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Aminoacids in Sea-Water by Gas-Chromatography.

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The difficulties concerning the analysis of organic materials in sea water arise from the high salt content, as previously mentioned by many authors. Reports have appeared describing the isolation, separation and identification of amino acids from ~~unhydrolysed~~ sea water samples [1-8]. The methods include coprecipitation of organic materials with heavy metal hydroxides; evaporation combined with filtering off the inorganic salts and ionexchange of the concentrate [3], the use of ion retardation technique [7], extraction of aminoacids as their dinitrophenyl derivatives after reaction with dinitrofluore-benzen and thin layer chromatography [4], extraction of aminoacids as their dansyl-derivatives after reaction with dansyl cloride and thin layer chromatography [9] (unpublished data). The use of ligand exchange chromatography [6], combined with a Technicon Aminoacid Analyser [8] seems to be the best method so far. The analysis in the aminoacid analyser, however, takes many hours and needs a fairly great sample. So in the search for a more rapid method combined with smaller samples, we have done som work where we have applied gas chromatography as the final step in the analysis. This has made it possible to use samples in the order of 50-200 mls, and it is possible to use samples in the order of 10 mls in connection with the use of electron capture detector instead of flame ionization detector.

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1.. Apparatus.

A Perkin-Elmer 900 gas chromatograph, equipped with a dual flame ionization detector and an electron capture detector, employed with a Hitachi Perkin-Elmer 165 recorder.

A Perpex peristaltic pump from LKB-Products, Sweden.

A Büchi Vacuum Rotary Evaporator-R from W. Büchi Glassapparatefabrik, Flawi, Switzerland.

An ultrasonic mixer, type Son Blaster series 200 from the Narda Ultrasonic Corp., Westbury, L.I., New York.

Millipore filtration apparatus and H.A. Millipore filters HAWP 47 mm (0,45u) from Millipore Filter Corporation, Bedford, Massachusetts, USA.

Syringes for injection in the gaschromatograph, Hamilton Type 7101N.

2. Reagents.

All reagents were purchased from E. Merck, A.G. Darmstadt, Germany, unless otherwise stated.

Chelex 100, analytical grade chelating resin, 50-100 mesh sodium form from Bio-Rad Laboratories, Richmond, California, USA.

Dry HCl gas min. 99,0% purity was obtained from J.T. Baker Chemical Co., Phillipsbury, New Jersey, USA.

Dry methanol was made according to Vogel,[10] by refluxing over magnesium turnings for 2 to 3 hours and destilled.

Anhydrous methylen chloride was refluxed over anhydrous $CaCl_2$ for 30 minutes and destilled.

Highly purified nitrogen gas, purchased from Norsk Hydro, Norway was used as carrier gas.

Silicic acid 0,2-0,5 mm was purified according to Waterfield and Del Favero[11], to prevent the formation of gas during elution with ether, water jacketed columns were used.

Molecular sieve 5 A was used to dry the N_2-gas used to evaporate samples.

3. Columns for gas liquid chromatography.
The column packing used was Tabsorb Cat. No. TA-33, Lot. No. P32-103, a specially developed polyester coated column packing for N-trifluoroacetyl amino acids n-butylesters, purchased from Regis Chemical Company, Chicago, Illinois, USA.

Columns used were of stainless steel 200 cm long, with inner diameter 2 mm. The weight of Tabsorb per column is approx. 2,5 grams.

4. Instrument setting.
Initial column temperature 100°C, final temperature 210°C.
Program rate 4°C/min.
Injector block 290°C, manifold 270°C.
Nitrogen carrier gas flow, 24 ml/min.
Hydrogen flow, 35 ml/min.
Air flow, 400 ml/min.
Chart speed, 10 mm/min.

5. Outline of the procedure.
The method combines ligand-exchange chromatography as the isolation step and gas liquid chromatography modified after Gehrke et. al.[12] or direct evaporation and gas liquid chromatography as the final step.

Isolation of the amino acids.

I 1. The sea water sample was filtered through H.A. Millipore filter (0,45µ) and adjusted to pH 2, the free fatty acids were extracted with 3 x 10 mls of ethylacetate per 100 ml sample. Ethylacetate remaining in solution was evaporated in a round bottomed flask on an all glass vacuum evaporator, the pH of the sample was adjusted to 9,5 and passed through a column of 10 mls copper-chelex at a flow rate of 4 mls per minute. The column was washed with distilled water untill the wash water was free of chloride ions. Elution of the sorbed aminoacids was performed using 3,0 M ammonia. According to Siegel and Degens a short column of chelex-100 in NH^+-form was put in series with the copper-chelex column at this stage of the procedure in order to capture the liberated copper ions. The ammonia effluent was evaporated to dryness in a round bottomed flask and to ensure complete removal of water it was further dried azeotropically with dry aceton or stored overnight in a dessicator over phosphorous pentoxide. The sample was then ready for part two; preparation for gas liquid chromatography.

2. The sea water sample, after filtration and extraction of free fatty acids, was adjusted to pH 7,0 and evaporated to dryness and further dried azeotropically with aceton, and prepared for gas liqued chromatography.

II The preparation of the amino acids for gas liquid chromatography.

1. Methylation.

To the dry sample, still in the round bottomed flask is added 5 ml of anhydrous methanol containing 1,25 meq/ml of dry HCl gas. The flask is stoppered with a ground glass stopper and mixed manually followed by ultrasonic mixing for 30 seconds and left for esterification 30 minutes at room temperature.
If isolation step 1 a is used, the methanol is evaporated by vacuum rotary evaporator at 60°C or the sample is transferred to a acylation tube with a little extra dry methanol and dried on a sand bath at 100°C with a stream of dry N_2-gas.

2. Butylation.

a) To the sample in the acylation tube is added 2 ml of butanol containing 1,25 meq/ml dry HCl gas, the tube is closed and mixed manually and by ultrasonics, and the interesterification is left to completion for $2\frac{1}{2}$ hours at 100°C on an oil bath.

b) Alternatively according to the method of Coulter and Hahn[13] are using when preparing the n-propyl derivatives, 2 mls of butanol containing 8 meq/ml of HCl is added, mixed and left for 10 minutes at 100°C, evaporated by a stream of dry N_2-gas and the same procedure repeated once more. The butanol is evaporated with a stream of dry N_2-gas or on a Vacuum Rotary evaporator at 60°C.

3. Acylation.

To the sample, now containing amino acid-butylesters, is added 1,6 ml dichlormethane and 0,4 ml trifluoroacetic-acid anhydride. The tube is closed and mixing is effected manually and in the ultrasonic equipment.

The acylation procedure can be done in two ways; at 150°C in 5 minutes in an oilbath, or at 100°C in one hour on a sand-bath. After this procedure the tube is cooled down and evaporation is done very carefully (to prevent the trifluoroacylated butylamino-acids TAB to volatilise), or the mixture is purified on a silicic acid column as described by Waterfield and Del Favero[11] and evaporated.

The sample is then dissolved in a measured volume of dichlormethane (100 µl) and part of this (0,5 µl) is injected on the gas chromatograph. If nesessary concentrate further.

The volume is not critical if an internal standard is used.

6. Results and discussion.

The results of analysis so far obtained, using the procedure described of a standard mixture of amino acids (fig. 1), of samples of sea water both unhydrolysed (fig. 2) and hydrolysed (fig. 3), and the quantitative estimation of the contents of amino acids in the example given in fig. 3, (table I), are very promissing.

The calculation of the contents (table I) is based on the width at half height times the height of the peaks, ornithine used as the internal standard. In fig. 2, 17 amino acids with known retention times are found since lysine is hardly distinguishable. All the 18 amino acids with known retention times are found in the hydrolysed sample (ornithine was added as internal standard). In both samples there are one distinct peak between alanine and valine, four peaks between aspartic acid and glutamic acid. There are also four peaks between glutamic acid and tyrosine in the hydrolysed sample, but five in the unhydrolysed sample. In both samples a double peak is found after tyrosine and four peaks after tryptophane. Work on the identification of these peaks has now been initiated.

Considering that only 0,5 to 1% of the isolated and reacted sample is injected into the gaschromatograph, this method opens up enormous possibilities. All compounds that can be volatilized as such or reacted to give volatile compounds can be determined in a gas chromatograph.

By combining a split stream system to the gaschromatograph, and thereby take out 9/10 of the different peaks for analyses in an infra-red spectrophotometer it is possible to "fingerprint" the identification of the different compounds, as one micro gram or even 100 nanograms are enough to give a quantitative infra-red spectrum.

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Table I

The retention times and the retention temperatures of 18 amino acids, and the amount of the 15 amino acids found in the sample shown in fig. 3. The table also shows the relative molar response of the amino acids towards ornithine.

Amino acids as their TAB* derivatives	Retention time (min.)	Retention temp. (°C)	Content µg/L	Relative Molar Response amino acid/orn.	
				Our Results	From Gehrke [12]
Alanine	4,10	116,5	6,50	0,808	0,683
Valine	5,20	120,8	3,30	0,982	0,901
Glycine	5,95	123,8	20,00	0,609	0,537
Isoleucine	6,60	126,4	4,50	1,074	1,025
Leucine	7,60	130,4	3,00	1,102	1,050
Proline	8,50	134,0	3,50	0,998	0,923
Threonine	9,25	137,0	7,25	0,879	0,841
Serine	11,05	144,2	1,30	0,681	0,802
Cysteine	13,65	154,6	-	0,460	-
Methionine	14,20	156,8	0,85	0,973	0,863
Hydroxyproline	14,75	159,0	5,10	0,962	1,088
Phenylalanine	15,70	162,8	4,80	1,414	1,494
Aspartic acid	16,50	166,0	10,50	1,143	1,192
Glutamic acid	20,00	180,0	24,10	1,273	1,367
Tyrosine	22,30	189,2	-	0,394	1,298
Ornithine	24,50	198,0	25,00	1,000	1,000
Lysine	26,25	205,0	8,50	1,167	1,168
Tryptophane	27,35	209,4	1,25	0,961	-

* TAB = N-trifluoroacetyl-n-butyl ester

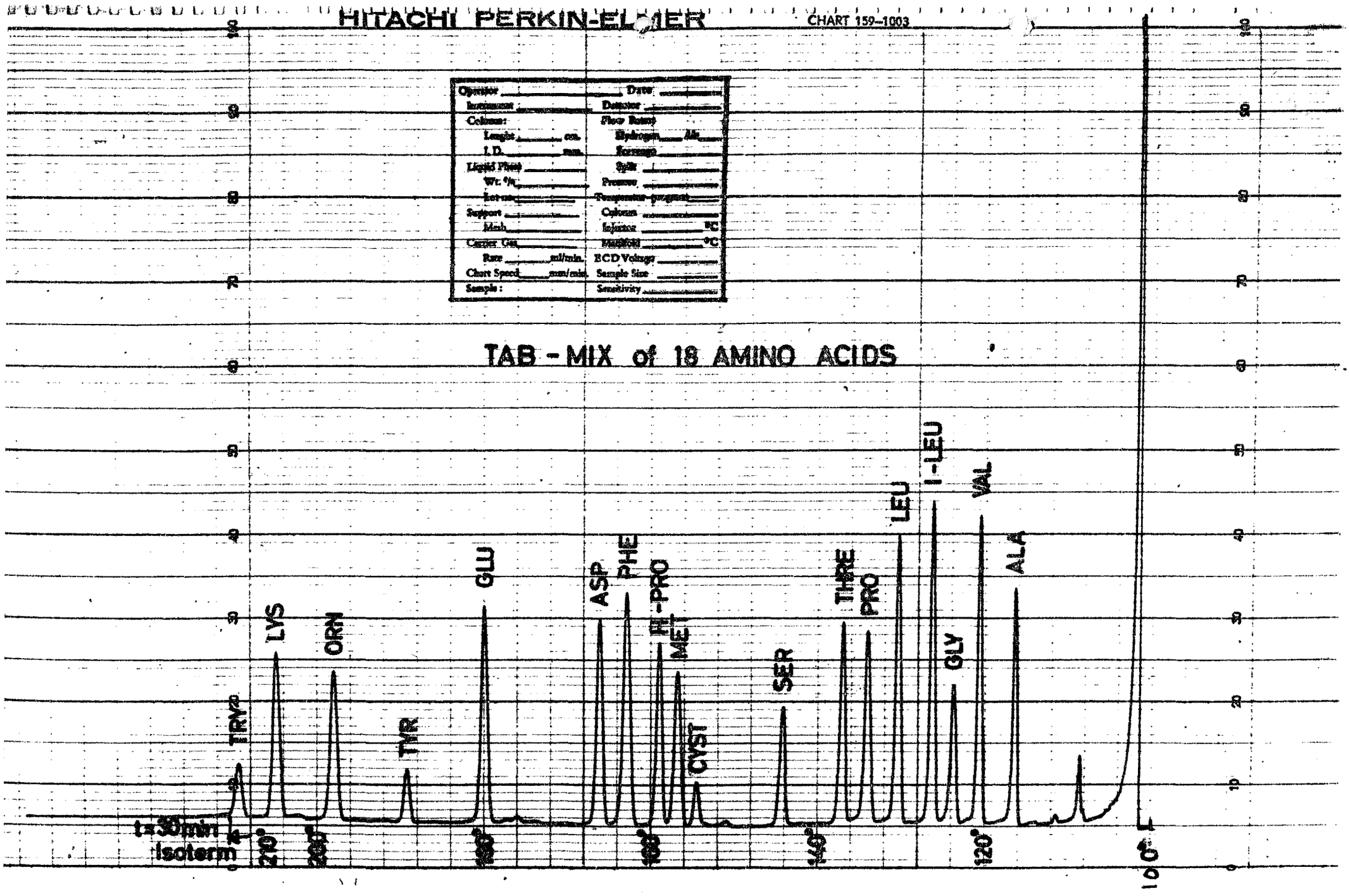
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Fig 1

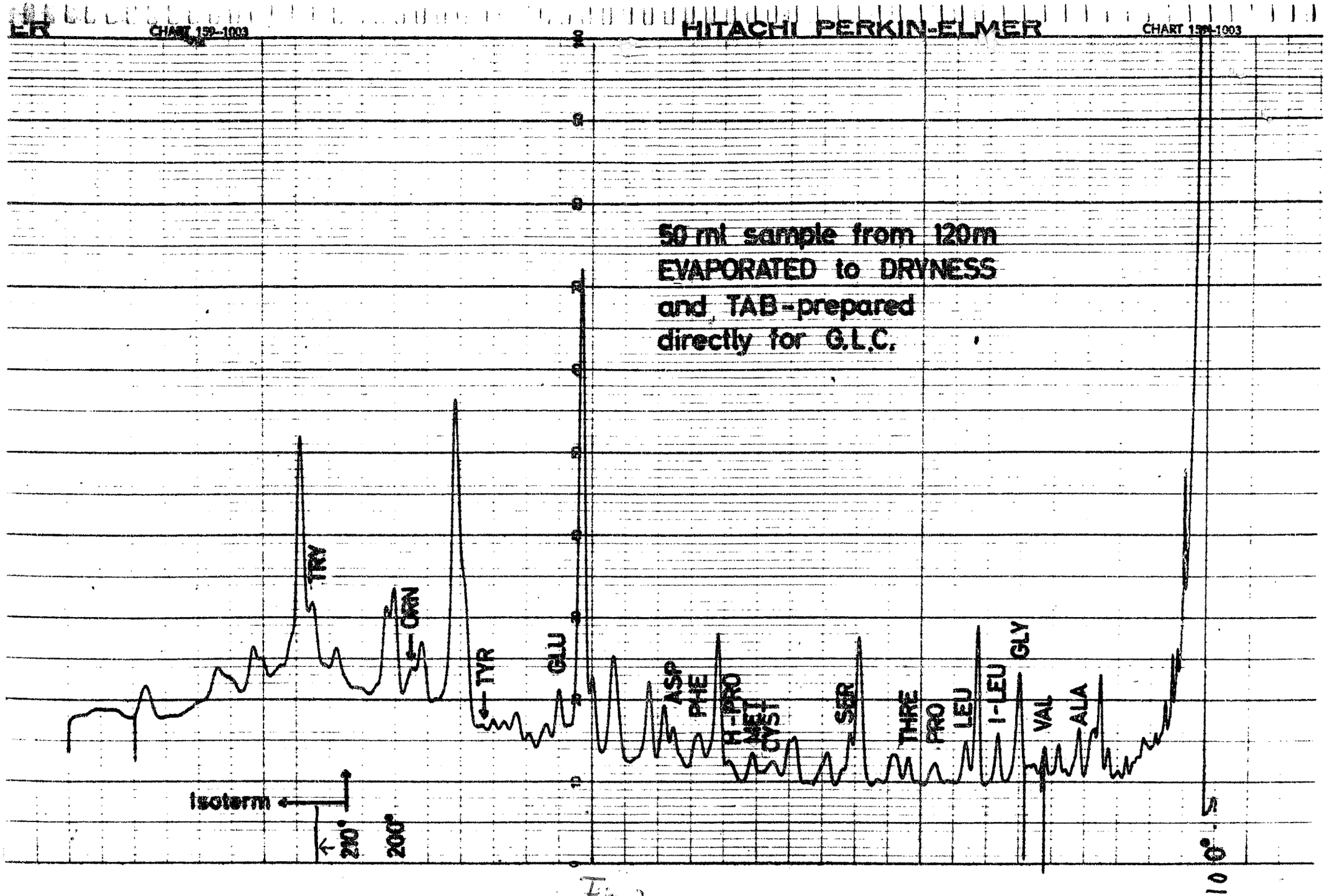
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Fig. 2