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Determination of amino acids using *o*-phthalaldehyde–2-mercaptoethanol derivatization Effect of reaction conditions

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Abstract

This paper presents a systematic approach to determine the optimal reaction conditions, with respect to accuracy and sensitivity, of the quantitative determination of primary amino acids in a single run, using *o*-phthalaldehyde–2-mercaptoethanol derivatization. To this end an experiment was designed in which the effects of reaction time, concentration of 2-mercaptoethanol and type of solvent were determined simultaneously. The response of all parameters tested was found to be interrelated: the effect of a change in one reaction condition also depended on the other reaction conditions. The reaction conditions determined in this research resulted in an accuracy better than 0.25 μM, an average reproducibility of 0.6% and an average sensitivity of 136 fmol.

Keywords: Derivatization; Amino acids; *o*-Phthalaldehyde; 2-Mercaptoethanol

1. Introduction

In animal cell cultivations it is common practice to analyze large amounts of interrelated samples over long periods of time. Since trend analysis is very important, the method used for analysis has to give highly reproducible results during several days. Moreover, the method should be able to determine all primary amino acids in a single run and should be accurate over a wide concentration range. This excludes optimization methods that focus on single amino acids.

The derivatization of amino acids using *o*-phthalaldehyde (OPA) is the most frequently applied HPLC method for the quantitative determination of primary amino acids. This method was introduced by Roth [1] in 1971. Since then, many modifications have been proposed. These involved the composition of the mobile phase consisting of either methanol [2–6] or acetonitrile [7–10], different combinations of OPA with 2-mercaptoethanol [1,3–6,8,10,11], ethanethiol [5,9] or 3-mercaptopropionic acid [2,7], and either pre-column [2–10] or post-column [11] derivatization. In addition, a wide range of reaction conditions has been applied with respect to the amount of 2-mercaptoethanol (0.8–2.5 μl/mg OPA

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[5–8,10,11,13]), reaction time (0–3 min [3–6,8,10,13,14]) and the solvent used [dilution of the samples with water [3,7–10], borate buffer [10,13] or acid (commercial standards)]. This short summary of applied derivatization conditions indicates that it is still unclear what the optimal conditions are for this derivatization method. In spite of what might be expected, only little research has been done to establish these optimal conditions [1,12,15].

The reason for the wide range of derivatization conditions used, can be found in the possible reactions of OPA with amino acids and 2-mercaptoethanol. Fig. 1 shows a simplified reaction mechanism for the three possible reactions that can occur: hydrolysis of OPA, stabilization of OPA by 2-mercaptoethanol and reaction of OPA with 2-mercaptoethanol and amino acids, producing fluorescing derivatives that spontaneously decompose. The complexity of this reaction mechanism makes it difficult to predict the optimal derivatization conditions. Three conditions can be considered crucial: the pH of the reaction mixture, the concentration of 2-mercaptoethanol and the reaction time. The pH is important since the reaction rates may depend on the pH. Since the pH-dependency of each reaction may

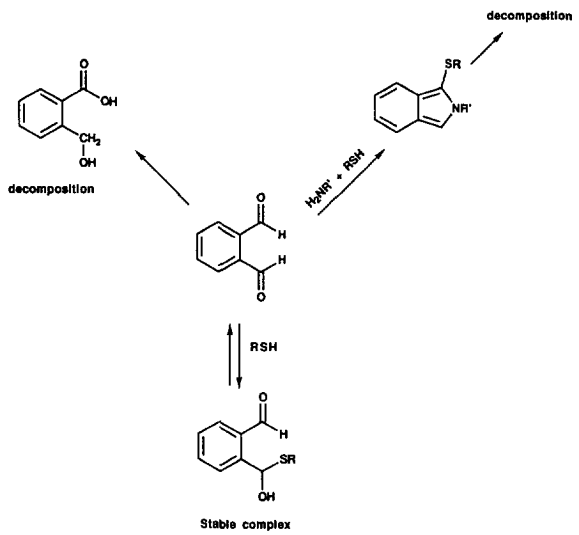

Fig. 1. Simplified reaction scheme of the possible reactions of *o*-phthalaldehyde (OPA) with amino acids (H_2NR') and 2-mercaptoethanol (RSH): OPA hydrolyzes, it is stabilized using 2-mercaptoethanol and it reacts with 2-mercaptoethanol and amino acids, producing fluorescing derivatives that spontaneously decompose.

be different, it can be expected that there is an optimal pH at which the production rate of the fluorescing derivatives is much higher than its decomposition rate. The 2-mercaptoethanol concentration determines the stability of the OPA–2-mercaptoethanol complex. Since this is an equilibrium reaction, addition of 2-mercaptoethanol results in a reduction of the amount of OPA that is directly available for the reaction with amino acids. Finally, the reaction time is important to allow the reaction of OPA with amino acids to complete.

This paper presents a method to determine the settings for the OPA derivatization that give an optimal average response for all important amino acids. To this end an experimental set-up was designed in which the interaction was determined between the accuracy and the sensitivity of the analysis method and the reaction time, the 2-mercaptoethanol concentration and the pH. All three parameters were found to be essential in the optimization of the OPA method.

2. Experimental

2.1. Equipment

The HPLC system used was a Waters system (Waters, Division of Millipore, Milford, MA, USA) consisting of a Model 600E system controller with a single pump gradient system, a Model 715 Ultra WISP sample processor and a Model 470 fluorescence detector. The detector was operated using an excitation wavelength of 335 nm and an emission wavelength of 425 nm. The column was kept at 35°C using a Model CHM column heater module and a Model TCM temperature control module. The system was coupled to a NEC Powermate 80386SX 16 MHz computer fitted with Maxima 820 Chromatography Workstation software, version 3.30 (Millipore).

A flow-rate of 1 ml/min was used throughout all experiments. To determine the response, the peak area was used.

2.2. Column

The amino acids were separated using a 150×3.9 mm I.D. 85711 Waters Resolve 5 μm spherical C_{18}

column, protected by a Waters 15220 Nova-Pak 4 μm spherical C_{18} guard-column.

2.3. Reagents

LiChrosolv-quality methanol, 2-mercaptoethanol and *o*-phthalaldehyde were all purchased from Merck (Darmstadt, Germany). Borate buffer (0.4 *M*) and a standard solution of amino acids containing Asp, Glu, Ser, Gly, Arg, Ala, Tyr, Met, Val, Phe, Ile, Leu, and Lys were purchased from Hewlett-Packard (Waldbrunn, Germany). Individual amino acids were purchased from Sigma (St. Louis, MO, USA). A Milli-Q water purification system (Millipore) was used to obtain HPLC-grade water.

2.4. Sample preparation

2.4.1. Preparation of the OPA derivatization solution

To 100 ml borate buffer, 500 mg OPA and the required amount of 2-mercaptoethanol were added. The mixture was allowed to dissolve overnight, after which it was filtered through a 0.45-μm membrane filter. The OPA solution was stored in the dark at 4°C.

2.4.2. Preparation of the mobile phase

The mobile phase consisted of 32% (v/v) methanol and 20 mmol/l phosphate, dissolved in Milli-Q water (pH 6.8).

2.4.3. Preparation of the samples

A mixture containing 20 μmol/ml of Asp, Ser, Gly and Ala dissolved in 0.1 *M* H_2SO_4, was diluted with either borate buffer, Milli-Q water or 0.1 *M* H_2SO_4 until the required concentration was reached. From these samples 15 μl was injected onto the column, together with 30 μl OPA reagent using the auto-addition method of the WISP. This auto-addition method involves: rinsing the needle; acquiring OPA-reagent in the needle; rinsing the outside of the needle; acquiring sample in needle; injection of mixture onto the column.

2.4.4. Choice of experimental conditions

It was our intention to determine the optimal settings for the OPA derivatization during a routine analysis run of samples containing primary amino acids. This means that not the response of a single amino acid is important, but the average response of all primary amino acids that are to be determined. This paper presents the results of an experiment in which three reaction conditions have been varied. Since the effect of varying one reaction condition can depend on the other reaction conditions, the experiment has been set up in matrix form. This results in 36 different combinations of the reaction conditions to be tested. Therefore, if the combined effect of the amount of 2-mercaptoethanol, the reaction time and the pH is to be determined, this will result in extensive experimental work. Side effects like decomposition of OPA in the stock solution would probably disturb these experiments because of this long run time. Therefore, an alternative method was applied, using samples containing only Asp, Ser, Gly and Ala in a range from 1 to 40 μM. This significantly reduced the total analysis time from 1 h to 9 min. Since the overall time required for these experiments could now be limited to days rather than weeks, both the OPA solution and the mobile phase could be withdrawn from one stock solution, ensuring identical conditions throughout the experiments.

3. Results and discussion

3.1. Selection of amino acids

An important demand for this experimental set-up is that the average behaviour of the four selected amino acids is comparable to the average behaviour of a sample containing most amino acids to be determined. Fig. 2 shows that this is indeed the case. In this figure, the effect of reaction time (bars), amount of 2-mercaptoethanol (x-axis) and sample volume (three different sections in the figure) on the R.S.D. (y-axis) is displayed for the four selected amino acids (top section) and for a commercially available amino acid standard containing Asp, Glu, Ser, Gly, Arg, Ala, Tyr, Met, Val, Phe, Ile, Leu, and Lys (bottom section). Fast-degrading amino acids

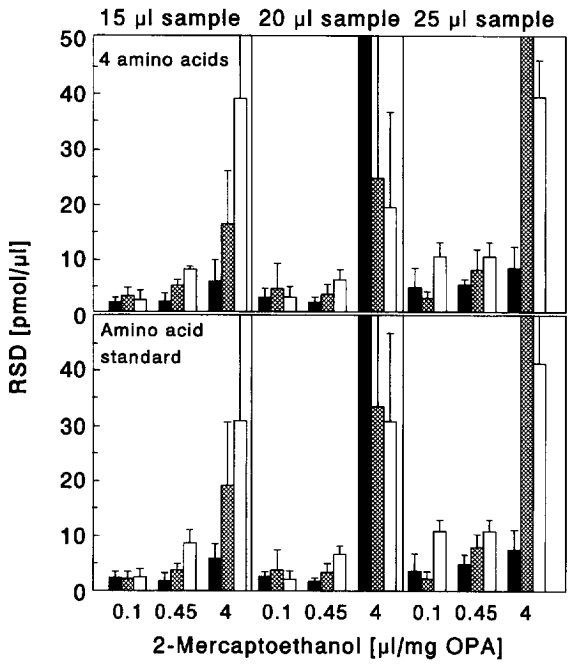

Fig. 2. Comparison of the average response of a sample containing Asp, Ser, Gly and Ala with the average response of a standard solution, containing Asp, Glu, Ser, Gly, Arg, Ala, Tyr, Met, Val, Phe, Ile, Leu and Lys. In both cases Milli-Q water was used as solvent. For both sets of amino acids, the effect of reaction time, 2-mercaptoethanol concentration and injection volume was determined on the R.S.D. Reaction times: black column, 2 min; cross-hatched column, 4 min; white column, 6 min.

like Gln were not included, since their response would disturb this experiment. To compare both sets of amino acids, the residual standard deviation (R.S.D.) was used. The R.S.D. is a measure for the accuracy of a calibration curve, and therefore for the absolute error of a measurement. It is determined from the absolute deviation between the true concentration of a known sample and the concentration obtained with the calibration curve, using Eq. 1:

$$\text{R.S.D.} = \sqrt{\frac{\text{residual sum of squares}}{\text{degrees of freedom}}}$$

$$= \text{S.D.}(y)\sqrt{\frac{n-1}{n-2}(1-r^2)} \qquad (1)$$

where S.D.(y) is the standard deviation of the y-value of the calibration curve, n is the number of measurement points, and r is the correlation coefficient. To determine the R.S.D., samples with amino acid concentrations of 10, 25, 100, 250 and 800 μM were analyzed in duplicate. In this case, Milli-Q water was used as solvent. Fig. 2 shows that the trend in R.S.D. of the average response of the four amino acids does not markedly differ from the trend in R.S.D. of the average response of the commercial standard containing 13 amino acids. Therefore, the mixture containing Asp, Ser, Gly and Ala can be used to determine the optimal settings for the complete set of amino acids.

3.2. Effect of 2-mercaptoethanol

In the literature it is stated that the amount of 2-mercaptoethanol does not markedly influence the fluorescence of alanine [1]. Because it is assumed that this is also true for the other amino acids, small amounts of 2-mercaptoethanol are added to the OPA reagent every few days to stabilize the OPA reagent [4,5,8,10]. This assumption was verified for a sample containing 25 μM of each of the four amino acids. To be sure the reaction of OPA with the amino acids was complete, a reaction time of 3 min was selected. Performing the experiments with borate buffer, Milli-Q water and 0.1 M H_2SO_4, the 2-mercaptoethanol concentration was varied in a range from 0.01 to 4.8 μl/mg OPA. These three solvents were selected for two reasons. Firstly, in the literature these three solvents are used most often. Secondly, applying these solvents results in a different pH of the complete reaction mixture. Thus the effect of the pH on the overall reaction of OPA with amino acids and 2-mercaptoethanol can be determined. Fig. 3 shows the effect of the 2-mercaptoethanol concentration on the normalized peak area of the individual amino acids. The normalization was achieved by defining the maximum peak area of the individual amino acids in the different solvents as 100%. Fig. 3 shows that the effect of the 2-mercaptoethanol concentration depends on the solvent used. Furthermore, Fig. 3 clearly shows that the response is different for each amino acid. However, for each amino acid the maximum peak area is reached at a low concen-

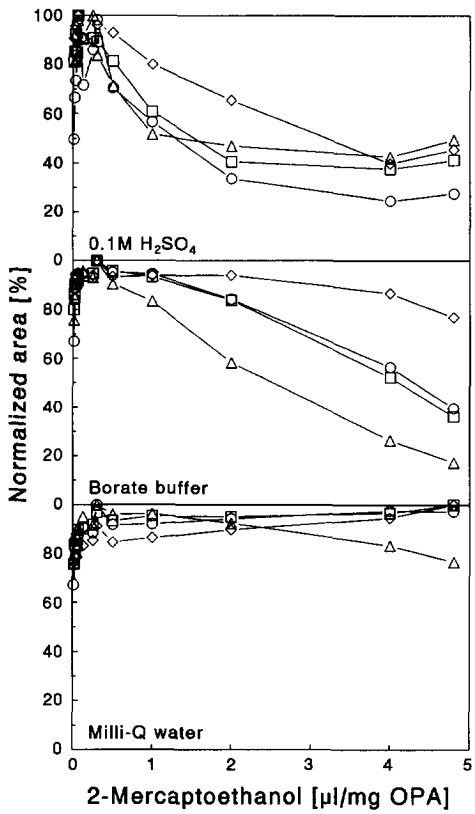

Fig. 3. Effect of the 2-mercaptoethanol concentration and the solvent on the fluorescence of the OPA-derivatives of Asp (△), Ser (○), Gly (◇) and Ala (□) at a reaction time of 3 min. The response has been normalized by defining the maximum peak area of the individual amino acids in the different solvents as 100%.

tration of 2-mercaptoethanol. The behaviour of the OPA derivatives can be explained by the stabilizing effect of 2-mercaptoethanol on OPA (Fig. 1). With a large excess of 2-mercaptoethanol the equilibrium resides at the stabile OPA–mercaptoethanol complex. If an amino acid is added to this complex, OPA is not eager to react with this amino acid. Therefore, with an increasing amount of 2-mercaptoethanol, OPA reacts more readily with 2-mercaptoethanol than with the amino acid. The amount of fluorescence will therefore decrease with increasing concentration 2-mercaptoethanol. From Fig. 3 three concentrations of 2-mercaptoethanol were selected to test the accuracy and the sensitivity of the analysis method: 0.14, 1.6 and 4.8 µl/mg OPA.

3.3. Determination of the sensitivity of the analysis method

Apart from the variation in the 2-mercaptoethanol concentration, also the reaction time and the solvent were varied to determine the sensitivity of the analysis method. For the experiments, reaction times of 0, 1, 3 and 9 min were tested, where 0 min stands for instant injection onto the column. Finally, borate buffer, Milli-Q water and 0.1 *M* H_2SO_4 were used as solvent. The pH values of the reaction mixtures are shown in Table 1. Fig. 4 shows the relative area of a sample containing 5 µ*M* of the four amino acids. This relative area is determined by defining the maximum peak area of each amino acid as 100%. The amino acids were not normalized per solvent since this would make comparison of the sensitivity between the different solvents impossible. To check the reproducibility, the experiments were performed in sixfold. The standard deviation of these samples is presented as error bars in Fig. 4. Fig. 4 clearly shows that the relative area depends on the solvent, the reaction time and the amount of 2-mercaptoethanol. The solvent affects the pH of the reaction mixture (Table 1), and therefore the reaction conditions. Fig. 4 shows that using a solvent with an increased pH results in an increase of the relative area. This is probably due to the increase in reaction rate of the reaction of OPA with amino acids. At a reaction time of 0 min, the reaction of OPA with amino acids in the presence of borate buffer is already completed, whereas in Milli-Q water and in acid the reaction still continues. Also the decomposition rate increases

Table 1
pH Values of the reaction mixtures using 0.1 *M* H_2SO_4, Milli-Q water and borate buffer as solvent

Solvent	Concentration 2-mercaptoethanol (µl/mg OPA)		
	0.14	1.6	4.8
Borate buffer	9.74	9.21	8.88
Milli-Q water	9.68	9.15	8.77
0.1 *M* H_2SO_4	8.32	8.17	8.02

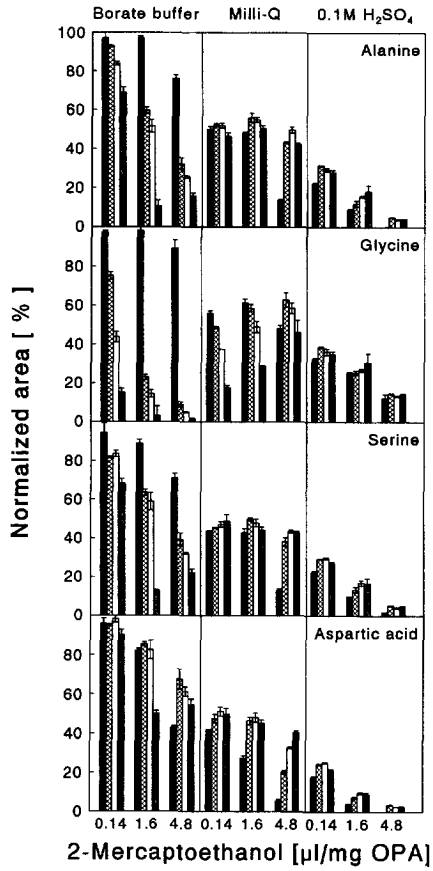

Fig. 4. Effect of reaction time, 2-mercaptoethanol concentration and solvent on the normalized area of Asp, Ser, Gly and Ala. The error bars show the standard deviation of each experiment ($n=6$). Reaction times: black column, 0 min; cross-hatched column, 1 min; white column, 3 min; hachured column, 9 min.

with increasing pH. This is best shown if the decrease in area with increasing reaction time in borate buffer is compared to the response in Milli-Q water and in acid. In borate buffer, a very rapid decrease of the peak area is observed with increasing reaction time. In 0.1 M H_2SO_4 the area is less dependent on the reaction time. Finally, the stabilization reaction of OPA with 2-mercaptoethanol depends on the pH and on the 2-mercaptoethanol concentration. At a high concentration 2-mercaptoethanol, less OPA is available to react with amino acids, causing the area to decrease. Also, with

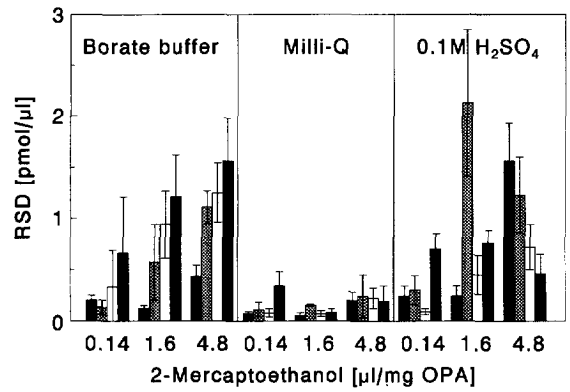

Fig. 5. Effect of reaction time, 2-mercaptoethanol concentration and solvent on the average residual standard deviation (R.S.D.) of Asp, Ser, Gly and Ala. The R.S.D. is obtained from a quadratic curve fit. Reaction times: black column, 0 min; cross-hatched column, 1 min; white column, 3 min; hachured column, 9 min.

decreasing pH, more OPA reacts with 2-mercaptoethanol. This also causes the area to decrease.

3.4. Determination of the accuracy of the analysis method

Fig. 5 shows the average R.S.D. of the four calibration curves as a function of the 2-mercaptoethanol concentration, the reaction time and the solvent. For this, the same conditions were used as in the previous experiment. The calibration curves were generated with a quadratic curve fit and were based on six different samples containing 1.25, 2.5, 5, 10, 20 and 40 μM of the different amino acids. All samples were analyzed in duplicate. To obtain a reliable calibration curve, the R.S.D. should be as small as possible. Fig. 5 shows that the smallest R.S.D. values are obtained using Milli-Q water as a solvent. In borate buffer, the R.S.D. increases with increasing reaction time and with increasing 2-mercaptoethanol concentration, whereas in Milli-Q water the R.S.D. is independent of reaction time. Also the 2-mercaptoethanol concentration has no significant effect on the R.S.D. In 0.1 M H_2SO_4 the effect on the R.S.D. is more complicated. The R.S.D. increases with increasing 2-mercaptoethanol concentration, but the effect of the reaction time also depends on this concentration. These effects can be explained look-

ing at Fig. 4, where it is shown that for a specific solvent, the R.S.D. increases with decreasing peak area. So, to obtain a high accuracy and sensitivity, the peak area should be as high as possible, but as independent as possible from the reaction time, the 2-mercaptoethanol concentration and the solvent.

3.5. Linearity of the calibration curves

As mentioned before, Fig. 5 shows the R.S.D. values of quadratic curve fits. As can be seen in Table 2, especially the calibration curve in Milli-Q water shows a non-linear behaviour. In Table 2, the average R.S.D. values for each solvent are shown, together with their standard deviation. The R.S.D. values are presented for both quadratic and linear curve fits. Table 2 shows that the calibration curves in borate buffer and in 0.1 *M* H_2SO_4 are linear, whereas in Milli-Q water they are quadratic. However, even the linear calibration curve through the Milli-Q data provides very accurate results. This non-linear behaviour of Milli-Q water is an important observation, since it means that the shape of the calibration curve depends on the solvent used.

3.6. Real-life test

From the data presented, the optimal settings for our analysis method could be determined accurately. Milli-Q water was preferred because the results were, compared to borate buffer and H_2SO_4, relatively independent of the amount of 2-mercaptoethanol and the reaction time. A 2-mercaptoethanol concentration of 1.6 μl/mg OPA was selected because the OPA reagent is more stable with an increased concentration of 2-mercaptoethanol. However, since the reaction of OPA with amino acids is slowed down by 2-mercaptoethanol, a further increase of the amount of 2-mercaptoethanol would mean a loss of sensitivity. Stability experiments showed that an OPA stock solution containing 1.6 μl 2-mercaptoethanol/mg OPA is stable for at least one month, provided it is kept dark and cool. Finally a reaction time of 1 min was selected, because after 1 min all amino acids have reached their maximum fluorescence. Selecting a reaction time of 0 min would mean that some amino acids have not finished their reaction with OPA. On the other hand, selecting 3 or 9 min reaction time would mean that the amino acid-OPA derivatives have more time to decompose. These settings lead to an accuracy better than 0.25 μM (Fig. 5).

Fig. 6 shows two typical chromatograms applying the reaction conditions defined in this paper. The reproducibility and sensitivity of the method were determined using standard solutions of 5 and 1.25 μM, respectively. Results are shown in Table 3. It shows that the average limit of sensitivity (at a signal-to-noise ratio of 3) and reproducibility of this method are 136 fmol and 0.6%, respectively.

Table 2
Average R.S.D. values for the quadratic and linear curve fits of the calibration curve (μM)

	Quadratic		Linear	
	R.S.D.	S.D.	R.S.D.	S.D.
Borate buffer	0.71	0.49	0.80	0.44
Milli-Q water	0.15	0.09	0.49	0.26
0.1 *M* H_2SO_4	0.74	0.61	0.81	0.66

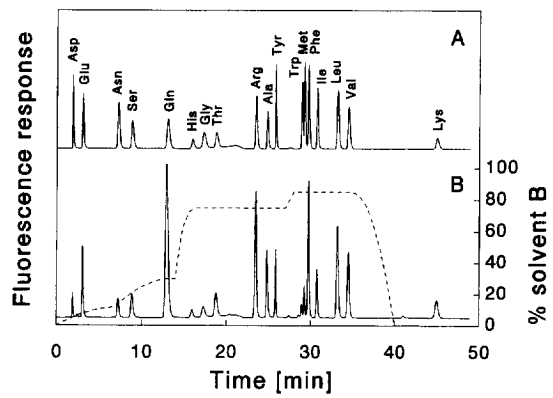

Fig. 6. Typical chromatograms of a 5 μM amino acid standard (A) and DMEM cultivation medium containing 7.5% bovine serum and 2.5% foetal calf serum (B), applying the reaction conditions defined in this paper. Reaction time, 1 min; solvent, Milli-Q water; 2-mercaptoethanol concentration, 1.6 μg/ml OPA. Chromatographic conditions: flow-rate, 1 ml/min; temperature, 35°C; injection volume, 15-μl sample+30-μl reagent. Eluent A, 1/6 dilution of eluent B with MilliQ-water; eluent B, 65% (v/v) methanol in 20 m*M* phosphate buffer (pH 7.5). The dashed line indicates the solvent gradient composition.

Table 3
Limit of sensitivity determined on a 1.25 μM sample ($S/N=3$) and confidence interval of a stock solution containing 5 μM of each amino acid ($n=14$)

Amino acid	Limit of sensitivity (fmol)	Confidence interval (μM)
Asp	49	5±0.010
Glu	71	5±0.011
Asn	89	5±0.015
Ser	125	5±0.025
Gln	136	5±0.033
His	426	5±0.023
Gly	224	5±0.092
Thr	266	5±0.040
Arg	78	5±0.016
Ala	107	5±0.017
Tyr	47	5±0.012
Trp	64	5±0.046
Met	50	5±0.012
Val	50	5±0.013
Phe	70	5±0.027
Ile	71	5±0.016
Leu	101	5±0.016
Lys	426	5±0.111

4. Conclusions

The determination of amino acids using HPLC in combination with OPA as a derivatization agent provides a very useful method. However, to obtain a reliable method, care must be taken with the treatment of the samples and the standard solutions, because the linearity of the calibration curve depends on the solvent being used. The use of linear calibration curves does not always coincide with the real response of the OPA amino-acid derivatives. If Milli-Q water is used as solvent, a quadratic calibration curve is required to produce accurate results. All three
parameters tested, i.e. the 2-mercaptoethanol concentration, the reaction time and the solvent used, were found to be crucial for the optimization of the amino acid analysis method. Therefore, a regular addition of 2-mercaptoethanol to the OPA solution should be avoided. This addition will lower the peak area which will result in a decrease of the sensitivity. Since the calibration curves will also change by this addition, the reproducibility of the analysis method will also decrease.

Applying the optimal reaction conditions for the quantitative determination of amino acids as determined in this research results in an accuracy better than 0.25 μM. The average limit of sensitivity (signal-to-noise ratio of 3) and reproducibility of this method are 136 fmol and 0.6%, respectively. Table 4 shows a summary of sensitivity and reproducibility data obtained from the literature cited, together with

Table 4
Comparison of sensitivity and reproducibility obtained in this research and extracted from cited literature

	Sensitivity (pmol)	Reproducibility (%)
Literature ($S/N=2$–2.5)	0.2–10 (avg=2.30)	0.4–8 (avg=4.8)
This method ($S/N=3$)	0.05–0.43 (avg=0.14)	0.2–2.2 (avg=0.6)

the results presented in this paper. Compared to the literature cited, the method developed in this research is both sensitive and reproducible.

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