Journal of Chromatography, 204 (1981) 143–148
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,182

NEW DETECTION AND SEPARATION METHOD FOR AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple system for simultaneous fluorescence detection of primary and secondary amines has been developed. The optimum conditions for fluorescing secondary amines in the two-stage reaction technique using sodium hypochlorite and *o*-phthalaldehyde have been studied, and then the switching flow method (sodium hypochlorite is added only to secondary amines) and the non-switching flow method (sodium hypochlorite is added to all the amino acids) have been compared. It was concluded that the non-switching flow method ensures high efficiency and reliability. This newly-developed system was applied to the detection of amino acids and gave satisfactory results.

INTRODUCTION

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of 2-mercaptoethanol, to form fluorescent substances. This reaction is often utilized for the post-column derivatization in trace analysis of amino acids by high-performance liquid chromatography (HPLC). It has recently been effectively used for close examination for inborn errors of amino acid metabolism. However, OPA does not react with amino acids such as proline and hydroxyproline to form fluorescent compounds.

Roth[1] reported, in his paper concerning the fluorescing reaction of primary amines, that proline can be derivatized by treating it with sodium hypochlorite (NaClO) to give a compound that reacts with OPA.

Then, St. John[2] tried fluorescence detection of proline and other amino acids, using a two-stage reaction coil system, as shown in Fig. 1A. In the first reaction coil, the sample was made to react with NaClO, while in the second reaction coil, the reaction with OPA was made. He used the switching flow method so that NaClO was added only to the part of the effluent that contained proline or hydroxyproline. As for the other part of the effluent, some buffer solution was added so that the flow-rate of the mobile phase was kept constant in the second reaction coil. Such a complicated method was adopted in order to prevent the trouble that might be caused if NaClO had been added to the primary amines. This switching method is costly and not very easy to operate.

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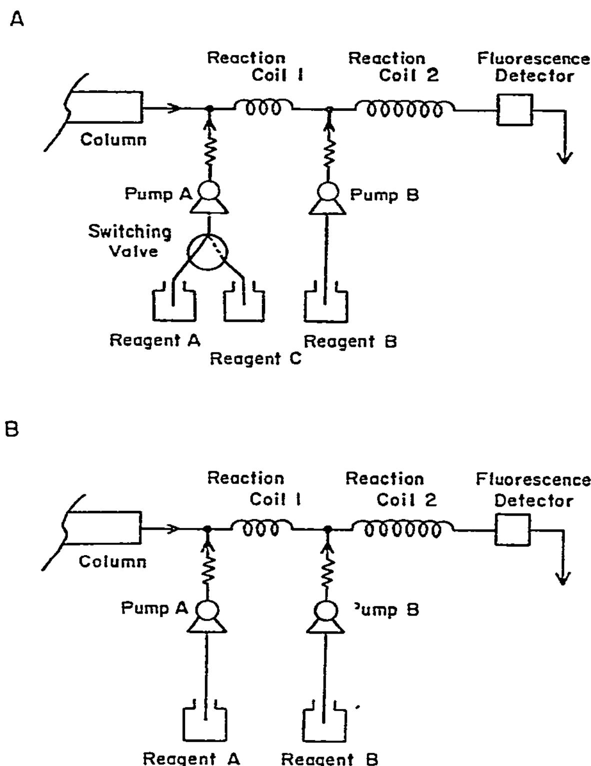

Fig. 1. Diagrams of (A) the switching flow method and (B) the non-switching flow method. Reagent A, buffer with sodium hypochlorite; reagent B, OPA reagent; reagent C, buffer without sodium hypochlorite.

We tested the non-switching flow method in analysis of amino acids, in which NaClO is added to all the effluent, and found it practically useful.

EXPERIMENTAL

Equipment

The HPLC system used was the Shimadzu LC-3A which incorporates a liquid pump of CDQR system[3]. The column was housed in the Shimadzu CTO-2A column oven. The flow of the mobile phase was changed by means of the Shimadzu SGR-1A step gradient elution unit. In the SGR-1A, the mobile phase changeover is performed with a rotary six-port valve which is operated according to the program set on digital timers. Samples were introduced by the Shimadzu SIL-1A variable injector.

As for the detector, the Shimadzu CRD-5A[4] chemical reaction detector was used. The CRD-5A consists of a chemical reaction bath, a pump for reaction reagent, and a filter-type fluorometric detector. The chemical reaction bath consists of a temperature controller and a thermostatted box containing a tin block in which a stainless steel reaction coil, a heater, and a temperature sensor are embedded. The

temperature of the reaction coil is controlled at any temperature between ambient temperature plus 15°C and 150°C, to a control accuracy of ±0.2°C.

A commercial, low-cost reciprocating plunger pump was used for supplying reaction reagents. The Shimadzu FLD-1 filter-type fluorometric detector was used in the CRD-5A.

A mercury lamp generating a light of 300–400 nm wavelength and having the peak intensity at 360 nm was used as the excitation lamp. A fluorescent filter that cuts off the wavelengths shorter than 400 nm was used for the fluorometry.

The switching between the buffer solution that contained NaClO and the buffer solution that did not contain NaClO was carried out by a three-port valve which was driven manually or automatically by a motor according to a preset program.

Column

Amino acids were separated on a Shimadzu LC column ISC-07/S1504. It is a strongly acidic cation-exchange resin of styrene–divinyl benzene copolymer, 10% in cross-linkage and 7.5 μm in the average particle diameter, and is packed in a stainless-steel tube (150 × 4.0 mm I.D.).

Reagents

Sodium hypochlorite (NaClO) was purchased from Shimakyu Yakuhin, Osaka, Japan, and its chlorine concentration was 10%; *o*-phthalaldehyde (OPA) was purchased from Wako, Osaka, Japan. Sodium citrate, sodium hydroxide, perchloric acid, boric acid, 2-mercaptoethanol and the standard amino acid samples, were also purchased from Wako. Grape juice and wine samples were kindly offered by Research Institute of Fermentation, Yamanashi University (Yamanashi, Japan).

RESULTS AND DISCUSSION

Fluorescing reaction of proline

Length and temperature of reaction coil. Using the flow system shown in Fig. 1B, we investigated the influence of the dimensions of the reaction coil 1 (*i.e.* the length of reaction time) and its temperature, upon the fluorescence intensity of the derivative of proline produced. The reagent A was 0.1% NaClO solution in 0.3 *M* borate buffer (pH 10.5); the reagent B was OPA reagent (400 mg of OPA and 1 ml of 2-mercaptoethanol in 500 ml of 0.3 *M* borate buffer (pH 10.5). Distilled water was used as the mobile phase, its flow-rate adjusted to 0.5 ml/min. The flow-rates of the reagents A and B were adjusted to 0.75 ml/min. The reaction coil 2 was 1000 × 0.3 mm I.D., and was kept at 50°C.

Our experiments showed that the optimum size of the reaction coil 1 at temperatures 50–60°C was 1000–2000 × 0.3 mm I.D. The same results were obtained when different buffers, phosphate buffer for example, were used in place of borate buffer. It was found that use of a longer reaction coil 2 resulted in a higher fluorescence intensity of the proline derivative. But use of a longer reaction coil resulted in dispersion of the sample bands, hence broadened peaks. In our experiments, we used the reaction coil of the Shimadzu CRD-1D chemical reaction detector system which is now effectively used in various fields. It was also found that the optimum length of the reaction coil 1 was not dependent on the length of the reaction coil 2.

Concentration of reagent. The optimum content of NaClO in the reagent A depends on the amount of 2-mercaptoethanol in the reagent B. When the amount of 2-mercaptoethanol in the OPA reagent is adjusted to 0.2%, the optimum concentration of NaClO is 0.1–0.2%. When the NaClO content is equal, a higher concentration of 2-mercaptoethanol will result in lower fluorescence intensity of the proline derivative. (The reaction coil 1 is 1000 × 0.3 mm I.D., and the other conditions are the same as described under *Length and temperature of reaction coil.*

Switching flow method

In the switching flow method, shown in Fig. 1A, the reagent C, which is borate buffer containing no NaClO, is allowed to flow till the glutamic acid fraction is eluted out, after which the switching valve is turned to the position of solid line to allow the reagent A to flow. Thus, the proline is detected. After the proline has been detected, the switching valve is turned back to the position of dotted line, to allow the reagent C to flow again. This valve-switching step had been thought necessary because, if the reagent A is added to the entire effluent, the fluorescing reaction of the amino acids having primary amine radicals might be hindered. The switching of the valve, however, inevitably causes fluctuation of the baseline, because of the different fluorescent background between the reagent A and the reagent C. In order to prevent the elution of proline during the baseline fluctuation, it is necessary to adjust the operational conditions so that glutamic acid and proline are separated to an otherwise unnecessary degree.

The biggest disadvantage of the switching flow method is the high cost of the switching valve and the programmer for driving it. Thus we proceeded to investigate the practicality of the non-switching flow method.

Non-switching flow method

We investigated how the primary amines are affected when the non-switching flow method shown in Fig. 1B is used. Tables I and II show the influences of the reaction temperature and the amount of NaClO on the fluorescence intensities of the four amino acids. These data show that the influences are negligible if the reaction

TABLE I

INFLUENCE OF THE REACTION COIL TEMPERATURE ON THE DETECTION OF AMINO ACIDS

Sample solution: Asp, 2.4 mg; Ser, 2.1 mg; Glu, 2.1 mg; Gly, 2.5 mg; in 50 ml of mobile phase. Injection volume: 5 μl. Detector attenuation: × 32. Separation conditions: column, LC column ISC-07/S1504; mobile phase, 0.2 *N* sodium citrate (pH 3.20); flow-rate, 0.5 ml/min; column temperature, 55°C.
Other conditions are the same as in Table III. Except coil temperature and NaClO concentration.

Amino acids	*Peak height (relative response)*								
	Reaction coil temperature (°C)								
	16	*30*	*40*	*50*	*55*	*60*	*65*	*70*	*80*
Asp	8.0	21.7	30.0	37.5	35.0	31.0	22.5	13.0	4.0
Ser	28.2	49.0	52.0	53.5	49.0	44.5	35.0	24.0	7.0
Glu	8.0	18.0	22.0	25.0	23.5	20.0	14.5	9.0	2.0
Gly	65.0	74.0	69.5	70.0	68.0	69.0	—	65.5	58.5

TABLE II

INFLUENCE OF THE CONCENTRATION OF SODIUM HYPOCHLORITE ON THE DETECTION OF AMINO ACIDS

For conditions, see Table I.

NaClO concn. (μl/ml buffer)	*Peak height (relative response)*			
	Asp	*Ser*	*Glu*	*Gly*
0.5	44	61	29	86
1.0	44	60	28	81
2.0	42	57	26	77
4.0	37	52	23	66
8.0	30	40	17	47

TABLE III

COMPARISON OF THE SENSITIVITY OF VARIOUS AMINO ACIDS IN THE SWITCHING AND THE NON-SWITCHING FLOW METHOD

Separation conditions: column: LC column ISC-07/S1504, strong acidic cation-exchange resin, 7.5 μm, 150 × 4.0 mm I.D.; mobile phase: A, 6% ethanol in 0.2 *N* sodium citrate (pH 3.20); B, 0.2 *N* sodium citrate (pH 4.25); C, 0.6 *N* sodium citrate (pH 8.0); D, 0.6 *N* sodium citrate (pH 10.0); E, 0.2 *N* NaOH; stepwise gradient program of mobile phase: A, 14 min; B, 16 min; C, 5 min; D, 30 min; E, 3 min; flow-rate: 0.5 ml/min; column temperature: 55°C.

Reaction conditions: reagent: A, 0.1% sodium hypochlorite in 0.4 *M* borate buffer (pH 10.0), flow-rate: 0.75 ml/min; B, 400 mg of *o*-phthalaldehyde and 2 ml of mercaptoethanol in 500 ml 0.3 *M* borate buffer (pH 10.0), flow-rate: 0.75 ml/min; reaction coil: 1, 1000 × 0.3 mm I.D.; 2, 2000 × 0.3 mm I.D.; reaction temperature: 55°C.

R = (response in non-switching flow method)/(response in switching flow method).

Amino acid	*R*	*Amino acid*	*R*
Asp	0.63	Met	0.66
Thr	0.95	Leu	0.93
Ser	0.83	Ileu	0.87
Glu	0.74	Tyr	0.86
Pro	1.00	Phe	0.97
Gly	0.83	His	0.75
Ala	0.81	Lys	0.94
Cys	1.88	Arg	0.99
Val	0.60		

temperature is within 50–60°C and the NaClO concentration is 0.1–0.2%, though it is possible that the results may depend on the reaction time in the reaction coil 2. We determined the optimum reaction conditions and LC conditions as shown in Table III, for analysis of amino acids.

The switching flow method and the non-switching flow method were compared under these operational conditions, and the results are shown in Table III. The data show that, contrary to the reports presented before, the non-switching flow method ensures satisfactory results provided that the operational conditions are appropriate. The data for the primary amino acids are not noticeably affected by the continuous supply of NaClO.

The calibration curve for each amino acid is satisfactorily linear from a few picomole level to a few tens of nanomole level. This shows that the primary amino acids undergo quite stable reaction when the non-switching flow method is used. The

detection limit for proline is a few tens of picomole level. If the Shimadzu RF-500 LC fluorescence spectromonitor, which employs a xenon lamp, is used as the detector, the sensitivity can be enhanced by five times for most amico acids.

Applications

From the results of our experiments described above, we have come to a conclusion that the non-switching flow method is quite simple to perform and provides satisfactory performance, and we have developed an amino acid analysis system which utilizes the non-switching flow method. The system was successfully applied to various analyses. Fig. 2 shows an example of analyses.

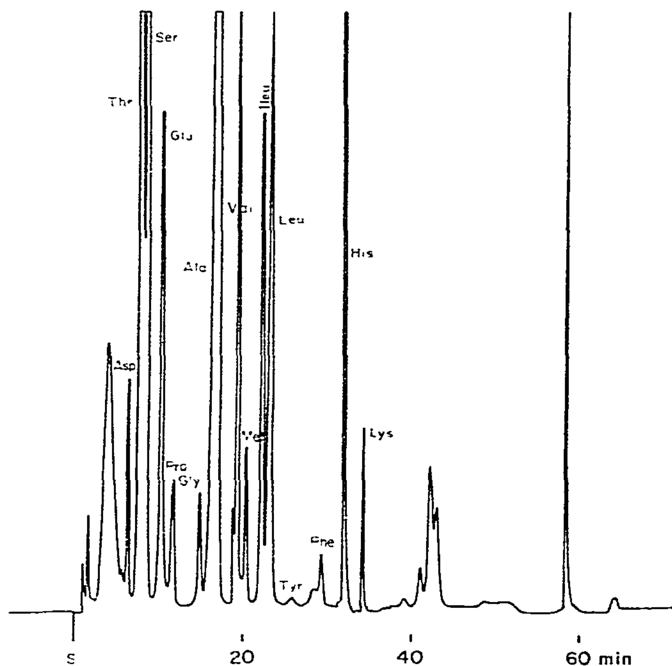

Fig. 2. An analysis of the amino acids in grape juice. Operational conditions are the same as in Table III.

CONCLUSION

The switching flow method has been so far used for adding sodium hypochlorite (NaClO) to detect secondary amino acids in the post-column derivatization for amino acids with *o*-phthalaldehyde.

The non-switching flow method was tested for the same purpose and has proved to provide satisfactory results, using a much simpler system.

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