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SOLVENT EXTRACTION CLEAN-UP FOR PRE-TREATMENT IN AMINO ACID ANALYSIS BY GAS CHROMATOGRAPHY

APPLICATION TO AGE ESTIMATION FROM THE D/L RATIO OF ASPARTIC ACID IN HUMAN DENTINE

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SUMMARY

Solvent extraction, a clean-up method for samples for the determination of amino acids by gas chromatography, was investigated and compared with a conventional ion-exchange purification. Amino acids were esterified with isopropanol and extracted with various organic solvents. The solubilities of the amino acid isopropyl esters increased with increasing solvent polarity and the size of the amino acids. The optimum pH was found to be 10.5. The method was applied to the estimation of ages by measurement of the D/L ratio of aspartic acid in human dentine. The D/L ratios so determined were slightly lower than from the ion-exchange method with respect to all dentines examined. However, there were little or no significant differences in the ages estimated by both methods, and the correlation coefficient of this method was 0.982. The method is suitable for the enantiomeric analysis of amino acids, and has several advantages in the technique and time.

INTRODUCTION

Analysis of amino acids by gas chromatography (GC) is an universally accepted method for the determination of mixtures of biochemical, clinical, geological and other interest. The method complies with the demands for more precise, sensitive, automatic and inexpensive techniques.

In 1966, Gil-Av *et al.*[1] discovered an enantioselectivity in N-trifluoroacetyl (TFA)-L-isoleucine lauryl ester which when coated on a glass capillary enabled the resolution of enantiomeric alanine derivatives. After continuous development of this type of stationary phase for improvement of the enantioselectivity and thermal stability[2–8], Frank *et al.*[9] reported that a well known Chirasil-Val enables the resolution of all protein amino acids in the form of their N-pentafluoropropionyl-isopropyl esters within 28 min.

GC enantiomeric analysis of amino acids has been applied in various fields of research for the determination of the D/L ratio in synthetic[10] and naturally occurring[11]

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mixtures. The age-dating from the D/L ratio of amino acids is of interest. Helfman and Bada[12] reported that human age could be estimated from the extent of aspartic acid racemization in dentinal collagen. This has been recognized as a convenient technique for deducing human age, especially in forensic medicine[13].

With regard to the practical GC analysis of amino acids, the sample clean-up process is indispensable prior to derivatization. This process is required not only for desalting the sample, but also for the removal of various kinds of materials which sometimes interfere with the amino acid peaks as they are or in the form of decomposition products. Conventionally, the ion-exchange method is used with a strong cation- and (or) anion-exchange resin in a short glass column of 5–10 ml in volume. However, the limitations and disadvantages of this method have been reported by several authors[14,15]. The method requires the passage of hydrochloric acid and sodium hydroxide for the activation of the resins. This activation is laborious and lengthy in the case when numerous samples have to be analyzed.

Enantiomeric analysis of amino acids has been applied essentially to the determination of D/L ratios, or the percentages of the D-enantiomers, of individual amino acids, not to the quantitation of the total amounts. Therefore, slight losses of constituents during the formation of derivatives are not taken into account. In consideration of these practical requirements, we investigated a solvent extraction purification of samples and applied it to the age estimation from the D/L ratio of aspartic acid contained in human dentine ranging in ages from 18 to 60 years.

EXPERIMENTAL

Reagents

All amino acids and hydroxyapatite were obtained from Sigma (St. Louis, MO, U.S.A.). Isopropanol and dichloromethane were obtained from Wako (Osaka, Japan), and once distilled from analytical grade. Biphenyl was from Tokyo Kasei, Japan. Dowex 50W-X8 (50–100 mesh) was from Muromachi Kagaku (Tokyo, Japan). All other reagents were of analytical grade (Wako). The 6 *M* hydrochloric acid was prepared by distilling twice diluted concentrated hydrochloric acid in an all-glass apparatus and finally adjusting the concentration by addition of doubly distilled water. The normality was verified by withdrawing a 1.00-ml aliquot, mixing with 20 ml of water and titrating with standardized sodium hydroxide solution. The buffer solution was prepared by the gentle addition of 0.2 *M* ammonium hydroxide to 0.2 *M* ammonium chloride and adjusting the pH to 9.0, 9.5, 10, 10.5 and 11, respectively. The measurement of pH was carried out by means of a Corning Digital 112 pH meter.

Amino acid stock solution

A standard amino acid stock solution was prepared by the dissolution of nine amino acids (L-Ala, L-Val, Gly, L-Leu, L-Pro, L-Hyp, L-Asp, L-Phe, L-Glu) in 0.1 *M* hydrochloric acid to a concentration of 10 μmol/ml of each. The solution was stable for at least 2 months upon storage in a refrigerator.

Ion-exchange column

The cation-exchange column was prepared by adding 5 ml of Dowex 50W-X8 (50–100 mesh) to a glass column fitted with a quartz-wool plug. The column was

activated by thorough washing with 6 *M* hydrochloric acid (30 ml), water (50 ml), 2 *M* sodium hydroxide (30 ml). water (50 ml), at least twice. Subsequently, 6 *M* hydrochloric acid (30 ml) was passed through the column, followed by washing washed with water to neutrality.

Biphenyl was used as an internal standard for the determination of the distribution ratio of amino acid esters. A standard solution was prepared by dissolving it in dichloromethane to a concentration of 4 μmol/ml. The solution was stored in a refrigerator.

Apparatus

Gas chromatography (GC) was performed using a Shimadzu GC-7AG instrument equipped with dual flame ionization detectors. A digital integrator, Shimadzu C-R1A was used for the determination of peak areas. Both packed and capillary columns were employed. In the case of the packed column, 0.5% EGA on Chromosorb W AW, 100–120 mesh (Gasukuro Kogyo, Tokyo, Japan) in a 2 m × 2.6 mm I.D. coiled glass column was used. The laboratory-made silylated glass capillary column (25 m × 0.3 mm I.D.) was statistically coated with 0.3% chirally modified silicone GE-XE-60[16].

Determination of the distribution ratio and extraction efficiencies of amino acid esters

A 100-μl volume of the amino acid standard solution was pipetted into a PTFE-lined screw cap Pyrex tube (100 mm × 13 mm). The solvent was removed by use of a nitrogen flow and the contents were esterified with 2.00 ml of acetyl chloride–isopropanol (2:8, v/v)[17]. After the solution had been evaporated to dryness, 1.00 ml of the buffer and 1.00 ml of solvent were added successively by a Pipetman (Gilson, France). The tube was tightly capped and shaked vigorously for 3 min. The tube was centrifuged at 2300 *g* for about 3 min, and the mixture was cleanly separated into two layers. For the determination of the distribution ratio of amino acid esters between the aqueous and organic layers, 50 μl of each fraction were sampled by a microlitre syringe, 10 μl of TFA were added to both fractions, which were vacuum dried suspended in 1 ml of dichloromethane and acylated with 200 μl of trifluoroacetic anhydride (TFAA). The solution was heated at 100°C for 10 min, and the excess of reagents was carefully evaporated. The dried residue was dissolved in 50 μl of the biphenyl internal standard solution. Finally, 2–3 μl aliquots were injected into the 0.5% EGA packed column.

Treatment of dentine

The treatment of dentine was carried out according to a previous method[12,18] with slight modification. Fig. 1 shows the treatment process using the solvent extraction method(I) and the ion-exchange method(II), respectively. The dentine isolated was washed successively with 0.1 *M* hydrochoric acid, water and acetone with ultrasonication, and dried in a vacuum desoiccator. About 5 mg of the dried dentine were weighed into a PTFE-lined screw cap Pyrex tube (100 mm × 13 mm) and 2 ml of distilled 6 *M* hydrochloric acid were added. The tube was tightly capped and heated in a laboratory-made block heater at 100 ± 0.5°C for 6 h to hydrolyse the dentinal collagen. After cooling to room temperature, the excess of hydrochloric acid was removed by rotary evaporation below 40°C. The dried residue was treated as follows.

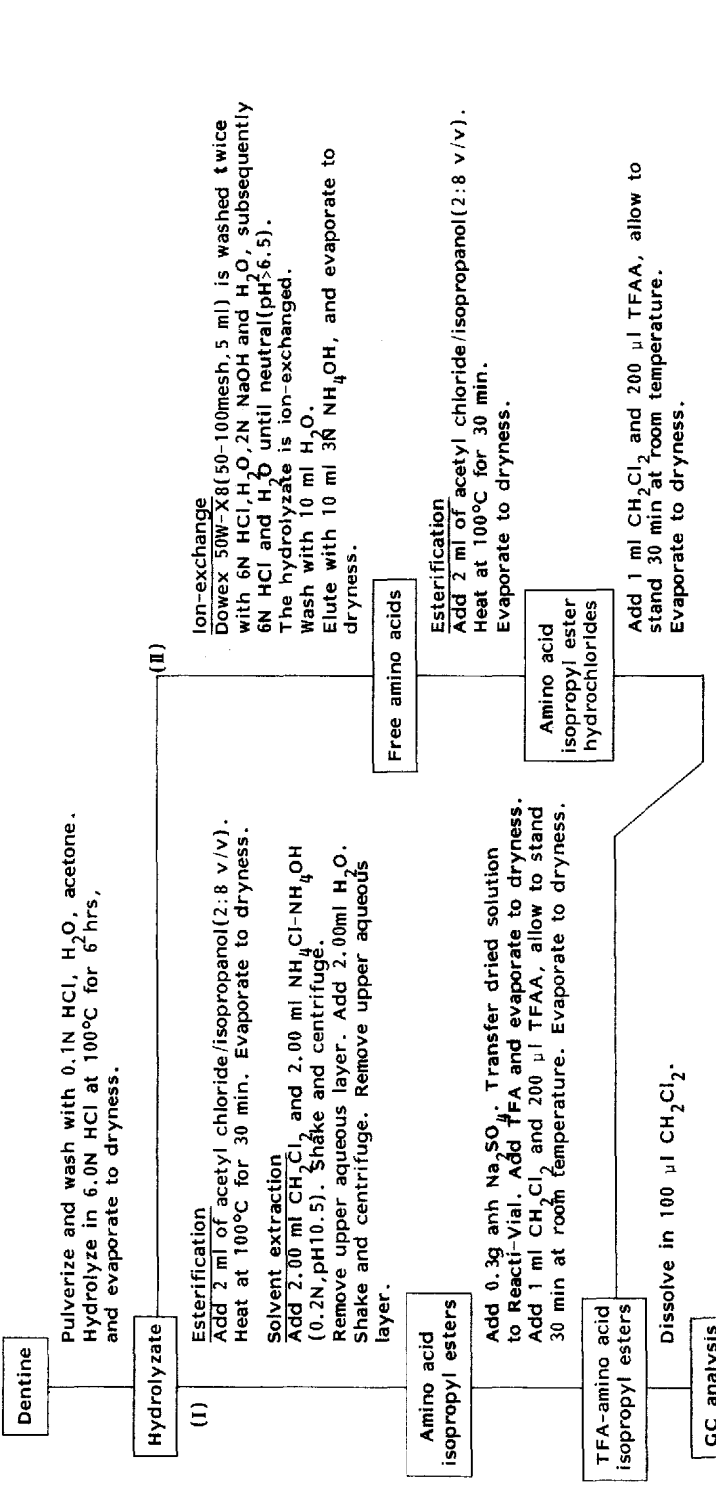

Fig. 1. Procedure for the treatment of dentine; (I) solvent extraction method; (II) ion-exchange method.

Solvent extraction method (SEM). The dried residue was subjected to direct esterification without any pre-treatment in 2.00 ml of esterification reagents at 100°C for 30 min. The excess of reagents was removed by evaporation under a stream of nitrogen. Following the addition of 2.00 ml of dichloromethane, and an equivalent volume of the buffer (pH 10.5), the tube was tightly capped and shaked vigorously for 3 min. After centrifugation at 2300 *g* for 3 min, the upper aqueous layer was discarded. The extraction was repeated with the addition of a 2.00 ml of water. A 0.3-g amount of anhydrous sodium sulphate was added to the residual solvent for the dehydration. The solution was transferred to a Reacti-Vial by a Pasteur pipette taking care not suck up the sodium sulphate. A 10-μl portion of TFA was applied to the solution to convert amino acid isopropyl esters into non-volatile TFA salts. After the excess of reagents had been removed under a stream of nitrogen, the dry residue was dissolved in 1 ml of dichloromethane, ultrasonicated and acylated with 200 μl of TFAA at room temperature for 30 min. The excess of reagents and solvent were removed under a gentle stream of nitrogen at 40°C. A 100-μl volume of dichloromethane was added to dissolve the final derivatives and 1–3 μl aliquots were injected into glass capillary column.

Ion-exchange method (IEM). The dry residue was dissolved in 10 ml of 0.1 *M* hydrochloric acid, passed through the column of Dowex 50W-X8, washed with 10 ml of water and the amino acids were eluted with 10 ml of 3 *M* ammonium hydroxide. The solvent was evaporated to dryness in a rotary evaporator at below 40°C. A 2.00-ml volume of esterification reagent was added to the dry residue and the mixture was heated to 100°C for 30 min. The vial was opened and the excess of reagents was removed under a stream of nitrogen with heating at 100°C. A 1-ml portion of dichloromethane and 200 μl of TFAA were added to the dry amino acid ester hydrochlorides and the mixture was left at room temperature for 30 min. The excess of reagents was evaporated to dryness under a gentle stream of nitrogen and the residue was dissolved in 100 μl of dichloromethane.

Operational conditions for GC

Typical operating conditions for GC using the EGA packed column are as follows: injector temperature, 250°C; carrier gas, helium; inlet pressure, 2.5 kg/cm^2; oven temperature, 80°C, programmed to 190°C at 7.5°C/min.

In a case of the capillary column the conditions were as follows: injector temperature, 250°C; carrier gas (helium) flow-rate, 45 ml/min; splitting ratio, 1:40; oven temperature, 80°C, held for 4 min and then programmed to 190°C at 4°C/min.

RESULTS AND DISCUSSION

Distribution ratio and pH

Since the amino group is a water-miscible function, the distribution ratios of amino acid esters depend largely on the pH of the buffer added. The relationship between the distribution ratio, *D*, and the pH of the buffer can be approximated as[19]

$$\log D = \log K_2 - \log K_1 + \text{pH} \quad (1)$$

where K_1 is the equilibrium constant associated with the protonation of the amino acid

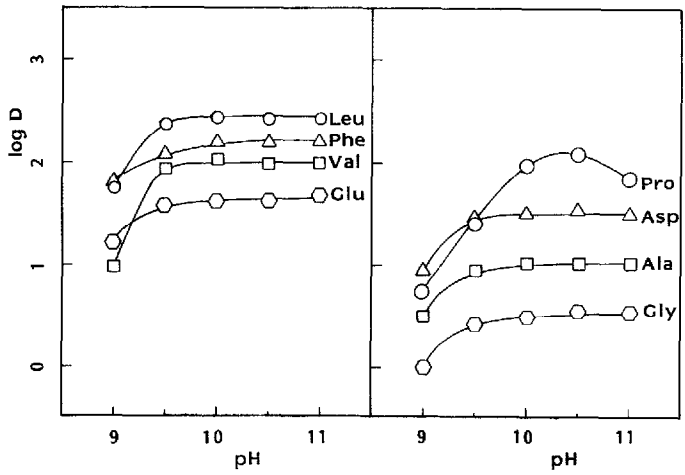

Fig. 2. Influence of pH on the distribution ratio of amino acid isopropyl esters.

isopropyl ester in the aqueous layer, and K_2 is that related to the distribution of the amino acid isopropyl ester between the organic and aqueous layers. Fig. 2 illustrates this relationship. The distribution ratio, D, becomes constant at pH >10, except for Pro which shows a maximum in D at pH 10.5. The optimum pH for the buffer extraction was 10.5. Table I shows the extraction efficiencies (EE) for the amino acid isopropyl esters in diethyl ether, chloroform, carbon tetrachloride and dichloromethane as the extraction solvents. EE was calculated from

$$\text{EE}\ (\%) = 100 \cdot \frac{C_{A,S}V_S}{C_{A,S}V_S + C_{A,B}V_B} = 100 \cdot \frac{D}{D + V_S/V_B} \tag{2}$$

where $C_{A,S}$ and $C_{A,B}$ are the concentrations of amino acid isopropyl ester in the solvent and buffer, respectively, V_S and V_B are the volumes of solvent and buffer, respectively. The values of EE increased with increasing solvent polarity. Therefore, chloroform or dichloromethane, the high polarity solvents, enabled efficient extraction of Val, Leu, Pro, Asp, Phe and Glu, a little lower with Ala and Gly. Carbon tetrachloride was the next most effective, except for Ala, Gly and Hyp. Diethyl ether was least effective among the solvents tested. From these data, dichloromethane is the most suitable solvent for extraction, considering also its low boiling point.

TABLE I

EXTRACTION EFFICIENCIES (%) FOR AMINO ACID ISOPROPYL ESTERS

NM = Not measurable.

Solvent	*Ala*	*Val*	*Gly*	*Leu*	*Pro*	*Hyp*	*Asp*	*Phe*	*Glu*
Diethyl ether	27	87	12	70	56	5	87	94	96
Chloroform	85	99	50	99	99	33	99	97	99
Carbon tetrachloride	67	96	42	99	98	NM	92	99	96
Dichloromethane	91	97	80	98	99	24	98	98	99

TABLE II

RECOVERIES (%) OF AMINO ACIDS

Procedure as shown in Fig. 1.

Treatment method	*Ala*	*Val*	*Gly*	*Leu*	*Pro*	*Hyp*	*Asp*	*Phe*	*Glu*
Solvent extraction	39	79	11	90	90	7	95	92	96
Ion exchange	81	80	77	80	82	96	93	77	90

Recovery of amino acids

Table II shows the recoveries (%) of amino acids by SEM and IEM. The recoveries were obtained by dividing each datum by the quantitative value obtained directly from a derivatized standard amino acid mixture. The recoveries of Ala, Gly and Hyp were low in SEM, but the other amino acids were recovered comparably or even more effectively than with IEM. The recoveries in IEM were not constant for all amino acids. Although the recoveries in SEM were not quantitative, with respect to Leu, Pro, Asp, Phe and Glu, this method is considered to be better in this respect than IEM. For the quantitative anaysis of these amino acids or for enantiometric analysis of all amino acids except Hyp, SEM is preferred.

Esterification of amino acids in the presence of salt

Since amino acids are functionalized with amino (imino) and carboxyl groups, their simultaneous extraction into an organic solvent without any pre-treatment is impossible. In this study, amino acids were esterified to give lipophilic properties before extraction. We investigated the influence of salt on the esterification of amino acids with isopropanol. Hydroxyapatite was used as a salt which constitutes human teeth. Table III shows the esterification yield of amino acids with isopropanol in the presence of various amounts of hydroxyapatite. The procedure is as follows.

A 2 *M* hydrochloric acid solution of a hydroxyapatite was mixed with a 100-μl volume of amino acid stock solution in a Reacti-Vial in an appropriate ratio and dried under a stream of nitrogen with heating at 100°C. The mixture was esterified with isopropanol and acylated with TFAA as described. Finally, the derivatives were dissolved in a 100 μl of biphenyl internal standard solution and 2–3 μl aliquots were injected for GC. The esterification yields decreased gradually with increasing amount of hydroxyapatite. This is particularly masked for Val. Other amino acids show

TABLE III

ESTERIFICATION YIELDS (%) OF AMINO ACIDS IN THE PRESENCE OF HYDROXYAPATITE

Hydroxyapatite/amino acid (M/M)	*Ala*	*Val*	*Gly*	*Leu*	*Pro*	*Asp*	*Phe*	*Glu*
0	100	100	100	100	100	100	100	100
10	80	85	89	99	93	92	98	99
50	73	78	82	88	90	92	102	99
100	85	82	88	98	85	83	101	95
200	76	69	96	85	82	87	91	88

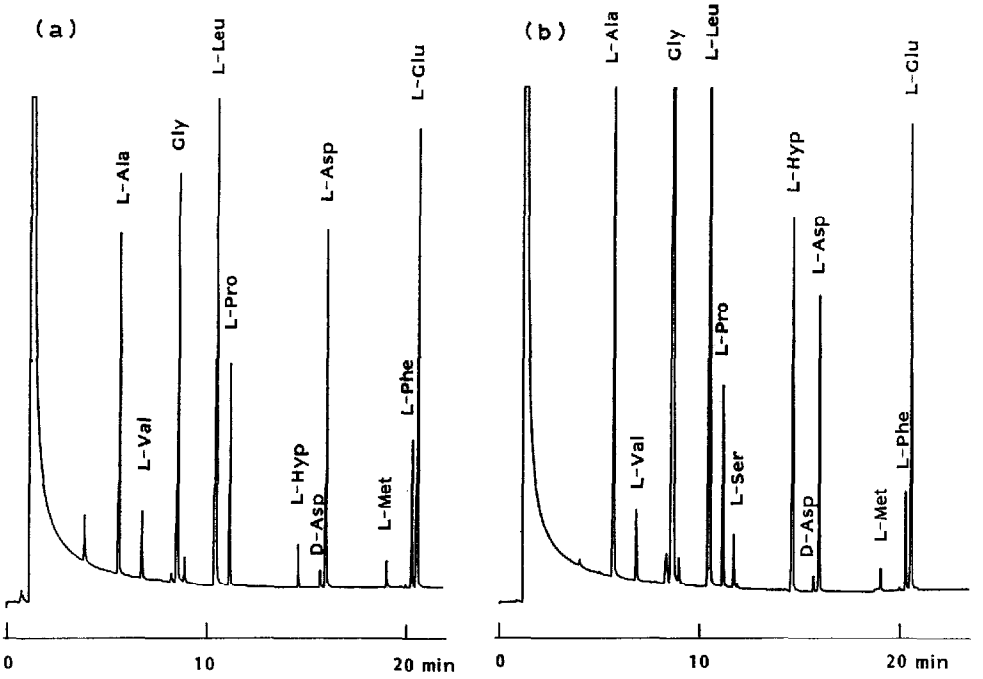

Fig. 3. Chromatogram of amino acids in dentine (41 years old, masculine): (a) solvent extraction method; (b) ion-exchange method. For other conditions see text.

76–96% of the yields in the case of a 200-fold (M/M) addition of hydroxyapatite per each amino acid.

Age estimation from the D/L ratio of Asp in human dentine

Fig. 3a shows a typical chromatogram of amino acids in dentine treated with SEM and Fig. 3b that with IEM. Comparing (a) with (b), the peak areas of Ala, Gly and Hyp obtained by SEM were smaller than those by IEM. However, the areas of Leu, Pro, Asp, Phe and Glu obtained by SEM were almost the same as those by IEM. The D-amino acid was observed only for Asp in (a) and (b). Table IV presents the D/L ratio of Asp and the coefficient of variation (C.V.) determined from seven types of teeth ranging in ages from 18 to 60 years. The D/L ratios obtained from SEM were slightly lower than those from IEM. The reason for this is not yet fully understood, but it might be due to a low degree of racemization when Asp is adsorbed on or desorbed from the ion-exchange resin and when evaporated from the ammonium hydroxide after elution from the ion-exchange column. Since the average C.V. obtained by SEM is somewhat smaller than that obtained by IEM, SEM is believed to be superior in reproducibility to IEM.

Fig. 4 shows a plot of

$$\ln \frac{1 + \mathrm{D/L}}{1 - \mathrm{D/L}} = 2kt + C \tag{3}$$

TABLE IV

RACEMIZATION OF ASP IN HUMAN DENTINE

Age (years)	*Number of samples*	*Solvent extraction*		*Ion exchange*	
		D/L	*C.V. (%)*	*D/L*	*C.V. (%)*
18	6	0.034	7.84	0.037	9.67
27	6	0.043	4.81	0.044	7.18
36	6	0.046	8.85	0.048	4.63
41	7	0.050	1.86	0.051	2.99
49	6	0.051	2.86	0.052	3.39
54	5	0.059	2.73	0.060	5.01
60	6	0.061	3.57	0.063	3.13

based on the data given in Table IV where t is the age of the dentine in years and k is the racemization rate constant of Asp. A least squares fit of the data yields for

$$\ln \frac{1 + \text{D/L}}{1 - \text{D/L}} = 2 \cdot 6.2 \cdot 10^{-4} t + 0.048 \tag{4}$$

$$\ln \frac{1 + \text{D/L}}{1 - \text{D/L}} = 2 \cdot 6.05 \cdot 10^{-4} t + 0.052 \tag{5}$$

for SEM and IEM respectively. The correlation coefficient, r, was 0.982 for SEM and 0.984 for IEM. It is thought that r needs to be improved in order to specify the kind of the tooth and the sampling site of the dentine in the tooth.

CONCLUSIONS

Solvent extraction clean-up, a purification method of samples for GC analysis, showed excellent recoveries of amino acids except Ala, Gly and hydroxy amino acids compared with the conventional ion-exchange clean-up. This method is of special interest for enantiomeric analysis. D/L ratios determined by SEM were smaller slightly

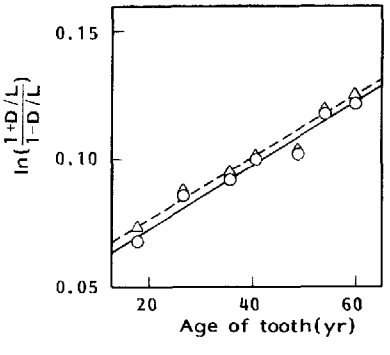

Fig. 4. Plot of $\ln[(1 + \text{D/L})/(1 - \text{D/L})]$ for Asp against the age of dentine: ——, solvent extraction method; – – –, ion-exchange method.

than those by IEM. The method is thought to be suitable for the treatment of apatite or calcareous materials. On the contrary, organic matter causes difficulties (interference with amino acid peaks).

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