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Sequential injection analysis system for on-line monitoring of L-cysteine concentration in biological processes

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Abstract

A sequential injection analysis (SIA) system was developed to monitor the concentration of L-cysteine in biological processes on-line. It is based on the redox reaction of L-cysteine with iron(III) in the presence of 1,10-phenanthroline (phen) and the detection of the red-iron(II)–phen complex with a spectrophotometry. The system was fully automated using software written in the LabVIEW^{TM} development environment. A number of system variables such as the flow rate of the carrier buffer solution, the volume ratio of the sample to the reagents, and the reaction coil length, etc., were evaluated to increase the sensitivity and performance of the SIA system. Under partially optimized operating conditions the performance of the SIA system was linear up to a concentration of L-cysteine of 1 mM ($R^2 = 0.998$) with a detection limit of 0.005 mM and a sample frequency of 15 hr^{-1}. The SIA system was employed to monitor the concentration of L-cysteine on-line in a continuously stirred reactor and a fermentation process of *Saccharomyces cerevisiae*. The on-line monitored data were in good agreement with the off-line data measured by a HPLC with a fluorescence detector ($n = 15$, $R^2 = 09899$).

Keywords: Biological process; L-Cysteine; Monitoring; Sequential injection analysis; *Saccharomyces cerevisiae*

1. Introduction

L-Cysteine is an amino acid containing a thiol group, which plays an important role in the metabolism of living cells. It participates in many biochemical processes, e.g. in the synthesis of tripeptides such as glutathione [1]. In several studies [2–4], the production of glutathione by microorganisms was reported to be influenced by the concentration of L-cysteine. Therefore, it is important to monitor and control the concentration of L-cysteine in fermentation processes used for the production of glutathione.

Traditionally, the concentration of L-cysteine has been determined by colorimetric methods [5], a high performance liquid chromatography (HPLC) using a photometric [6] and fluorometric [7] detector or electrochemical detection methods with mercury [8] or mercury amalgam electrodes [9]. However, the HPLC system is expensive and time consuming for use in a biological process, where the concentrations of L-cysteine change with time. Therefore, a selective, simple and rapid method is required for the on-line monitoring of L-cysteine in bioprocesses.

The flow injection analysis (FIA) technique has several distinct advantages in terms of its simplicity, cost performance, flexibility and rapidity. L-Cysteine has been determined using FIA systems with many different detection systems, e.g. photometric [10], chemiluminescent [11] and amperometric [12,13]. However, sequential injection analysis (SIA) system has several advantages over FIA, due to its versatility and reliability along with its low frequency of maintenance, as described in several reports [14–18]. This technique also consumes very little of the sample and

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reagent solution and has been used to on-line monitor the concentrations of glucose, penicillin, ethanol and glycerol in biological processes [19,20]. However, it has not previously been used to monitor the concentration of L-cysteine in a biological process. Therefore, the objective of this work is to develop an SIA system for the on-line monitoring of L-cysteine in biological processes, e.g. in a continuously stirred tank reactor or in a fermentation process for the production of glutathione by *Saccharomyces cerevisiae*. The SIA system described in this study is based on the redox reaction of L-cysteine with iron(III) in the presence of 1,10-phenanthroline, which produces a red-iron(II)–phen complex [10].

2. Experimental

2.1. Reagents

All chemicals used in this study were of analytical reagent grade and purchased from Sigma Co. (Seoul, Korea) or Fluka Co. (Buchs, Switzerland). All solutions were prepared in deionized water. A stock solution of iron(III) (50 mM) was prepared by dissolving 2.703 g of ferric chloride hexahydrate in 200 ml of hydrochloric acid (10 mM). A stock solution of copper(II) (50 mM) was prepared by dissolving 2.5 g of copper(II) sulfate pentahydrate in 200 ml of hydrochloric acid (10 mM). The solutions of copper(II) and iron(III) were mixed in a volume ratio of 1:2 and used as reagent 1. A phenanthroline (phen) solution (50 mM) was prepared by dissolving 1.0 g of 1,10-phenanthroline monohydrate in 100 ml of hydrochloric acid (10 mM) and used as reagent 2. The stock solutions were stored in the dark at 4 °C. An L-cysteine solution (10 mM) was freshly prepared by dissolving 1.2116 g of L-cysteine in deionized water and diluting it to 1.0 l. Working solutions of L-cysteine, copper(II), iron(III) and phen were prepared by suitable dilution from the stock solutions with deionized water. An acetate buffer solution (100 mM) was prepared at pH 4.8 and used as the carrier solution.

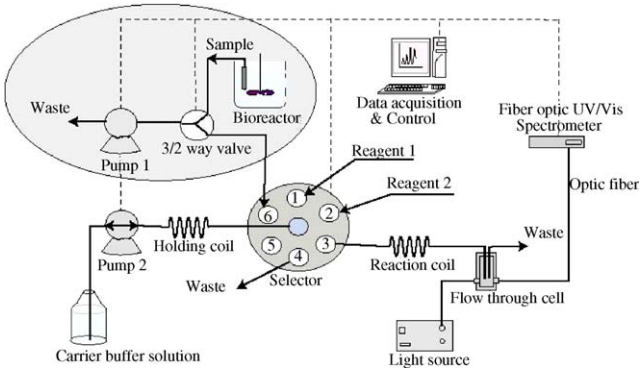

Fig. 1. Schematic diagram of a SIA system used to monitor the concentration of L-cysteine.

2.2. The SIA system

Fig. 1 shows the SIA system used to monitor the concentration of L-cysteine. It consists of a Minipuls peristaltic pump (Gilson Co., Villiers-le-Bel, France), a 6 port electrical selection valve (Knauer GmbH, Berlin, Germany) and a fiber optic spectrophotometer (Model S2000; Ocean Optics Inc., Florida, USA) connected to a flow-through cell (with an inner volume of 25 μl and an optical path length of 10 mm, Hellma GmbH, Muelheim, Germany) and a deuterium-tungsten lamp (DH-2000, Ocean Optics Inc.) by two optic fibers (2 m in length with a fiber diameter of 200 μm, Ocean Optics Inc.). The absorbance of the red-iron(II)–phen complex solution was measured at 510 nm. The control of the system was accomplished by means of a PC equipped with a model PCI-6024E A/D interface board (National Instruments Co.). The software used for the data acquisition and system control was written using the graphical programming language, LabVIEW™ (version 6.1, National Instruments Co., Texas, USA). The operating sequence of the SIA system is described in Table 1.

2.3. Biological process

In this study L-cysteine is used as one of the precursors for the biosynthesis of glutathione during the fermentation

Table 1
Operating sequence of the SIA system for one cycle

Time (s)	Pump 1	Valve	Description
0	Off	Reagent 2 (phen)	Select reagent 2 stream (valve position 2)
1	Reverse		Draw up reagent 2 solution
3	Off		Pump stop
4		Sample (L-cys)	Select sample stream (valve position 6)
5	Reverse		Draw up sample solution
13	Off		Pump stop
14		Reagent 1 (Fe + Cu)	Select reagent 1 stream (valve position 1)
15	Reverse		Draw up reagent 1 solution
17	Off		Pump stop
18		Detector	Select detector line (valve position 3)
19	Forward		Pump stack of zones to detector
199	Off		Pump stop
200		Home	Return valve to starting position (valve position 2)

of *S. cerevisiae* [1]. In this work *S. cerevisiae* ATCC7754 (American Type Cell Collections, USA) was employed to produce glutathione in a 7.5 l bioreactor (KoBiotech Co., Incheon, Korea) with a working volume of 5.0 l. The fermentation medium consisted of 10.0 g l^{-1} glucose, 2.4 g l^{-1} KCl, 0.012 g l^{-1} NaCl, 6.0 g l^{-1} $(NH_4)_2SO_4$, 3.0 g l^{-1} KH_2PO_4, 2.4 g l^{-1} $MgSO_4{\cdot}7H_2O$, 1.0 v/v% trace elements solution (0.12 g l^{-1} $CaCl_2{\cdot}2H_2O$, 0.12 g l^{-1} $ZnSO_4{\cdot}7H_2O$, 0.024 g l^{-1} $MnSO_4{\cdot}5H_2O$. 0.006 g l^{-1} $CuSO_4{\cdot}5H_2O$), and 0.3 v/v% vitamin stock solution (0.002 g l^{-1} folic acid, 0.2 g l^{-1} riboflavin, 0.4 g l^{-1} thiamine–HCl, 0.4 g l^{-1} nicotinic acid, 0.4 g l^{-1} pyridoxine–HCl, 2.0 g l^{-1} Ca–panthothenate, 0.02 g l^{-1} biotin, 10.0 g l^{-1} inositol and 0.2 g l^{-1} *p*-aminobenzoic acid) [4]. Two precursors, viz. 7 g l^{-1} glycine and 7 g l^{-1} glutamic acid were also added to the medium for the biosynthesis of glutathione in the cells. The pH value of the medium was adjusted to 4.5 by adding 1 M HCl or 14 wt.% NH_4OH solution, as necessary. A 0.2 μm polypropylene microfiltration tubular on-line sampling module (ABC Co., Puchheim, Germany) was used to take samples of cell free medium [21]. Before applying the SIA system to a real biological process, the concentration of L-cysteine in a continuously stirred reactor was on-line monitored by the SIA system.

3. Results and discussion

3.1. Optimization of the SIA system

The optimal conditions of the SIA system for the monitoring of L-cysteine were investigated by studying the influence of various parameters on its sensitivity and precision. Prior to the univariate optimization the following reference operating conditions were used in the concentration range of L-cysteine of 0.0–2.0 mM: a buffer flow rate of 0.6 ml min^{-1}, a reaction coil length of 103 cm (i.d. 0.75 mm), concentrations of copper(II), iron(III) and phen of 25 mM, an equal volume ratio of reagent 1 (Cu(II) and Fe(III)) and reagent 2 (phen), a volume ratio of the sample to the reagents (reagent 1 and reagent 2) of 2:1, and a total volume of sample and reagents of 150 μl. A number of parameters were optimized by measuring their relative peak heights, i.e. subtracting the absorbance of the samples from the absorbance measured for a blank solution (deionized water). The measurement for each sample was repeated at least four times.

The formation of iron(II)–phen complex is mainly influenced by the zone penetration time and the reaction among L-cysteine, copper and iron(III) in the presence of phen. The zone penetration time depends upon the flow rate of the buffer solution. Therefore, the buffer flow rate was varied from 0.2 to 1.0 ml min^{-1}, while the total volume of sample and reagents and the volume ratio of sample to reagents were kept constant by varying the draw-up time of each solution accordingly. This resulted in a lower sampling rate for lower flow rates and a higher sampling rate for higher flow rates. In Fig. 2, the absorbance decreased with increasing flow rate, due to the smaller zone penetration time between the sample and reagents and the reduction of the reaction time. In the range of 0.5–0.8 ml min^{-1} there was little difference in either the peak heights or in the change of the slope of linear calibration curves between 0.0 and 1.0 mM ($R^2 > 0.990$). Therefore, as a compromise between the sensitivity and sampling rate, a flow rate of 0.6 ml min^{-1} with a sampling rate of 15 h^{-1} was used in the remainder of this work.

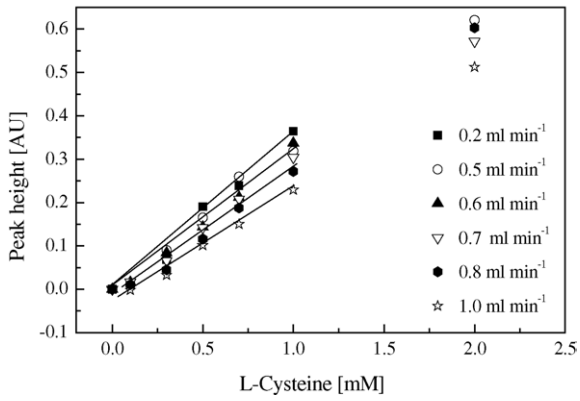

Fig. 2. Effect of the buffer flow rate on the peak height.

While the total volume of the sample and reagents, i.e. reagent 1 and reagent 2 was kept at 150 μl, the volume ratio of sample to reagents, i.e. ratio of sample volume to reagents volume was varied between 1:5 (25 μl (sample):125 μl (62.5 μl (reagent 1) + 62.5 μl (reagent 2))) and 5:1 (125 μl:25 μl (12.5 μl + 12.5 μl)). In Fig. 3(a) the peak height decreased as the volume ratio of sample to reagents was increased from 1:1 to 1:5 in the linear L-cysteine concentration range of 0.0–1.0 mM ($R^2 > 0.990$). In Fig. 3(b) the peak height increased with increasing volume ratio of the sample to reagents (from 1:1 to 4:1), but the linear concentration ranges of L-cysteine with the volume ratio of the sample

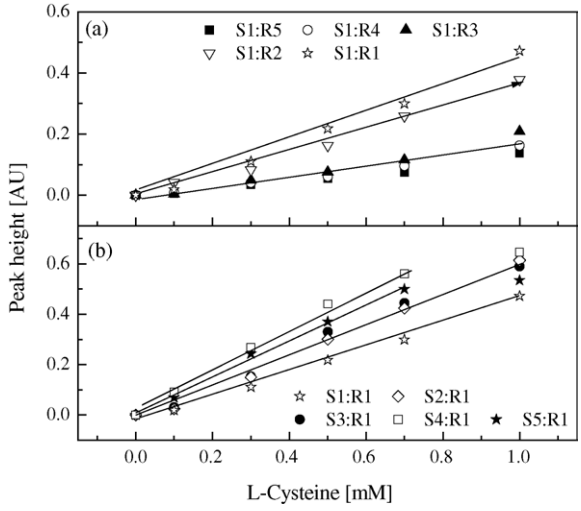

Fig. 3. Effect of the volume ratio of sample to reagents on the peak height.

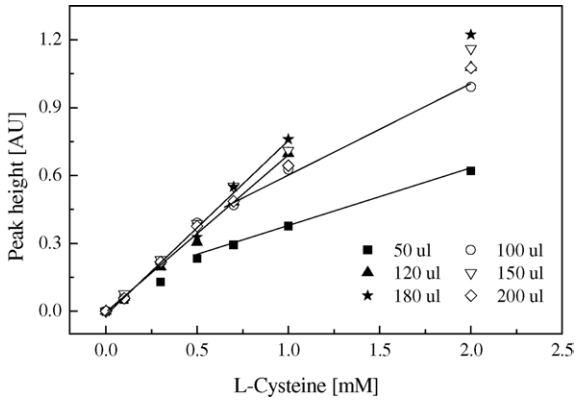

Fig. 4. Effect of the total volume of sample and reagents on the peak height.

to reagents of 4:1 and 5:1 were only between 0.0 and 0.7 mM ($R^2 > 0.990$). From Fig. 3, a volume ratio of 2:1 was considered to provide high sensitivity and good performance and was therefore used in all further experiments.

The total volume of the sample and reagents was optimized in the range of 50–200 μl, while the volume ratio of sample to reagents, i.e. 2:1 was kept constant. In Fig. 4, the peak height increased with increasing total volume up to 180 μl in the concentration range of L-cysteine of 0.1–2.0 mM. A linear calibration graph was obtained in the concentration range of L-cysteine of 0.5–2.0 mM ($R^2 > 0.990$) with total volumes of 50 and 100 μl, and in the concentration range of L-cysteine of 0.0 to 1.0 mM L-cysteine ($R^2 > 0.990$) with total volumes of 120, 150 and 180 μl. When the total volume was greater than 120 μl, the sensitivity (slope of the peak heights) no longer increased significantly, and so the total volume of 120 μl was taken as the optimum value.

In this work, equal volumes of the two reagents were separately injected into the holding coil of the SIA system. The optimal concentrations of Cu(II), Fe(III) and phen were investigated in the concentration ranges of 10–30 mM with steps of 5 mM. In the case where the concentrations of all three components were varied between 10 and 30 mM, the peak heights increased as the concentrations of reagents were increased up to 25 mM and the highest sensitivity was obtained in the linear concentration range of L-cysteine of 0.0–1.0 mM. Table 2 shows the effect of varying the concentrations of Cu(II), Fe(III) and phen on the change in the peak heights at a concentration of L-cysteine of 0.5 mM, when the concentration of one of the three components was varied and those of the other two components were kept constant at 25 mM. The concentrations of Cu(II) and Fe(III) did not significantly affect the peak heights of the system, however the peak height increased as the concentration of phen was increased up to 25 mM. Therefore, a concentration of 25 mM was used for each of the three components in all further experiments.

Table 2
Effects of the concentrations of Cu(II), Fe(III) and phen on the peak heights at a concentration of L-cysteine of 0.5 mM

Cu(II) (mM)	Absorbance	Fe(III) (mM)	Absorbance	Phen (mM)	Absorbance
10	0.525	10	0.515	10	0.444
15	0.531	15	0.518	15	0.505
20	0.589	20	0.512	20	0.571
25	0.602	25	0.602	25	0.602
30	0.543	30	0.531	30	0.603

The concentrations of the other two components were both kept at 25 mM.

The dimensions of the reaction coil length affect the degree of zone penetration and the reaction. In this work, the effect of varying the length of the reaction coil on the peak height was studied with lengths of 15, 59, 103, and 147 cm, for a constant i.d. of 0.75 mm. For each reaction coil length, the peak height increased with increasing concentration of L-cysteine. That is, the reaction did not have sufficient time to reach completion with the reaction coils used in this work. It was also found that the peak height did not increase significantly in the concentration range of L-cysteine between 0.0 and 1.0 mM ($R^2 > 0.995$), when the reaction coil length was increased from 15 to 147 cm. Therefore, a reaction coil length of 59 cm was chosen as the optimum value in our further experiments.

From our prestudy, it was also found that the holding coil length did not have a significant influence on the sensitivity either, so that a value of 103 cm (i.d. 0.75 mm) was used in this work.

The proposed SIA system was evaluated with regard to its accuracy, precision, detection limit, linear range, etc. The linear calibration graph was obtained in the concentration range of L-cysteine from 0.0 to 1.0 mM (Peak height = 0.5596 × [L-cysteine]−0.00664; $R^2 = 0.998$, $n = 30$), under optimum conditions, i.e. a flow rate of 0.6 ml/min, a reaction coil length of 103 cm, concentrations of copper(II), iron(III) and phen of 25 mM, a volume ratio of sample to reagents (reagent 1 and reagent 2) of 2:1 and a total volume of sample and reagents of 120 μl. The detection limit gives an indication of the lowest L-cysteine concentration that can be distinguished from the background signal with 99% certainty [14]. The value of the detection limit was found to be 0.005 mM, which indicated that the SIA system functions well above the detection limit. A sampling rate of 15 samples per hour was obtained.

3.2. *On-line monitoring of* L-*cysteine in biological processes*

In the study of a biological process that consumes L-cysteine, various kinds of salts, metabolites and substrates were included in the sample, and these reacted with the reagents in the SIA system. These components either activate or inhibit the redox reaction. The effects of some of these components (nutrient salts, substrates and metabolites) on the peak heights of the SIA system were investigated in the case of an L-cysteine concentration of 0.5 mM, and the results are presented in Table 3. The kinds and concentrations of the nutrient salts and substrates studied here were based on the maximum amounts present in the culture medium of certain fermentation processes by *S. cerevisiae* [4]. In Table 3,

Table 3
Effects of various components (nutrient salts, substrates and metabolites) contained in the sample of L-cysteine with a concentration of 0.5 mM on the peak height

Components added to sample	Relative peak height (%)	Components added to sample	Relative peak height (%)
None	100	6.0 g l^{-1} $(NH_4)_2SO_4$	114.5 ± 3
0.012 g l^{-1} NaCl	100.5 ± 4	7.0 g l^{-1} glycine	59.9 ± 5
3.0 g l^{-1} KH_2PO_4	77.1 ± 3	7.0 g l^{-1} glutamic acid	126.5 ± 4
2.4 g l^{-1} $MgSO_4 \cdot 7H_2O$	92.6 ± 5	3.0 g l^{-1} malt extract	102.7 ± 3
2.4 g l^{-1} KCl	93.3 ± 3	3.0 g l^{-1} yeast	100.7 ± 2
10.0 g l^{-1} glucose	96.6 ± 2	5.0 g l^{-1} tryptone	90.8 ± 3
1% (v/v) trace elements solution	116.4 ± 5	0.3 (v/v)% vitamin stock solution	99.5 ± 2
0.47 g l^{-1} succinic acid	103.5 ± 4	0.38 g l^{-1} acetaldehyde	46.3 ± 4
10.7 g l^{-1} glycerol	98.7 ± 2	1.8 g l^{-1} acetic acid	99.5 ± 2
1.95 g l^{-1} fructose	107.1 ± 4	10.7 g l^{-1} ethanol	94.3 ± 5
		All compounds	102.05 ± 5

The peak height of 0.5 mM L-cysteine without the addition of any nutrient salts, substrates, or metabolites was set to 100% as the reference value. "All compounds" refers to all of the nutrient salts, substrates, and metabolites listed in the table.

some of the components that were added to the sample, e.g. 0.38 g l^{-1} acetaldehyde, 3.0 g l^{-1} KH_2PO_4 and 7.0 g l^{-1} glycine, caused a considerable decrease in the peak height of more than 20% of the reference value. This large inhibitory effect may result from the change in the pH of the sample or the broadening of the peak. This effect can be overcome by controlling the pH value in the sample and/or using the peak area for the calibration of the L-cysteine concentration. However, other components, such as the 1% trace element solutions, 7.0 g l^{-1} glutamic acid and 6.0 g l^{-1} $(NH_4)_2SO_4$ increased the peak height more than 10% of the reference value. When all of the compounds, i.e. salts, substrates and metabolites, listed in Table 3 had been added to the sample solution (0.5 mM, L-cysteine), there was little overall change in the peak height, i.e. no significant activating or inhibitory effect on the SIA system.

The inhibitory or activating effects of sample matrices therefore have to be taken into consideration for the on-line monitoring of L-cysteine concentration in a biological process. They can be overcome by mixing the L-cysteine standard solution with some of the substances present in real processes and using this for the calibration of the SIA system.

The on-line monitoring of the L-cysteine concentration in a simulated process (continuously stirred tank reactor with influent water and effluent rates of 10.0 ml min^{-1}) was carried out to test the performance of the SIA system. At the beginning, 5 ml of a 10.0 mM L-cysteine solution was quickly injected into the reactor with a working volume of 500 ml containing distilled water. The concentration of L-cysteine in the effluent from the reactor was on-line monitored using the SIA system under the optimized operating conditions. After 6 h, the concentration of L-cysteine in the reactor was 0 and 4.5 ml of a 10.0 mM L-cysteine solution was reinjected into the reactor.

Fig. 5 shows the results of the on-line monitoring and a comparison of these results with the off-line data measured by HPLC with OPA precolumn derivatization (Shimadzu RF-$10A_{XL}$ fluorescence detector at 370 nm(ex)/420 nm(em), a sample injection volume of 10 μl, gradient supply of two carrier solutions with a flow rate of 1.0 ml min^{-1} and a

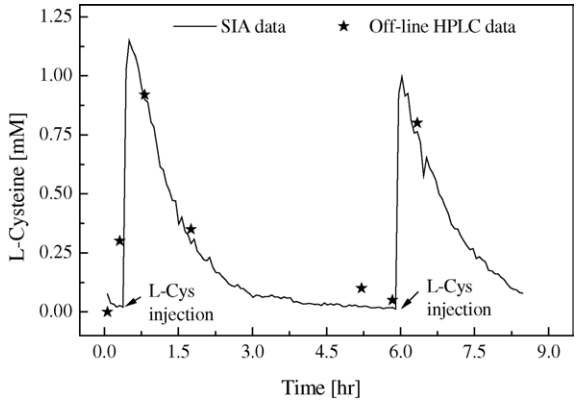

Fig. 5. On-line monitoring of L-cysteine concentration in a simulated process. The points correspond to the off-line analysis of L-cysteine with HPLC.

Resolve™ C18 column (with a pore size of 90 Å, a particle diameter of 5 μm at 60 °C). There were no significant differences between the data measured on-line and off-line.

During the fermentation of *S. cerevisiae* ATCC7754, the concentration of L-cysteine was monitored on-lines, and the results are shown in Fig. 6. Various compounds in the fer-

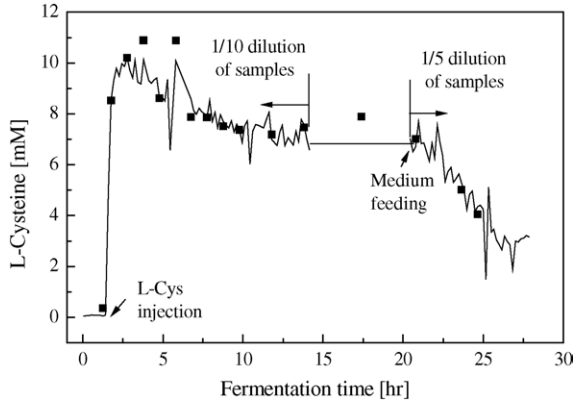

Fig. 6. On-line monitoring of L-cysteine concentration during the fermentation of *S. cerevisiae*. The points correspond to the off-line analysis of L-cysteine with HPLC.

mentation medium either activated or inhibited the redox reaction of L-cysteine, as shown in Table 3. Consequently, a standard solution was prepared by dissolving L-cysteine in the fermentation medium, and this was then used to calibrate the peak heights of the SIA system. After 12 h of the fermentation, the operation of the SIA system under optimum conditions was started, in order to monitor the concentration of L-cysteine. After 2 h of the SIA operation, 50 ml of concentrated L-cysteine solution (100 mM) was added to the bioreactor and monitored continuously. After the injection of the L-cysteine solution, the samples taken for the SIA system were diluted 1:10 with deionized water. Between 13 and 20 h, the SIA system was washed and calibrated with standard L-cysteine solution. At 20 h, 500 ml of fermentation salts medium was added to the reactor and the samples taken for the SIA system were diluted 1:5 with deionized water and monitored on-line. The on-line monitored SIA data were in good agreement with the off-line data of L-cysteine measured by the HPLC system well ($n = 15$, $R^2 = 0.9899$). The linear calibration curve for the standard solution of L-cysteine obtained by the HPLC had a correlation coefficient of $R^2 = 0.995$ (7 data points) over the range of L-cysteine concentration of 0–1.2 mM.

4. Conclusion

Sequential injection analysis is simple, robust, and cost-effective. An SIA system was developed for the monitoring of L-cysteine, based on the reaction of L-cysteine with copper, iron, and phen. The calibration graph was linear in the L-cysteine concentration range from 0.1 to 1.0 mM with a detection limit of 0.005 mM. The SIA system was employed to monitor the concentration of L-cysteine in a biological process, viz. in a fermentation process by *S. cerevisiae*, at a sample rate of 15 h^{-1}. The good agreement obtained between the on-line monitoring data and off-line measurement data showed the good reproducibility and sensitivity of the SIA system.

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