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A HIGHLY EFFICIENT CARBOXYLIC ACID ANALYZER AND ITS APPLICATION

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SUMMARY

A new highly efficient carboxylic acid analyzer, employed with liquid chromatography and a specific detection method, has been designed for the quantitative analysis of carboxylic acids in foods and biological samples. This apparatus is particularly advanced in terms of its sensitivity and resolution as a result of the extensive modifications to our previous system. The applications of this analyzer to the analyses of juices, beer, human serum and urine are presented.

INTRODUCTION

The analysis of carboxylic acids in foods, fermentation broth, body fluid or other biological samples is cumbersome, because the lack of a specific method of detection necessitates tedious sample preparation. Gas, liquid, paper and thin-layer chromatography have all been applied to the determination of water-soluble aliphatic carboxylic acids, however, none of these methods is always satisfactory. Gas chromatography (GC) requires the extraction and derivatization of carboxylic acids as volatile compounds, such as methyl esters. The derivatization sometimes results in multipeak formation from a single carboxylic acid and is thus liable to introduce errors into quantitation[1]. Paper and thin-layer chromatography (PC and TLC) are widely used for qualitative analysis but they have limited value for quantitative analysis. Liquid chromatography has advantages in terms of the ease of sample preparation, but has rarely been applied to the analysis of biological samples because the separation from other types of compounds is not adequate to permit the use of non-specific detection methods such as UV or refractive index (RI) detection. Partition chromatography on silica gel[2] and anion-exchange chromatography[3,4] have been reported for the separation of water-soluble carboxylic acids, but the chromatograms in the literature are usually of artificial mixtures.

Our previous papers described the advantages of a specific method of detection

for hydroxamic acids formed by the coupling reaction between carboxylic acids and hydroxylamine by the dicyclohexylcarbodiimide (DCC)[5,6]. The method was later applied to the automatic determination of carboxylic acids in biological samples by anion-exchange chromatography[7]. The flow system for automatic anion-exchange chromatography has now been improved. The resulting carboxylic acid analyzer offers higher sensitivity and resolution with three reagent pumps. The satisfactory reproducibility is illustrated by the analyses of various kinds of biological samples.

EXPERIMENTAL

Reagents

A 1 *M* solution of hydroxylamine perchlorate (HAP) and ferric perchlorate were obtained from Kanto, Tokyo, Japan. DCC was purchased from Tokyo Chem. Ind., Tokyo, Japan. The purest commercial grades of carboxylic acids, in either the salt or acid form, were used for calibration standards (Kanto; Tokyo Chem. Ind.; Wako, Osaka, Japan; Nakarai, Kyoto, Japan; BDH, Poole, Great Britain; or Sigma, St. Louis, Mo., U.S.A.). All other reagents were of analytical grade.

A solution of 0.116 *M* HAP and 0.07 *M* triethylamine (TEA) was prepared by dissolving each reagent in anhydrous ethanol and then filtering through filter paper. The solution was stored in a brown bottle which was connected to a tube containing potassium hydroxide for absorption of carbondioxide and water. A 0.13 *M* solution of DCC was prepared by dissolving the calculated amount of DCC in anhydrous ethanol*. A 0.005 *M* solution of ferric perchlorate was prepared by dissolving the calculated amount of the hexahydrate in 0.5 *M* perchloric acid solution, which was prepared by diluting 70% perchloric acid with ethanol.

Preparation of samples

Juices were filtered (if necessary) from insoluble materials. Urine and serum were added with one fourth part of 10% perchloric acid and the resulting mixture was centrifuged to remove the precipitated protein. After degassing with freezing, beer was similarly treated with perchloric acid. If necessary the samples were diluted to appropriate concentrations with 0.2 *N* hydrochloric acid.

Instruments

The apparatus for the quantitative analysis of carboxylic acids (Fig. 1) was an extensively modified version of our previous system. The major modifications are as follows.

(1) Diaion CA-10S or CA-08S (Cl^-) (Mitsubishi Kasei Kogyo, Tokyo, Japan), a strongly basic anion-exchange resin (containing 10 or 8% divinylbenzene), particle size 11–14 μm, was substituted for Diaion CA-08 (particle size, 16–20 μm). The resolving power of an ion-exchange resin is improved as the mean particle diameter decreases. Consequently, the column length (1 m × 3 mm) was reduced to one third that of the previous column.

(2) The eluent and reagent pumps were of the reciprocating type (Kyowa Seimitsu; KSU-16 ⓐ) with sapphire plunger, ruby balls and Diflon inner surface which

* DCC and its solutions cause skin irritation and should be handled with care.

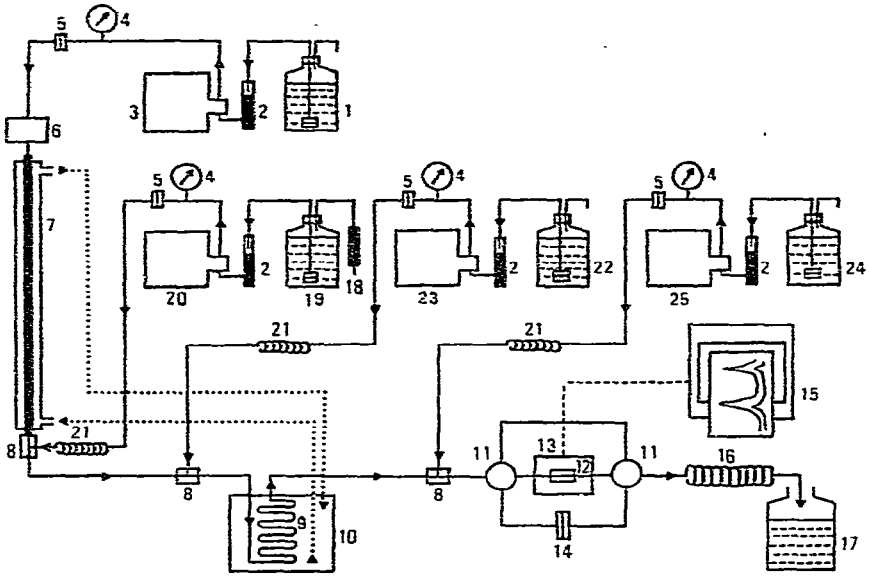

Fig. 1. Diagram of the system for carboxylic acid chromatography. 1, Eluent reservoir (0.2 *N* HCl); 2, glass trap; 3, eluent pump (flow-rate, 0.13 ml/min); 4, pressure gauge; 5, filter; 6, loop injector (volume of sample loops, *ca.* 133 μl as determined by titration); 7, chromatographic column (1 m × 3 mm), (column temperature, 50°); 8, mixing port; 9, reaction coil (30 m); 10, water-bath (50°); 11, four-way valve; 12, flow cell (20 mm path length); 13, photometer; 14, sediment filter; 15, two-pen recorder; 16, back pressing coil (10 m); 17, waste; 18, potassium hydroxide packing tube; 19, reagent reservoir (0.116 *M* HAP in 0.07 *M* TEA solution); 20, reagent pump I (flow-rate, 0.6 ml/min); 21, resistance tube; 22, reagent reservoir (0.13 *M* DCC solution); 23, reagent pump II (flow-rate, 0.4 ml/min); 24, reagent reservoir (0.005 *M* ferric perchlorate solution); 25, reagent pump III (flow-rate, 0.3 ml/min).

are resistant to the reagents used. A glass trap was connected to the inlet of each pump in order to remove air bubbles and sediments. A lower flow-rate results in a higher separation efficiency. The reciprocating cycle of the eluent pump was therefore reduced to 15 strokes per min in order to obtain a lower constant flow-rate.

(3) In the previous paper, the TEA solution was mixed with the HAP solution by means of two separate pumps and the resulting mixture was then fed into the effluent stream. Since the mixed solution of HAP and TEA is stable for at least one month at room temperature, the number of reagent pumps was reduced to three.

(4) In order to minimize diffusion, the line between the loop injector and the top of the column was made as short as possible using PTFE tubing of 0.25 mm I.D. The mixing port for combining the column effluent and the HAP and TEA solution was connected directly to the outlet of column. In addition, the column head and end fittings were disigned to so as to give a smaller dead space.

(5) In order to minimize peak broadening, the mixing coils were eliminated in the present system without appreciable increase in the base-line noise. The length of the reaction coil in the water-bath was also reduced to 30 m (0.5 mm I.D.).

(6) The photometric detector was equipped with a 530-nm interference filter, a flow cell of 20-mm light path and a two-pen recorder. It had a satisfactory signal to

noise ratio and four ranges of sensitivities, *i.e.*, 0–100 (× 1), 75–100 (× 4), 90–100 (× 10) and 95–100% (× 20) transmittance full scale.

(7) Each reagent passes through a resistance tube, as the device for damping the pulsation of the pumps, which consisted of a PTFE tube (20 m × 0.25 mm I.D.). Such tube is not needed in the eluent delivery line because the chromatographic column introduces an adequate damping.

(8) The flow circuit, in which the flow cell and the sediment filter are connected by means of two four-way valves, is shown in Fig. 2. The valves could be turned synchronously by means of a motor which was driven by a timer. Usually, effluent stream passed through the flow cell and then the sediment filter (Fig. 2a). However, when the sediment was produced in the line, the valves were operated so as to make the effluent pass through the circuit in the reverse direction, *i.e.*, through the sediment filter and then the flow cell (Fig. 2b). Thus the sediment is held by the filter and is prevented from entering the flow cell. In both cases, the direction of the flow in the flow cell is the same, but that in the sediment filter is reversed, when no more sediment was produced, the valves were switched backward to their original positions and the sediment in the filter was washed out to the waste.

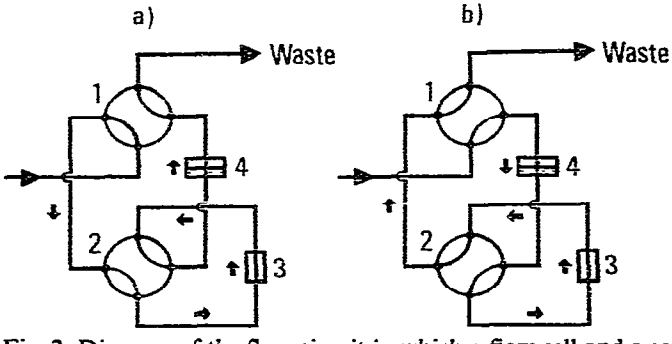

Fig. 2. Diagram of the flow circuit in which a flow cell and a sediment filter are connected by means of two four-way valves. 1, Four-way valve I; 2, four-way valve II; 3, flow cell; 4, sediment filter.

RESULTS AND DISCUSSION

Fig. 3 shows a typical chromatogram of a mixture of 10 carboxylic acids analyzed with the present system. This chromatogram is substantially better than that of a previous paper[7], and the retention times were about half those of the previous study. The peaks were sharp and base-line separation was achieved. Lactic and acetic acids, which could not be separated satisfactorily in the previous study, were completely separated. The peak areas obtained by multiplying the peak heights and the half-widths were proportional to the amounts of the carboxylic acids (Fig. 4). The detectability of a constituent is a function of the peak height and the noise level of the base-line. The peak height in absorbance units were five times greater in the present system, and the base-line noise was decreased several times. Consequently, the limit of determination for various carboxylic acids was improved from 0.2 μmoles in the previous paper to *ca.* 4 nmoles.

Table I gives the retention times and *C* values (the areas of the chromatographic

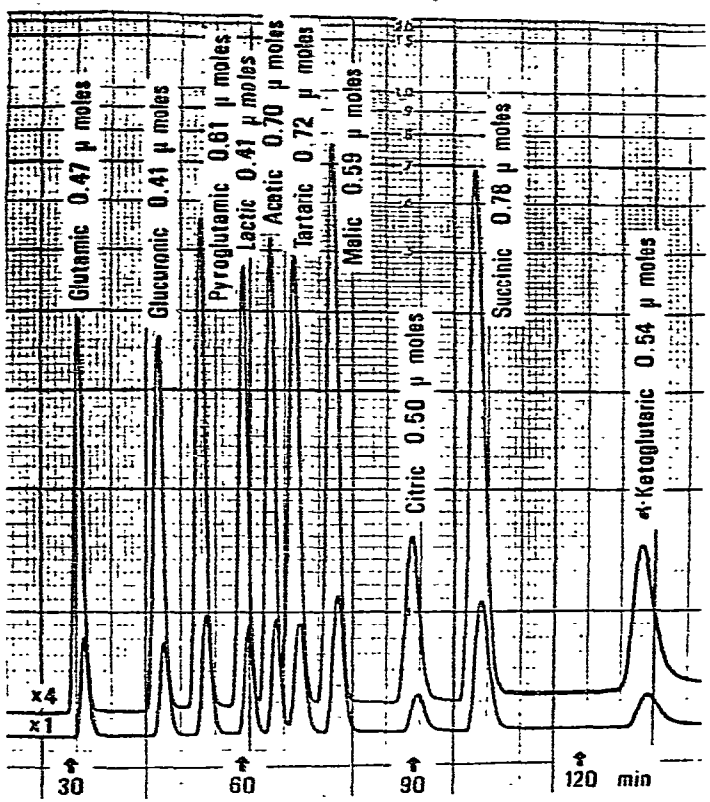

Fig. 3. Typical chromatogram of a standard mixture of water-soluble carboxylic acids. Resin, Diaion CA-10S. Sample size, 133 μl.

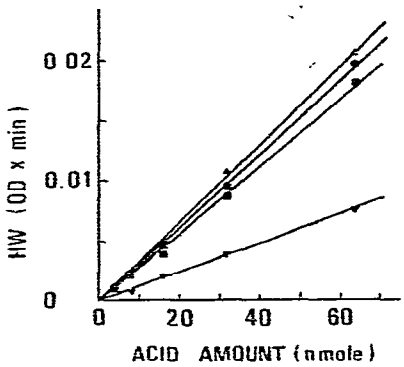

Fig. 4. Working graphs of some carboxylic acids. Resin, Diaion CA-08S. HW = Peak area, where H is the absorbance at peak maximum and W is the peak at half-height expressed in min. Acids: malic (▲); lactic (●); succinic (■); citric (▼).

TABLE I

THE RETENTION TIMES AND *C* VALUES OF VARIOUS WATER-SOLUBLE ALIPHATIC CARBOXYLIC ACIDS

Resin, Diaion CA-10S or CA-08S. Column (1 m × 3 mm) temperature, 50°. Eluent, 0.2 *N* HCl; flow-rate, 0.13 ml/min. Sample size, 133 µl. For definition of *C* see text.

Acid	*Retention time (min)*		*C value*
	CA-10S	*CA-08S*	
Glutamic	35	31	0.211
Gluconic	42	43	0.149
Glucuronic	48	47	0.316
Pyroglutamic	55	53	0.230
β-Hydroxybutyric	61.8	60	0.240
Lactic	62.4	60	0.330
Glycolic	64	59	0.277
Acetic	66	64	0.145
Levulinic	69	66	0.026
Tartaric	70	70	0.236
Pyruvic	73	71	0.148
Formic	75.6	73	0.061
Malic	76	76	0.386
Citric	89	89	0.175
Succinic	100	97	0.375
Isocitric	110	108	0.376
α-Ketoglutaric	128	125	0.227
Methylmalonic	138	139	0.190
Fumaric	309	304	0.829
cis-Aconitic	511	495	0.401

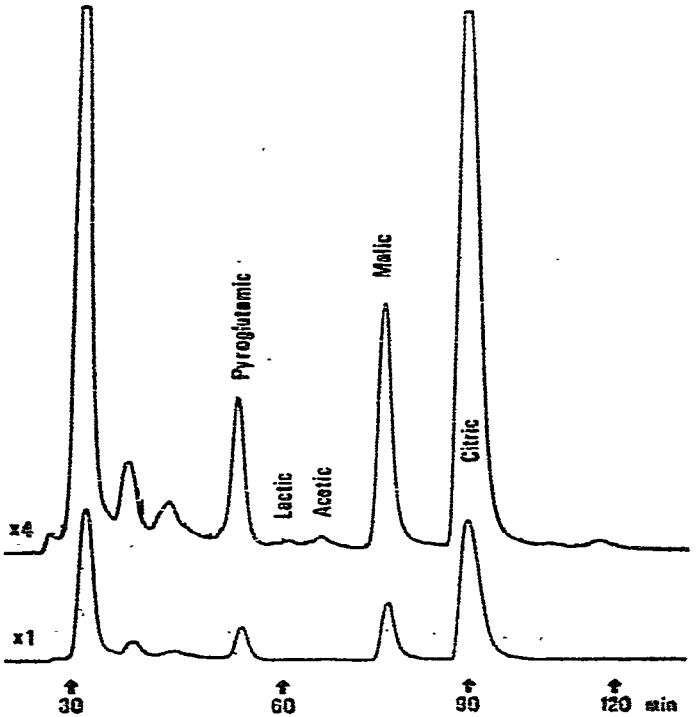

Fig. 5. Chromatogram of carboxylic acids present in tomato juice. Resin, Diaion CA-10S. Sample size, 133 µl.

peaks in time–absorbance units divided by the concentration in μmoles of the carboxylic acids). Among the acids studied, *cis*-aconitic and fumaric acids required longer times for elution. For the analysis of these acids, a 20-cm column gave good results. The resolution of lactic and acetic acids was better on a resin containing 10% cross-linking than on a resin with 8% cross-linking. The effect of cross-linking was reversed for the resolution of pyruvic, formic and malic acids. Thus, the choice of resin is dependent on the constituent carboxylic acids in the sample. The recovery of each carboxylic acid ranged from 98 to 103% with the additional recovery tests for each carboxylic acid. Under these chromatographic conditions, the separation of β-hydroxybutyric acid and lactic acid was still incomplete. Furthermore, various uronic, aldonic and saccharic acids were not identified because of the similarity of their retention times.

Figs. 5 and 6 show typical chromatograms of the vegetable and fruit juices, tomato and mandarin orange, respectively. The acid peaks were sharp and the baseline was stable. The labelled peaks were tentatively identified from the retention times as compared to those of standard mixtures. For example, the contents of citric and

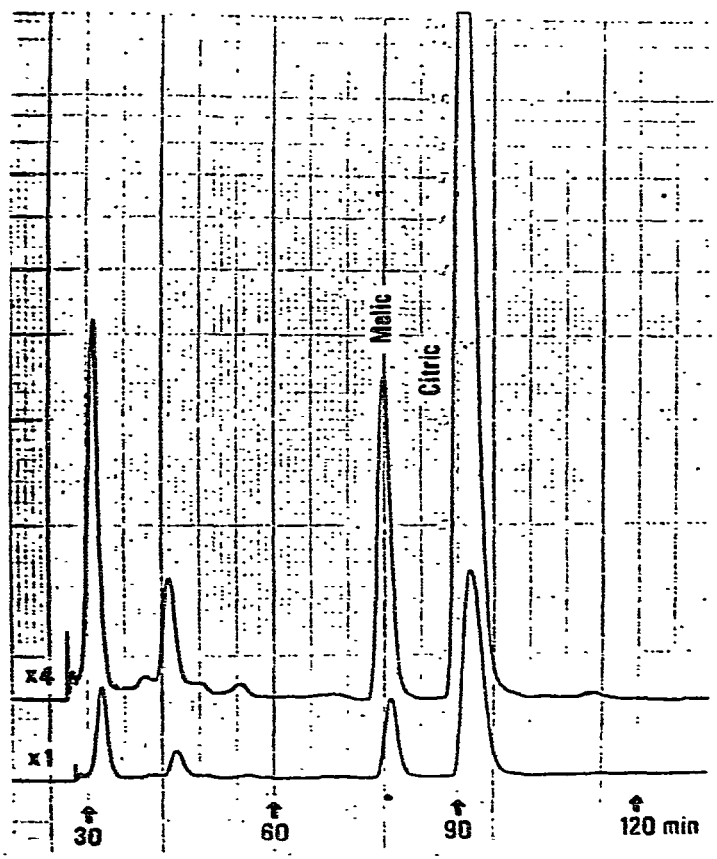

Fig. 6. Chromatogram of carboxylic acids present in mandarin orange. Resin, Diaion CA-10S. Sample, five-fold dilution. Sample size, 132 μl.

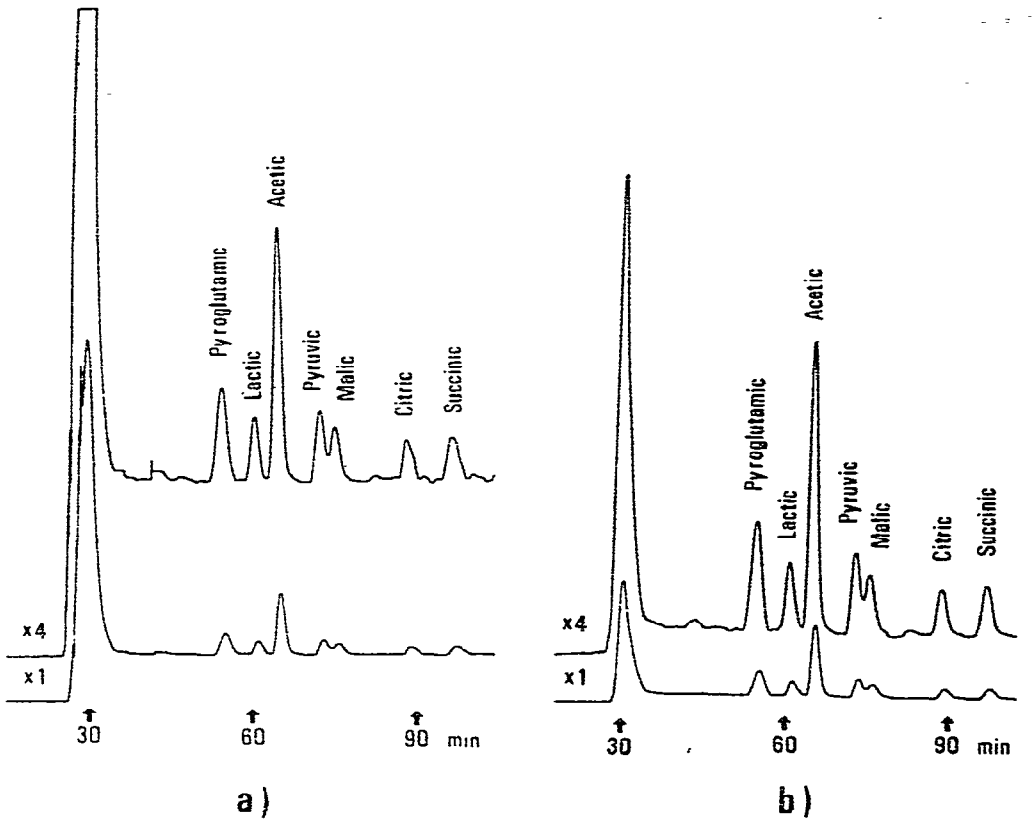

Fig. 7. Chromatogram of carboxylic acids present in beer. Resin, Diaion CA-08S. Sample size, 133 μl. The chromatogram was obtained without (a) or with (b) a sediment filter placed before the flow cell.

malic acids in fresh mandarin orange juice were determined as 2.76 and 0.38 mg/ml, respectively. Shimazu and Watanabe[8] reported the application of this new analyzer to the analysis of various wines. Kanbe *et al.*[9] also described the analysis of carboxylic acids in soy sauce.

In the analysis of beer, sediment is always produced in the line just after the void volume. When sediment was delivered into the flow cell, the base-line was considerably raised (Fig. 7a). Since sediment always seemed to be produced after 25 to 45 min, the two four-way valves were switched to change the direction of the effluent stream in the sediment filter at these times. Placing the sediment filter before the flow cell served to prevent the rise of the base-line (Fig. 7b). The sediment filter was not blocked with repeated use, and the analyses of samples which produced sediment gave good results.

Fig. 8 shows a chromatogram of human serum. In the analysis of serum, deproteinization was required in order to stabilize the base-line, and treatment with perchloric acid gave a satisfactory result. The predominant carboxylic acid in this serum sample was lactic acid. Fig. 9 shows a chromatogram of human urine from a patient suffering from hyperlactoaciduria. Smaller unknown peaks were still numerous;

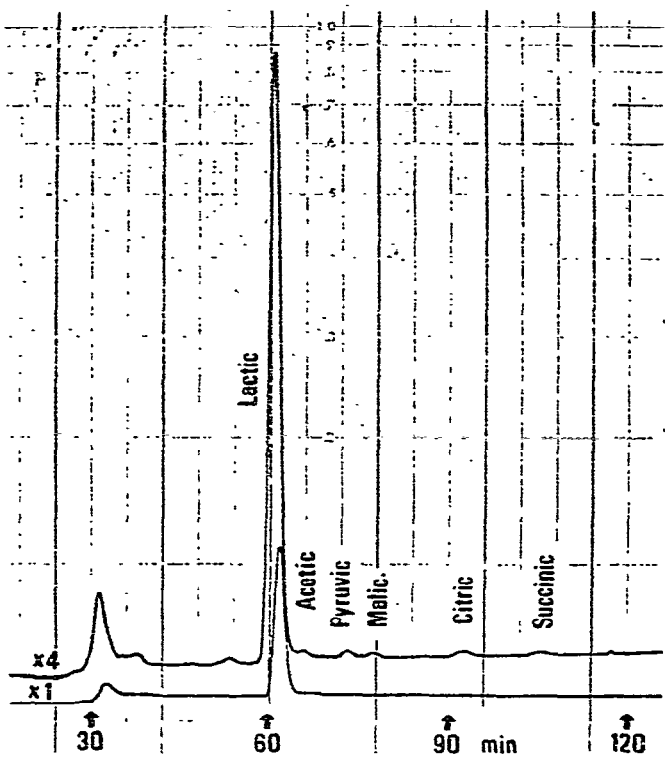

Fig. 8. Chromatogram of carboxylic acids present in human serum. Resin, Diaion CA-10S. Sample size, 132 μl.

however, in some cases a difference from a chromatogram of normal urine was easily detected by the appearance of large peaks. In recent years, many workers have demonstrated the analysis of urinary carboxylic acids by GC, and postulated that the analysis of urinary carboxylic acids will have an important role in the diagnosis of metabolic diseases[10–14]. This carboxylic acid analyzer is simple in operation and gives a quantitative result in the analysis of urinary carboxylic acids compared with conventional methods.

The capacity of the system to provide meaningful resolutions within 3 h is indicated by these chromatograms. The molar absorption coefficient of ferric hydroxamate (*ca.* 1000) is one twentieth of that of Ruhemann Purple in the ninhydrin reaction[7]. However, the sensitivity of this carboxylic acid analyzer has been increased to 4 nmoles from 0.2 μmoles for the previous system. This apparatus seems to be most suitable for the analyses of practical samples in various fields of biochemistry, agricultural chemistry, clinical chemistry and environmental science, in terms of its simplicity, sensitivity and resolution. The application of this analyzer to the analyses of various kinds of carboxylic acids, including aromatic and water-insoluble acids, is in progress in our laboratories.

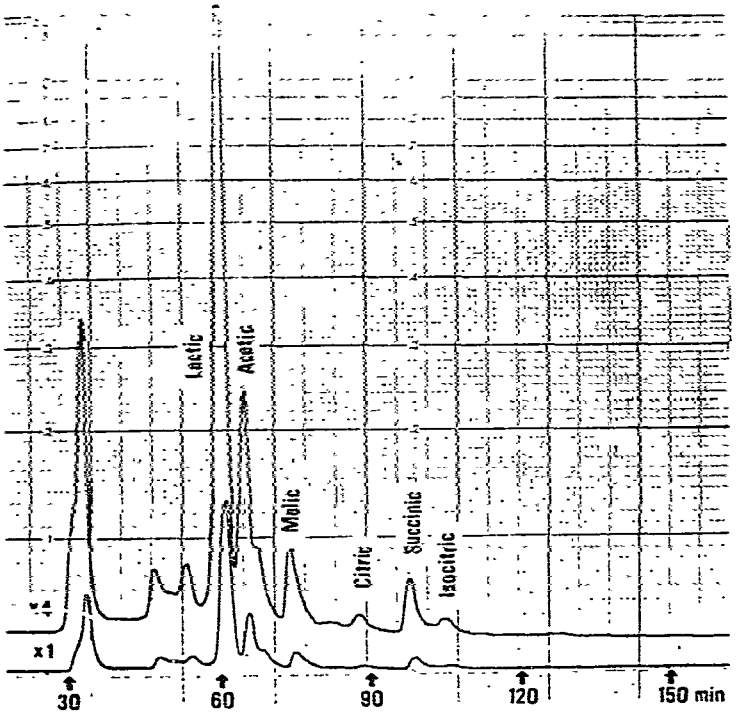

Fig. 9. Chromatogram of carboxylic acids present in human urine from a patient suffering from hyperlactoaciduria. Resin, Diaion CA-10S. Sample size, 133 μl.

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