbance was detected with an incorporated photometer. After method optimisation, it was possible to achieve linearity until 10 g/kg with no dilution step and with a detection level of 0.05 g/kg. Although some interferences were found, the performance of the method proved to be sufficiently reliable for on-line control purposes Commercially packed glucose oxidase (GOD) was used for the amperometric measurement of glucose. The method was linear up to 5 g/kg and it was possible to detect concentrations lower than 0.06 g/kg. For these measurements, no significant interferences were detected when the results were compared with other reference methods. The application of a simultaneous parallel configuration of the methods to a high cell density fed-batch *E. coli* fermentation was tested and reliable results were obtained within a 3 min delay. This information was made available to a supervisory computer running a developed LabVIEW™ programme via an Ethernet network, allowing the immediate implementation of control actions, improving the process performance.

Keywords: *Escherichia coli*; Bioprocess monitoring; Flow injection analysis; Glucose; Acetate; On-line

1. Introduction

Since its birth [1], flow injection analysis (FIA) has been widely used for the accurate characterisation of bioprocesses. During the last decade, a large number of works has been published emphasising the advantages of this technique when applied to the on-line monitoring of biotechnological processes [2,3], namely, its high sampling rate, small sample volumes, low reagent consumption, computer compatibility, high reproducibility and versatility.

The use of biosensors for on-line process monitoring [4], and the recent developments of the FIA systems towards decreasing reagent consumption and detection limits even further [5] states FIA as one of the most powerful tools for bioprocess quantitative monitoring.

Being the fed-batch culture of *Escherichia coli* one of the most widely used fermentation systems, especially for the production of high valued recombinant products, its sensing through FIA systems assumes great importance, mainly due to the accurate control required in these kind of processes.

* Corresponding author. Tel.: +351-253604407; fax: +351-253678986.
E-mail address: ecferreira@deb.uminho.pt (E.C. Ferreira).

0003-2670/02/$ – see front matter
PII: S0003-2670(02)00347-1

For this particular bioprocess, not only methods for the analysis of substrates and metabolites have been described, but also specific methods for the determination of recombinant proteins [6,7], and microbial activity [8,9] are available in the literature.

However, for general recombinant protein production systems, the control of substrates (usually glucose), and main by-product (acetate) concentrations represent the key for achieving better process performance. Usually, the main objective of this control action is to limit the accumulation of acetate, due to its negative effects in the process behaviour. These effects include the decrease of the biomass yield, the inhibition of growth when acetate is present at high concentrations (typically 10 g/l) and the decrease in the production of recombinant proteins [10,11]. Acetate is produced both when *E. coli* is grown under anaerobic or oxygen-limiting conditions and when carbon flux into the central metabolic pathways exceeds the biosynthetic demands and the capacity for energy generation within the cell [12,13], that is to say, when glucose is present above certain levels.

Due to the high cell densities generally obtained in these processes, the model-based prediction of variable values, with concomitant control actions, often fails in obtaining good results, motivating the development of on-line methodologies for the determination of these two key variables.

Thus, in this paper it is suggested the application of a developed two-channel FIA system to the on-line determination of glucose and acetate during the fed-batch recombinant fermentation of *E. coli*.

In recent years, the measurement of glucose with FIA has been intensively studied and is usually based on the reaction of the metabolite with immobilised glucose oxidase (GOD) according to the following reaction:

$$\text{Glucose} + O_2 + H_2O \rightarrow \text{Gluconic acid} + H_2O_2$$

The resulting H_2O_2 can be directly measured via electrochemical determination with a commercially available Pt-flow-through electrode [14] or indirectly via the chemiluminescence reaction [15,16]. It can also react with another enzyme (peroxidase) to produce a coloured product detectable photometrically [17,18]. In this work, a Pt-flow-through electrode was chosen, due to its commercial availability and simplicity.

On the other hand, acetate measurement with FIA is not yet accomplished in a routine basis, since the adaptation of its enzymatic determination method to a flow system is not straightforward.

The combination of the enzymes acetate kinase, piruvate kinase and lactate dehydrogenase immobilised in series with different detection methods has been described [19,20], while another approach was explored by Tservistas et al. [21], based on the oxidation of sarcosine by immobilised sarcosine oxidase, a reaction that is competitively inhibited by acetate. However, none of these methods is yet commercially available and its application to a routine basis would implicate the constant immobilisation of several enzymes (three in the first case). Moreover, the complexity of the earlier-mentioned methods makes them less flexible and causes difficulties with their integration in simultaneous determination of several analytes within the same FIA system.

For these reasons, a chemical method for the analysis of acetate was chosen, based on a simple and flexible method first described by Forman et al. [22]. Due to its nature, this method is not specific for acetate, also detecting other volatile acids present in the sample. However, in *E. coli* fermentations, this compound represents almost the total amount of detectable volatile acids produced as by-products of growth.

One of the major problems in adapting FIA measurement systems to the on-line monitoring of bioprocesses is related with the retrieval of liquid medium samples free of biomass and suspended particles; common problems are the clogging of the membrane system, and the possible introduction of errors in the measurements [23,24]. That issue is of particular difficulty when dealing with high cell densities (more than 40 g/kg of biomass). In fact, very few authors have described this kind of applications in the literature [25]. For low cell densities processes, both in situ probes [25] and external filtration modules [17,26] have been proposed, each with its particular drawbacks [16], but most of them excessively expensive and complex. In this work, the criteria for choosing this equipment were simplicity, low cost and robustness at high cell densities. The chosen external tangential filtration device proved to meet these requirements.

This paper is organised as follows: in Section 2, the experimental conditions are described, namely the fermentation conditions, the fermenter sampling

system, the FIA system, other analytical methods, and software. In Section 3, the main results obtained are presented, while Section 4 contains the major conclusions extracted from this study.

2. Materials and methods

2.1. Fermentation

Fermentations were conducted in a 5 l Biostat MD fermenter from B. Braun Biotech (Germany) connected to a digital control unit. The set points for the environmental properties were 37 °C for temperature, pH 7, and 30% of saturation for dissolved oxygen. In order to improve the accuracy of the measurements, the fermentation culture and feeding solution weights were continuously assessed with two balances and, as a consequence, mass based concentrations were used in this work.

A modified *E. coli* JM109 with the insertion of the interferon *α-2b* gene in the plasmid pET was used. The fermentation process started with a batch phase with an initial weight of 3 kg of a selected defined medium with the following composition: 5 g/kg of glucose, 6 g/kg of Na_2HPO_4, 3 g/kg of KH_2PO_4, 1 g/kg of NH_4Cl, 0.5 g/kg of NaCl, 0.12 g/kg of $MgSO_4{\cdot}7H_2O$, 0.34 g/kg of tiamine, 0.015 g/kg of $CaCl_2{\cdot}2H_2O$, 0.05 g/kg of kanamicin, 2 ml/kg of trace metals solution and 2 ml/kg of vitamin solution. Trace metal solution was of 27 g/l $FeCl_3$, 2 g/l $ZnCl_2$, 2 g/l $CoCl_2$, 2 g/l $NaMoO_4$, 1 g/l $CaCl_2$, 1 g/l of $CuCl_2$, 0.5 g/l H_3BO_3 and 100 ml/l HCl. Vitamin solution had the following composition: 0.42 g/l of riboflavine, 5.4 g/l of panthotenic acid, 6.1 g/l of nicotinic acid, 1.4 g/l of piridoxine, 0.06 g/l of biotine and 0.042 g/l of folic acid.

After glucose consumption during the batch phase (approximately 8–9 h), fed-batch phase started with the addition of the feeding solution, which had the composition of 250 g/kg of glucose, 10 g/kg of NH_4Cl and 4 g/kg of $MgSO_4{\cdot}7H_2O$. During the validation of FIA methods, the feeding profile was always exponential, with biomass data correction, as described elsewhere [27]. NaOH and phosphoric acid were used for pH control.

2.2. Sampling system

The sampling system is illustrated in Fig. 1. The bio-suspension was extracted from the fermenter using a peristaltic pump followed by the microfil-

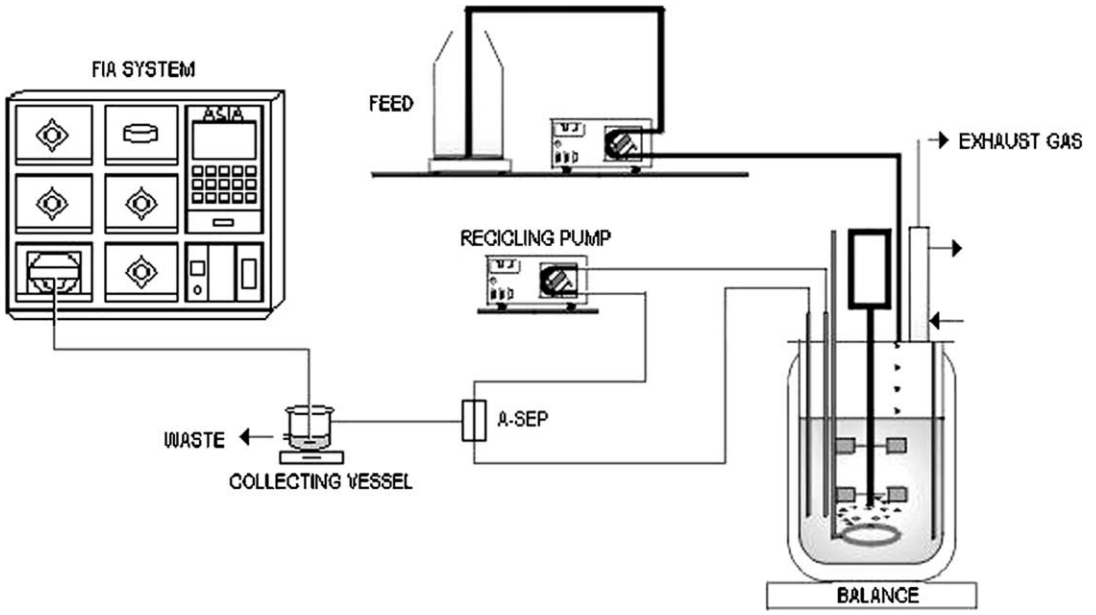

Fig. 1. Bioreactor sampling system.

tration unit A-SEP (Applikon, The Netherlands); inside this device, a small part of the bio-suspension passed through a standard 0.45 μm membrane with a 47 mm diameter (Pall Corporation, MI, USA), and the non-filtrated bio-suspension returned to the fermenter. In order to reduce dead volumes, which contribute to measurement bias, the sampling loop was kept as short as possible, and the peristaltic pump speed was maximised. Therefore, the retention time of a portion of bio-suspension outside the reactor never exceeded 1 min, and the corresponding volume never exceeded 40 ml. Also, to assure both a constant volume of filtrate for the FIA system and the correspondence of the samples collected to the most recent state inside the fermenter, a collecting vessel was designed to reject the filtrate volumes in excess. This vessel was permanently agitated with a magnetic stirrer and was always kept closed to avoid the evaporation of acetate.

2.3. FIA system

The FIA system used was the ASIA model from Ismatec (Switzerland). The set-up for the measurement of acetate was adapted from Forman et al. [22]. The sample containing the analyte was injected on a carrier stream, consisting of distilled water and suffered an acidification with diluted sulphuric acid. Consequently, the predominant form of acetic acid was the non-anionic form, which was able to cross a hydrophobic membrane into an indicator stream containing a phenol red solution. At the neutral pH of this stream, acetate could no longer cross the membrane back and dissolved in the indicator, causing a pH drop, and consequently a colour change, detectable photometrically at 555 nm. Acetate concentration was then proportional to the height of a negative peak. The method described by Forman et al. [22] also included an alkali stripper to eliminate the carbon dioxide (in the form of hydrogen carbonate) interference. However, initial experiments revealed that, probably due to the agitation of the sample, this metabolite did not interfere with the method.

Also, the phenol red concentration on the indicator solution proposed by Forman et. al. [22] (0.1 M) was found to be too high, leading to a small sensitivity. The concentration used was of 0.016 M. The acid concentration was kept at 1 M, while the phosphate concentration in the indicator stream was chosen to be an optimisation variable to increase the linear range or, on the other hand, to decrease the detection limit. The volumetric flow rates were 0.45 ml/min for the carrier and sulphuric acid streams and 1.05 ml/min for the indicator stream. The injection volume of the valve was of 50 μl. A sodium acetate solution containing 50 g/kg of acetate was used for calibration standards preparation. The reagents used for this measurement were all of analytical grade.

For glucose measurement, the sample containing the analyte was injected into a buffer carrier stream, which then reacted in a GOD enzymatic cartridge (Biocart Glucose LS, ref. 100012) from Anasycon (Hannover, Germany), that converted glucose into gluconic acid and hydrogen peroxide. The concentration of this compound was then measured electrochemically with an amperometric detector IS 2996 (Ismatec, Switzerland). The electric current was proportional to the glucose concentration present in the sample. The detector allowed three different sensitivities for the measurement: 1, 10 and 1000 μA/V. The composition of the buffer was initially of 17.9 g/l of Na_2HPO_4 and 15.6 g/l of $NaH_2PO_4{\cdot}2H_2O$. The volumetric flow rate was 0.45 ml/min. The injection volume of the valve was of 25 μl. Calibration standards were prepared diluting a glucose solution of 50 g/kg. The reagents used for this measurement were all of analytical grade.

Both glucose and acetate FIA methods are illustrated in Fig. 2, in a parallel configuration scheme that will be described in more detail in Section 3.

2.4. Other analytical methods

Cell growth was determined off-line using optical density (OD) measurements at 600 nm. One OD unit was equal to 0.32 g/kg of cell dry weight.

For comparative purposes, acetic acid was also measured off-line with HPLC and with an enzymatic kit. The HPLC measurements were conducted in a refractive index detector (Jasco, Canada) with a Chrompack organic acids column (Varian, USA) at 60 °C. The mobile phase consisted in a 0.01N solution of H_2SO_4 at a flow rate of 0.75 ml/min. Enzymatic measurements were done with a kit from R-Biopharm, Germany (ref. 148261) according to the manufacturer's instructions.

Due to the high level of interferences, analysis of glucose with HPLC was impossible, and the comparative methods selected for these measurements were

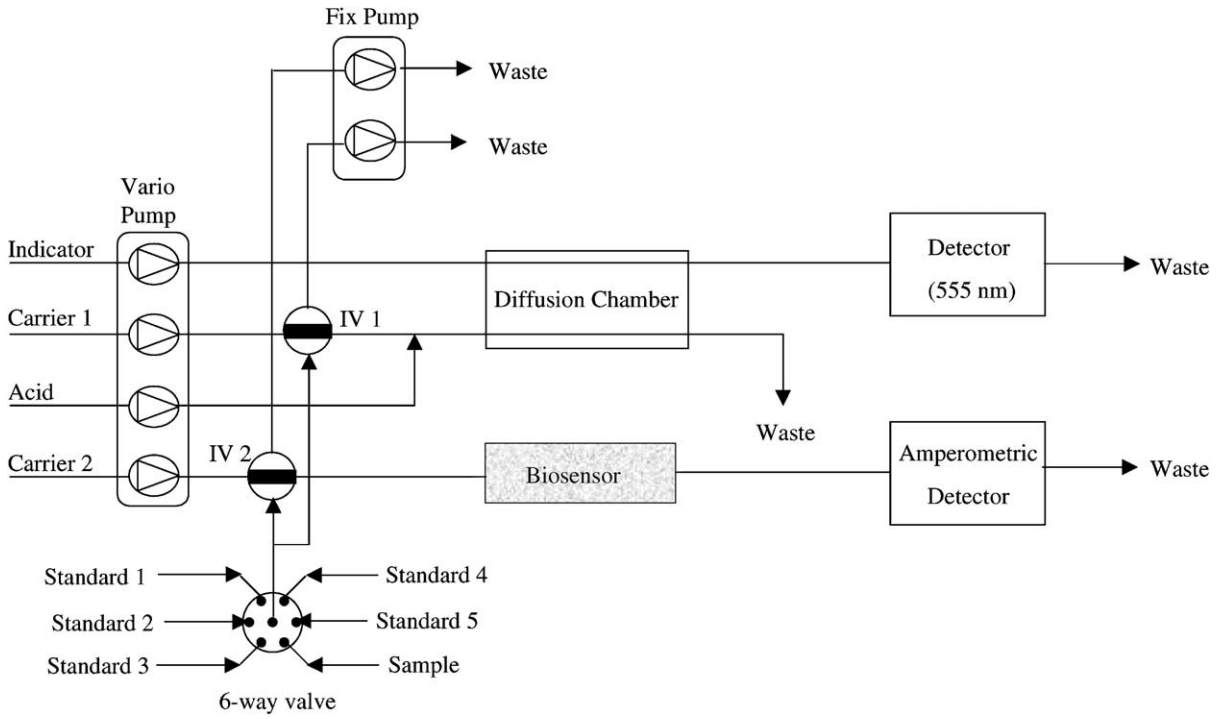

Fig. 2. Schematic representation of the system used for the analysis of acetate and glucose with FIA. IV is the injection valve, carrier 1 the distilled water and carrier 2 the buffer used for glucose measurement, as described in the text.

the di-nitrosalicylic acid (DNS) method and an enzymatic kit. For the first one, 500 μl of the DNS reagent (prepared according to [28]) were added to 500 μl of sample and boiled at 100 °C during 5 min. Afterwards, 5 ml of deionised water were added and the absorbance of the resulting solution was measured at 540 nm in a Sunrise modular absorbance microplate reader (Tecan, Austria). The enzymatic kit was also acquired from R-Biopharm, Germany (ref. 716251).

2.5. Software

Data from the FIA system (glucose and acetate concentrations) were acquired in one dedicated computer running the ASIA software version 2.10 (Ismatec, Switzerland) that also controlled the entire system (testing and recalibrating when necessary). These results were then captured in a supervisory computer via an Ethernet network and immediately integrated with the remaining state variables from the fermentation [27]. The graphical programming language LabVIEW™ (National Instruments, USA) was used to develop this integrated system. A virtual instrument, the basic element of a LabVIEW™ programme, was developed for the communication between both computers. The major tasks accomplished by this routine included identifying the last measurement, saving and finally making it available for any control algorithm running in the supervisory computer.

3. Results

3.1. Acetate measurement optimisation

During the optimisation of the acetate measurement with FIA, the main manipulated variable was the buffer capacity of the indicator solution, represented by its phosphate concentration. Several experiments were conducted at different buffer capacities of the indicator covering a wide range of concentrations. Table 1 shows how the linearity and detection levels

Table 1
Influence of the buffer capacity of the indicator solution on the linearity and detection level for acetate measurements with FIA

Phosphate concentration (mM)	Linearity limit (g/kg)	Equation of lines (signal (mV) × concentration (g/kg))	R^2	MDL (g/kg)	R.S.D. (%)
0.25	2.5	−89.79 × −7.97	0.9994	0.04	4.18
0.375	2.5	−125.38 × −18.69	0.9971	0.02	4.56
0.5	5	−93.50 × −26.04	0.9976	0.03	0.6
0.75	7.5	−76.41 × −26.96	0.9973	0.04	2.56
1	10	−53.20 × −7.39	0.9993	0.05	2.98
1.25	10	−46.23 × −10.54	0.9995	0.1	1.26

of the measurement can be influenced by that value. Method detection level (MDL) is defined as the constituent concentration that, when processed through the complete method, produces a signal that has a 99% probability to be different from the blank [29]. For the calculation of MDL in seven replicates of the sample, the condition was that mean had to be $3.14^*\sigma$ above the blank, being σ the standard deviation of the seven replicates. The R.S.D. was calculated, for each phosphate concentration, from 20 replicates of a standard in the middle of the linear range. The reference method for these experiments was the HPLC method.

The results obtained show the versatility of this method, and its applicability to processes where low acetate accumulations should be expected, such as batch or low cell densities fed-batch processes, and to other processes where acetate is present in a wide range of concentrations, like high cell density fed-batch fermentation.

For a batch fermentation, the recommended phosphate concentration would then be 0.25 mM. For this process, the interferences affecting the proposed method when analysing acetate on the fermentation bio-suspension were evaluated. The correlation obtained between FIA and HPLC for those experiments is given by, acetate(FIA) = 1.24 × acetate(HPLC) + 0.0085 with a correlation coefficient very close to unity (0.9879), indicating that the FIA method gives almost the same results as HPLC method.

For fed-batch processes, the most suitable phosphate concentration was considered to be 1 mM. For this case, interferences affecting the method performance were also evaluated. The first approach was to compare the results obtained using the FIA method off-line with other conventional methods (HPLC and enzymatic kit). The results obtained for samples from a fed-batch fermentation are represented in Fig. 3. It is clear that, for every sample, acetate concentrations obtained with FIA are slightly higher than those obtained with HPLC. This is mainly due to the already mentioned non-selectivity of the method for acetate in complex samples. However, the trend along the fermentation is similar. The corresponding relative differences between the three methods were also calculated. The average deviation between FIA and HPLC is about 16%, oscillating between 4 and 36%, much less then the difference observed between HPLC and the enzymatic kit (with a minimum of 16%, a maximum of 41% and an average of 27%). The magnitude of the deviation between HPLC and FIA was checked for other fed-batch fermentations and did not exceed 20%.

In order to test the reliability of the acetate measurements conducted with FIA, simulation studies were conducted (results not shown) applying a developed adaptive control algorithm for the acetate concentration [30]. As a first approach, the algorithm was applied to “real” values of acetate concentration; results show a very good convergence of acetate levels to the set point, and non-limited exponential biomass growth. To check the interference of FIA measurements, other simulations were conducted with the “real” acetate values affected by a 20% signal increase (the maximum difference obtained between HPLC and FIA) plus 5% of white noise (much higher than what is observed with acetate FIA measurements). The results show a good convergence and non-limited biomass growth, although the set point of “real” acetate suffers a negative deviation of 16–20%. This deviation was considered to be neglectable for control purposes and acetate measurements with FIA were considered to be reliable for on-line control applications.

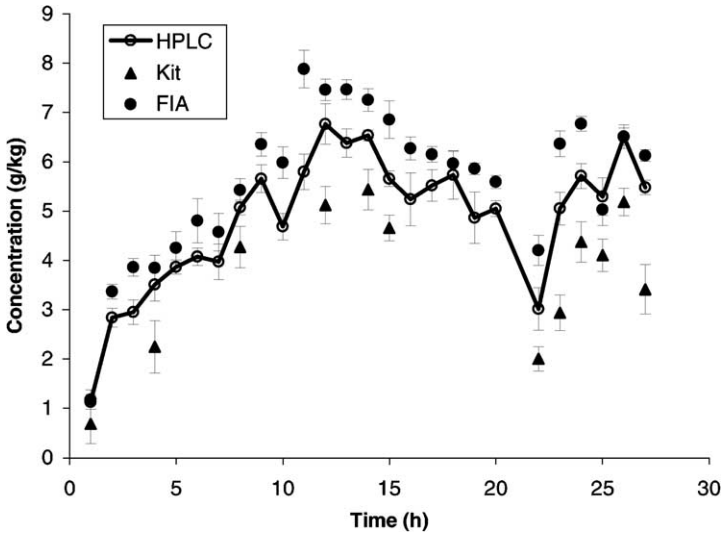

Fig. 3. Comparison between several methods for the measurement of acetate in the course of a fed-batch fermentation. Error bars account for standard deviation between two (HPLC and enzymatic kit) or three samples (FIA).

Finally, the interference of the sampling system in the method's behaviour was evaluated, by comparing the results obtained from FIA on- and off-line. These results are illustrated in Fig. 4. Before the introduction of the collecting vessel in the sampling system, the filtrate was accumulated inside an ordinary recipient, without agitation or filtrate rejection. The differences between on- and off-line analyses are notorious, due to the accumulation phenomenon. After the introduction of the collecting vessel, the on- and off-line results became very close, with no significant differences.

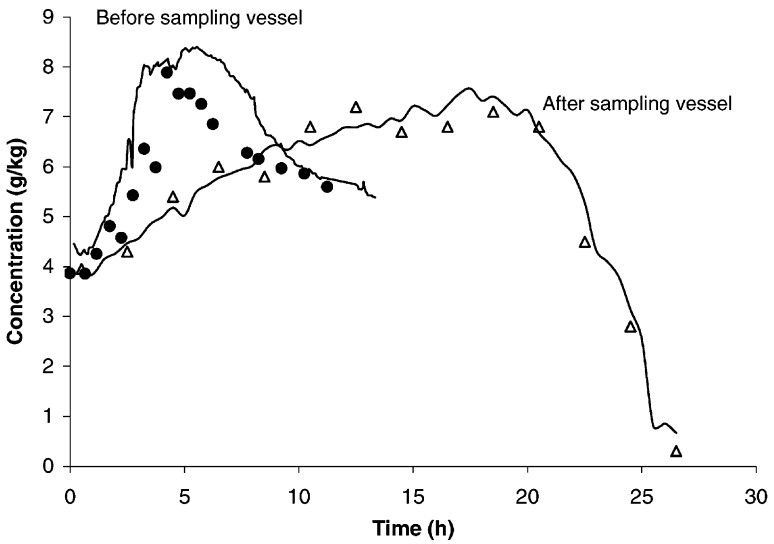

Fig. 4. Comparison between both on-line (solid lines) and off-line (●, △) FIA acetate analyses before and after the introduction of the sampling vessel in the system.

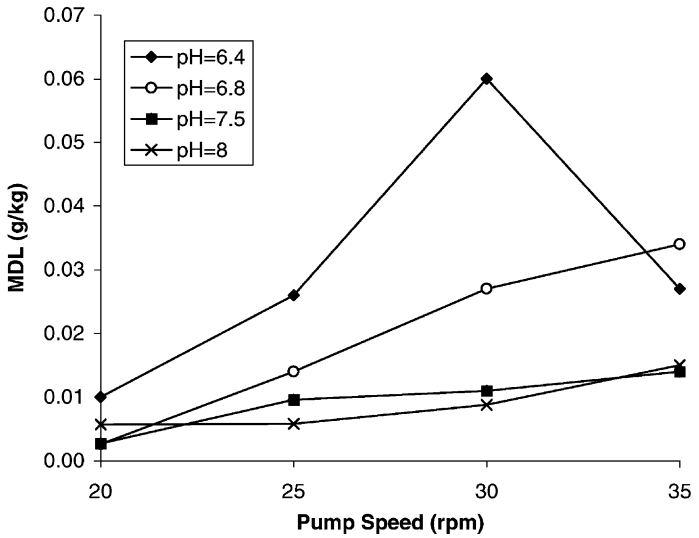

Fig. 5. Variation of MDL with pH and pump speed for the glucose method.

3.2. Glucose measurement optimisation

The main purpose of this optimisation procedure was to increase the linearity limit of the method, in order to be capable of determining both glucose and acetate in the same sample during a conventional *E. coli* fermentation. With a carrier composition of 17.9 g/l of Na_2HPO_2, 15.6 g/l of $NaH_2PO_4{\cdot}2H_2O$, and 2 ml/l of a 20% sodium azide solution, as recommended by the method described by Ismatec [31] both carrier pH and pump speed were varied and the MDL and linearity limit were determined. pH was varied by the addition of sodium hydroxide or phosphoric acid to the carrier. The results obtained are represented on Figs. 5 and 6.

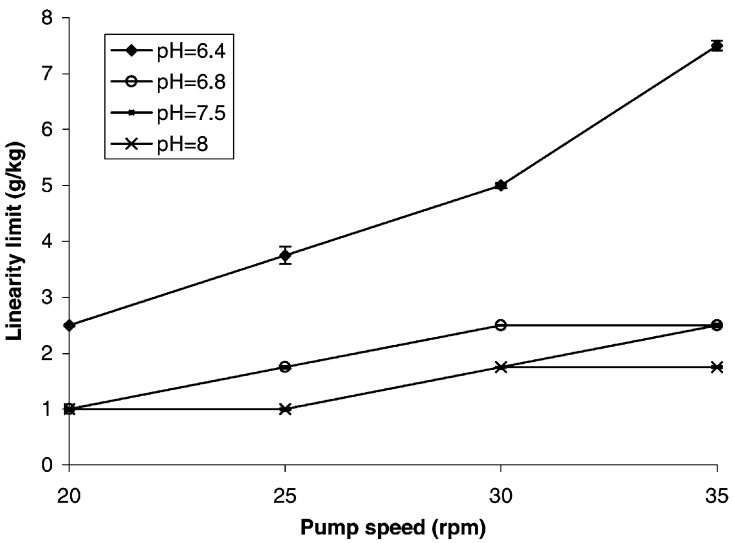

Fig. 6. Linearity limits of the method as a function of pH and pump speed for the glucose method. Error bars represent the standard deviation between five injections.

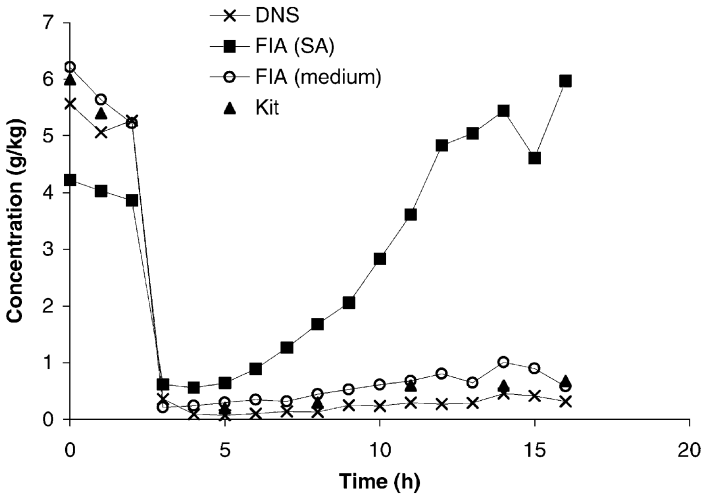

Fig. 7. Comparison of DNS, FIA and enzymatic kit glucose analyses in fermentation samples. FIA (SA) means that the carrier contained sodium azide. FIA (medium) represents the analyses taken with the carrier composition similar to the growth medium.

Results show that at pH 6.4 it is possible to achieve much higher linearity limits, avoiding the need of introducing a dilution step in the measurement system. Also, the corresponding MDL was considered to be sufficient for this process. Pump speed was fixed at 25 rpm.

However, when these conditions to were applied to real samples obtained from *E. coli* fed-batch fermentation, a very strong interference was observed (Fig. 7), that can not be explained by the complexity of the samples. After some experiments (results not shown), it was found that the presence of sodium azide in the carrier was the main cause of these discrepancies. In fact, this compound somehow inhibited the enzyme GOD and this effect was attenuated in the presence of sample components. This inhibitor was primarily added to the carrier in order to avoid the clogging of the system, and to inhibit growth in the tubing walls. However, at this point, sodium azide had to be excluded from the carrier, while some cleaning procedures had to be implemented in the system on a daily basis to avoid the aforementioned problems. The sensitivity of the detector had also to be changed from 1 to 10 μA/V.

Without sodium azide, further carrier optimisation had to be conducted in order to eliminate other interferences. It was found that the matrix effects of the samples were significant, and the contribution of each medium component to that interference was evaluated. In Fig. 8, the differences observed in terms of detector response for a 1 g/kg standard are shown. The first column of the chart corresponds to the signal obtained with a carrier composed of phosphates only. After that, the other medium growth components were added sequentially to check for individual interferences. It is clear that ammonium chloride is the major contributor for the observed signal reduction. To minimise those matrix effects, another carrier composition is proposed, combining the phosphates at the aforementioned concentrations and the medium components referred in Fig. 8. Thus, the aforementioned samples were analysed with this new carrier composition and the results are also shown in Fig. 7. Although the trend is similar to the ones observed for the other methods, there is still a significant difference, especially when comparing with DNS data. However, since these concentrations did not cover the whole range of glucose method and were not obtained on-line, the reliability of glucose method was checked during the simultaneous determination, which is described in the next section.

3.3. Simultaneous determination

After the optimisation procedure described in the previous sections, both methods were implemented in parallel. The only requirement of this configuration was that the pump speed had to be the same for both

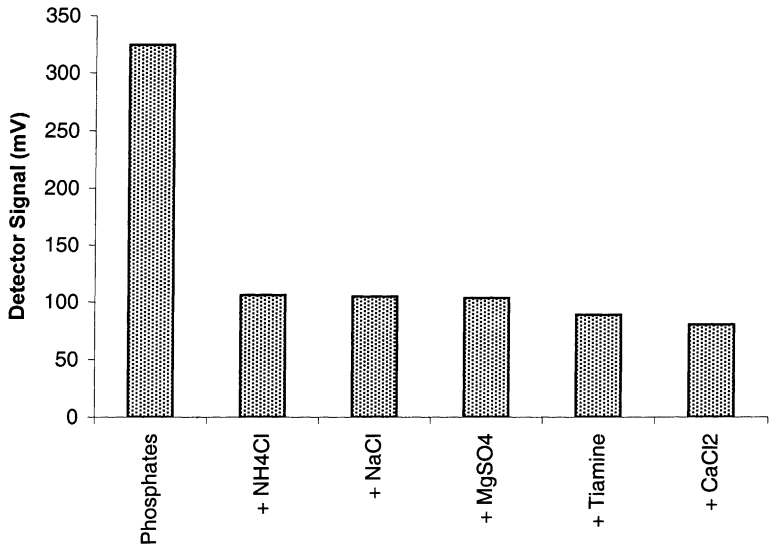

Fig. 8. Contribution of the individual medium components to the observed matrix effect during glucose analysis. Each component was added sequentially to the carrier at the concentrations presented in the growth medium (Section 2.1). The results presented correspond to the analysis of a 1 g/kg glucose standard.

analyses. This had been contemplated during optimisation, and the adopted value was 25 rpm. Sample injection was done simultaneously in both valves, with the help of a "T" configuration after the six-way valve. The major disadvantage of this configuration was the consumption of twice the sample volumes, when compared with the methods individually or with a possible serial implementation. However, the sampling system was always able to provide enough quantities for the analyses.

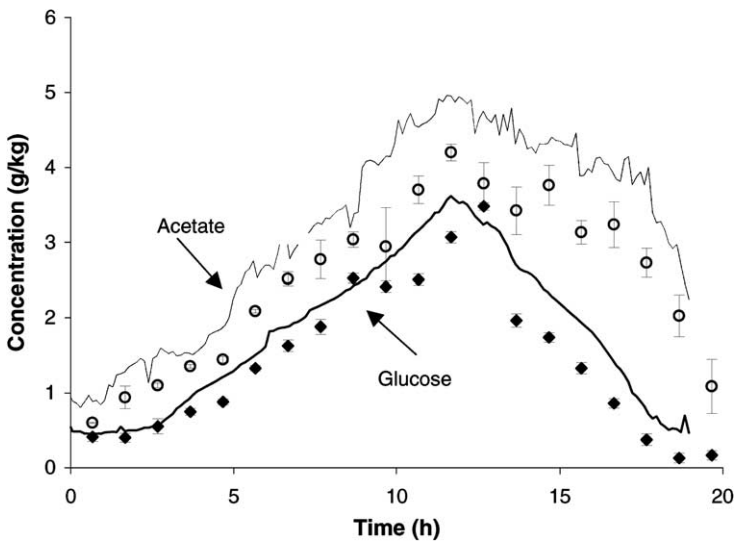

Fig. 9. On-line acetate and glucose measurement during a fed-batch fermentation obtained with the parallel simultaneous configuration (lines). The points correspond to the off-line analyses with the HPLC (for acetate) and DNS (for glucose) methods.

This configuration allowed both glucose and acetate measurements in one sample within 2 min. The concentration values could then be obtained with a total delay of 3 min (including 1 min for the sampling retrieval). Computer acquisition via LabVIEW^{TM} software was done immediately after analysis, allowing the performance of control actions in pseudo real time.

In order to validate the on-line simultaneous measurement of glucose and acetate, a fermentation was conducted and on-line results were compared with those obtained with conventional methods (HPLC for acetate and DNS for glucose). For this fermentation, the feeding was programmed with the purpose of obtaining glucose and acetate accumulation, in order to test both methods in a wide range of concentrations. When the metabolites accumulated in the medium at concentrations considered significantly high, the feeding was stopped to check for the measurements' robustness at high cellular concentrations, when certain fermentation by-products were present. Consumption of both components (and concomitant concentrations decline) started right after the stop of the feeding. The final cell dry weight obtained was of 40 g/kg, representing approximately 55 g/l. These results are illustrated in Fig. 9. For both methods, a good approximation between on-line FIA measurements and off-line reference methods was obtained.

4. Conclusions

A FIA method for the simultaneous on-line measurement of glucose and acetate was developed. Optimisation procedures allowed the elimination of major interferences by varying carrier and other reagents composition, while the developed sampling system permitted the analysis with minimised delays. Due the flexibility of both methods, it is possible, without a dilution step and, by only varying carriers compositions, to apply this system to a diversity of fermentation processes, where both the components can be present in a wide range of concentrations.

The low cost of reagents, together with the simplicity of the system, makes this technique an attractive alternative for the on-line monitoring of biotechnological processes, like the production of recombinant products with *E. coli*. Using this on-line information, it is possible to implement a model-based controller to regulate some of the relevant state variables during these fermentation processes. An adaptive controller was already developed to regulate acetate concentration using acetate measurements from FIA [30].

Acknowledgements

This work was supported by the PROTEXPRESS project (Agência de Inovação, Portugal). Fundação para a Ciência e a Tecnologia provided the financial support for I. Rocha through a doctoral research grant (PRAXIS XXI/BD/16961/98). The authors express their gratitude to Dr. J. Catita (Paralab, SA) for assistance in establishing the FIA system.

References

[1] J. Ruzicka, E.H. Hansen, Flow Injection Analysis, 2nd Edition, Wiley, New York, 1987.
[2] B. Sonnleitner, in: T. Scheper (Ed.), Advances in Biochemical Engineering/Biotechnology, Vol. 66, Springer, Berlin, 1999, pp. 1–64.
[3] J. Bradley, W. Stöcklein, R.D. Schmid, Process Control Qual. 1 (1991) 157.
[4] K. Schügerl, B. Hitzmann, H. Jurgens, T. Kullick, R. Ulber, B. Weigal, Trends Biotechnol. 14 (1996) 21.
[5] J. Ruzicka, Anal. Chim. Acta 261 (1992) 3.
[6] A. Benito, F. Valero, J. Lafuente, M. Vidal, J. Cairó, C. Solá, A. Villaverde, Enzyme Microb. Technol. 15 (1993) 66.
[7] J. Cairó, M. Vidal, A. Villaverde, F. Valero, J. Lafuente, C. Solá, Biotechnol. Tech. 5 (1991) 389.
[8] T. Ding, U. Bilitewski, R.D. Schmid, D.J. Korz, E.A. Sanders, J. Biotechnol. 27 (1993) 143.
[9] F. Valero, J. Lafuente, C. Solá, A. Benito, M. Vidal, J. Cairó, A. Villaverde, Biotechnol. Tech. 6 (1992) 213.
[10] D. Riesenberg, K. Menzel, V. Schulz, K. Schumann, G. Veith, G. Zuber, W.A. Knorre, Appl. Microbiol. Biotechnol. 34 (1990) 77.
[11] S.A. Rothen, M. Sauer, B. Sonnleitner, B. Witholt, Biotechnol. Bioeng. 58 (1998) 92.
[12] K. Han, H.C. Lim, J. Hong, Biotechnol. Bioeng. 39 (1992) 663.
[13] M. Walle, J. Shiloach, Biotechnol. Bioeng. 57 (1998) 71.
[14] U. Bilitewski, W. Drewes, J. Neermann, J. Schrader, R. Surkow, R.D. Schmid, J. Bradley, J. Biotechnol. 31 (1993) 257.
[15] J. Nielsen, K. Nikolajsen, S. Benthin, J. Viladsen, Anal. Chim. Acta 237 (1990) 365.
[16] L.H. Christensen, J. Nielsen, J. Viladsen, Anal. Chim. Acta 249 (1991) 123.
[17] F. Valero, J. Lafuente, M. Poch, C. Solá, A.N. Araújo, J.L. Lima, Biotechnol. Bioeng. 36 (1990) 647.

[18] S. Chung, X. Wen, K. Vilholm, M. Bang, G. Christian, J. Ruzicka, Anal. Chim. Acta 249 (1991) 77.
[19] T. Becker, R. Kittsteiner-Eberle, T. Luck, H.L. Schmidt, J. Biotechnol. 31 (1993) 267.
[20] X.-J. Tang, A. Tocaj, O. Holst, G. Johansson, Biotechnol. Tech. 11 (1997) 683.
[21] M. Tservistas, B. Weigel, K. Schügerl, Anal. Chim. Acta 316 (1995) 117.
[22] L.W. Forman, B.D. Thomas, F.S. Jacobson, Anal. Chim. Acta 249 (1991) 101.
[23] L.H. Christensen, J. Marcher, U. Schulze, M. Carlsen, R.W. Min, J. Nielsen, J. Viladsen, Biotechnol. Bioeng. 52 (1999) 237.
[24] K. Schügerl, J. Biotechnol. 31 (1993) 241.
[25] Y.L. Huang, T.J. Foellmer, K.C. Ang, S.B. Khoo, M.S.G. Yap, Anal. Chim. Acta. 317 (1995) 223.
[26] M. Garn, M. Gisin, C. Thommen, P. Cevey, Biotechnol. Bioeng. 34 (1989) 423.
[27] I. Rocha, E.C. Ferreira, in: D. Dochain, M. Perrier (Eds.), Proceedings of the Eighth International Conference on Computer Applications in Biotechnology, Que., Canada, 2001, pp. 353–358.
[28] G.L. Miller, Anal. Chem. 31 (1959) 426.
[29] L. Clesceri, A. Greenberg, A. Eaton (Eds.), Standard Methods for the Examination of Water and Wastewater, 20th Edition, American Public Health Association, Washington, DC, 1999.
[30] I. Rocha, E.C. Ferreira, in: Proceedings of the 15th IFAC World Congress on Automatic Control, Barcelona, 2002, accepted for publication and oral presentation.
[31] Ismatec, SA, ASIA Methods—Glucose, Ismatec, 1997.