Method of

# Analysis for Free Amino Acids

## In Conifer Bark and Needles Using Gas-Liquid Chromatography

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# METHOD OF ANALYSIS FOR FREE AMINO ACIDS IN CONIFER BARK AND NEEDLES USING GAS-LIQUID CHROMATOGRAPHY

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## INTRODUCTION

Experimental evidence has shown that a method using gas-liquid chromatography (g.l.c.) for analyses of biological materials for amino acids, and performed with standard laboratory equipment (Gehrke et al. 1968), is more rapid than the conventional ones, such as ion-exchange (amino acid analyzers), paper chromatography, thin layer chromatography (t.l.c.) and t.l.c. combined with high voltage electrophoresis. Blau (1968) and Blackburn (1968) have published reviews on the various methods, including g.l.c. for amino acid analysis.

Although g.l.c. has not, to my knowledge, been adopted by forestry research laboratories, it does warrant consideration because of the increasing demand for analysis of coniferous tissue for amino acids.

In the development of a method suitable for our requirements, the extraction method of Bielski and Turner (1966), as outlined by McMullan (1971), was adapted and combined with the g.l.c. methodology of Gehrke et al. (1968), as modified for direct esterification by Roach and Gehrke (1969).

This report outlines the materials and procedures used by us, with detailed comments on various aspects of the analysis. We have utilized the publications by Dr. Gehrke and his associates, with slight modifications to suit our purpose.

## THEORY

The development of satisfactory quantitative g.l.c. procedures for analysis of plant material, particularly conifer needles and bark, depends on:

a) Complete extraction of amino acids from the sample and the concentration and removal of interfering substances such as chlorophylls,

lipids, carbohydrates, fatty acids, etc.

b) Complete quantitative and stable derivatization procedure, required because amino acids are not sufficiently volatile to be analyzed directly.

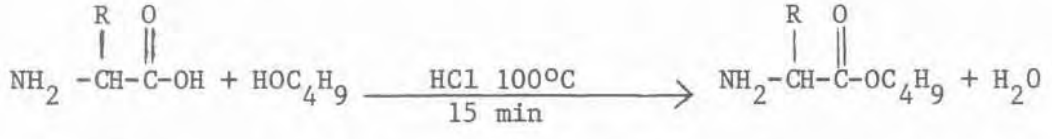
c) Good resolution of individual amino acids so that reproducible, accurate quantification of the peak areas can be obtained.

Extraction and sample cleanup

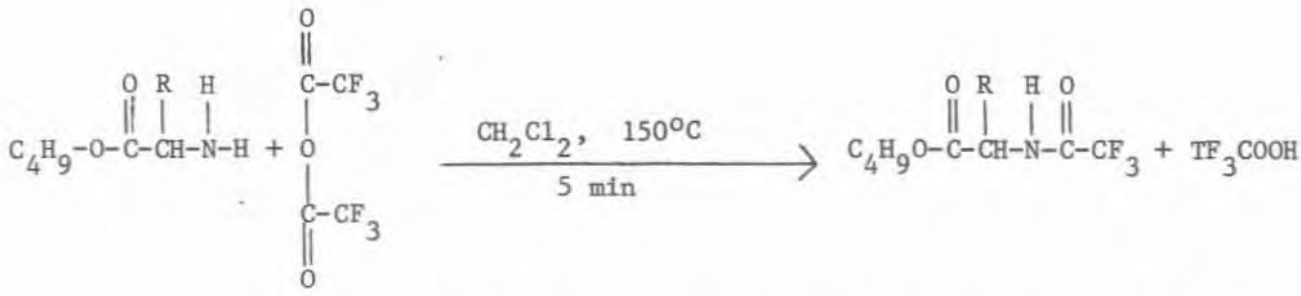
The free amino acids are extracted from bark or needle tissue in a mixture of chloroform and water, using an ultrasonic bath to aid in thorough mixing. After saturation with chloroform to produce two phases, the amino acids remain in the aqueous layer. The chloroform containing fatty materials and chlorophyll is discarded. Organic acids, sugars and other contaminants are removed by adsorbing the amino acids on a cation exchange column. Phenolics and other contaminants are removed by using a mixture of acetone and water. Amino acids are removed from the column by elution with 2N $NH_4OH$. A known amount of butyl stearate is added as an internal standard and the whole is dried under vacuum.

Derivatization

Volatile derivatives that can be successfully separated on a g.l.c. column are prepared by esterifying the amino acids to their n-butylesters.



Acylation with trifluoroacetic anhydride produces N-trifluoroacetyl-n-butylesters and trifluoroacetic acid (TFA).




These butylesters are less volatile than the methylesters used in previous methods, and losses due to evaporation during derivatization are less likely to occur.

Gas-liquid chromatographic analysis

To obtain separation of all amino acids, the derivative is injected into a dual-column gas chromatograph, each column containing a different packing. The resultant peak areas are electronically integrated, and the values obtained relative to the internal standard are then compared with those obtained from a standard mixture of amino acids relative to the same amount of internal standard. After calculation, the results are expressed as w/w% or µmol/gm (dry weight).

## REAGENTS AND EQUIPMENT

Reagents and materials

1. Amino acids (chromatographically pure)

   Mann Research Laboratories, New York, N.Y.

   The British Drug Houses Ltd.

2. 2N $NH_4OH$ - 133 ml conc (28%) $(NH_3)NH_4OH$ diluted to 1 liter with distilled water.

3. 3N HCl in n-butanol - obtained by passing dry HCl gas through n-butanol. The normality is checked by titration.

4. 0.1N HCl.

5. Methylene chloride (anhydrous)

   Reflux 1000 ml ACS reagent grade $CH_2Cl_2$ over 25 g of anhydrous $CaCl_2$ for 30 min. Distill in all-glass apparatus and store protected from atmospheric moisture.

6. Acetone/water mixture 1:1.

7. Dowex 50W-X8-strong acidic, cation exchange resin, 50-100 mesh spheres, "Baker Analyzed Reagent". An alternative resin is Amberlite IR-120-H (100-200 mesh) as used by Gehrke et al., though no comparative data were obtained.

8. Trifluoroacetic anhydride; Eastman grade.

9. Silanized spun glass wool.

Solutions

1. M.C.W. Solvent

   methanol/chloroform/water, 120:50:30 vol/vol.

2. Basic Standard Amino Acid Solution

   Contains 0.1 mg of each amino acid per ml water.

3. Working Standard Amino Acid Solution

   A 1:4 aqueous dilution of the Basic Standard contains 0.025 mg/ml of each amino acid.

4. Basic Butyl Stearate Solution of n-butanol

   Contains 1 mg butyl stearate per ml. Purchased from Regis Chemical Co., Chicago, Ill.

5. Working Butyl Stearate Solution (Internal Standard)

   A 1:40 dilution with n-butanol of the Basic Butyl Stearate Solution contains 0.025 mg/ml internal standard.

Equipment and glassware

1. Flat-bottom boiling flasks, 125 ml, $\bar{\S}$ 24/40 and other standard laboratory glassware (Note 1).
2. Acylation tubes, screw cap culture tube 10 ml. To minimize loss through leakage, the rims were ground flat and the Teflon coated lining was replaced with an all-Teflon lining. Practically no leakage has occurred since using this procedure.
3. Syringes. Hamilton 701N - 10 µl.
4. Esterification was carried out on a Multiple Magnetic hot plate stirrer at 100°C in 125 ml flat-bottom flasks fitted with drying tubes containing $CaSO_4$. The flasks were placed in individual beakers with paraffin oil and held down by placing lead rings around their necks. The temperature in each beaker should be observed to prevent it from drifting.
5. An all-Teflon rotary evaporator (CaLab[1], Model C) was used to remove the volatile solvents. Vacuum was produced by a Welch Duo-Seal vacuum pump (Model 405) with a capacity of 60 liters/min. A cold-finger condenser, a sodium hydroxide trap and $CaSO_4$ filters were placed between the evaporator and the pump to protect it from volatile and corrosive acidic compounds.
6. To provide the low temperature in the cold-finger condenser, the coil of a "Flexi-cool"[2] refrigeration unit was placed in the inside well of the condenser, filled with ethylene glycol monomethyl ether.
7. An infrared heat lamp suspended over the evaporator, aided in the evaporation. Care should be taken that the sample flask does not get too hot,

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[1] California Laboratory Equipment Company, 1399-64st St., Emeryville, California, 94608, U.S.A.

[2] FTS Systems, 31 Horsenden Rd, Newpaltz, New York, 12561, U.S.A.

particularly after all solvent is removed.

8. The closed tube acylation was carried out in an oilbath with a thermostatically-controlled heater.
9. A safety shield from Instruments for Research and Industry ($I^2R$) was used to provide protection from accidental breakage of the acylation tube.
10. Automatic pipettes are recommended for dispensing BuOH·HCl and $CH_2Cl_2$.
11. Ion-exchange column 150x9 mm (ID) Chromaflex Column #2 with "Varibor" Teflon stopcock adapter (Kontes Glass Co.).
12. A Mettler ultrasonic cleaner Model 1.5 was used for mixing during the extraction procedure and also in subsequent steps to loosen materials coated on the walls of the glassware.

Instrument

A Varian Aerograph Model 1520 gas chromatograph with small dual-column oven, equipped with dual flame-ionization detectors and dual differential electrometers, was used with a linear temperature programmer. The signal was fed through a Hewlett Packard digital integrator into a Westronics dual pen recorder Model LD11A.

Glass columns, 6 ft x ¼ inch OD (4 mm ID), were custom made to fit the oven-fitting configuration. A number of "Swagelock" fittings were used to mate the glass tubes to the 1/8-inch fitting of the transfer line to the detector. Double O-rings were used in the connections.

Substrates and Supports

Column A.-Ethylene glycol adipate (EGA) on Chromosorb W, 0.65w/w% for the separation of most amino acids.

Column B.-2.0 w/w% OV-17 + 1.0 w/w% OV-210 on Supelcoport for separation of Serine, γ-aminobutyric acid, histidine, arginine and tryptophan.

Both column packings were purchased from Analytical Bio Chemistry Laboratories, P.O. Box 1097, Columbia, Mo. 65201, U.S.A.

Preparation of the column materials is outlined by Zumwalt et al., 1971.

Column A should last about 2 months. Column deterioration shows in loss of the glycine-valine separation, loss of resolution in the methionine-hydroxyproline-phenylalanine region and loss of separation of ornithine and n-butylstearate.

Column B should last 3-6 months. Deterioration is indicated by reduced RWR aa/IS (see page 10) values for arginine and cystine.

Instrument Conditions

Injector temperature 225°C

Column Temperature 95°C-225°C

Program Rate 6°C/min.

Detector temperature 250°C

Electrometer Range x 1

Attenuator x 16

Carrier-gas flow ($N_2$): 40 ml/min

Air to detector 300 ml/min

Hydrogen 20 ml/min

Chartspeed 15 in/hr

Integrator recorder representation x 2

## PROCEDURE

### Sample Preparation (conifer bark and needles)

Field-collected samples were transported in dry ice to the laboratory. Bark was cut in slivers and samples were freeze-dried for 24 hours. The samples were ground in a Wiley mill to pass through a 32 mesh screen.

The powdered samples were again freeze-dried and stored below 0°C.

Extraction and purification

The sample was brought to room temperature in a dessicator. For the extraction, 500.0 mg of powdered tissue was placed in a 15 ml centrifuge tube to which 8 ml M.C.W. was added. After thorough stirring and mixing, the tube was placed in an ultrasonic bath for 10 min at room temperature. The mixture was centrifuged for 10 min at approx. 2900 rpm and the supernatant transferred to a 50 ml centrifuge tube. This extraction was repeated three times. To the combined supernatants were added 8 ml chloroform and, after stirring, 12 ml distilled water. After thorough mixing by inversion, the tubes were centrifuged for 10 min at 2900 rpm and the upper phase was transferred to a 125 ml flat-bottom flask. The bottom layer was discarded. In a waterbath at 60°C, the extract was evaporated to dryness under vacuum.

Ion-exchange cleanup

A 9 mm I.D. column was filled with water, and the Dowex 50W-X8 (ionic form H+) resin, which had stood overnight under water, was added to a height of 7 cm. The resin was washed with 30-40 ml water and drained so that the liquid level was at the resin surface. The dried extract was dissolved in a small amount of 0.1N HCl, carefully placed on the resin and slowly passed through the column max 20 drops per min. The flask was rinsed with 10 ml 0.1N HCl in several portions, followed by 10 ml distilled water, and the eluant discarded. Because of the small amounts involved, the flow through the column was kept slow. To remove certain phenolics and other contaminants, 5 ml of a 1:1 mixture of acetone and water was passed through the column, followed by a 5 ml water rinse. The amino acids were removed from the resin column with 30 ml of 2N $NH_4OH$ and the eluant was collected in a 125 ml flat-bottom flask.

Esterification

An exact amount (5.0 ml) of Working Internal Standard Solution (0.125 mg) was added to the above amino acid mixture. For the calibration mixture, the exact same amount (5.0 ml) of Working Internal Standard Solution was added to 5 ml of Working Calibration Standard Solution (0.125 mg of each amino acid). The solutions were evaporated at 60°C under vacuum to dryness; 2-3 ml $CH_2Cl_2$ was added to azeotropically remove the remaining water. After adding 1.5 ml n-butanol-HCl, a drying tube with $CaSO_4$ was attached and following mixing in an ultrasonic bath the flask, weighed down with lead rings, was placed in an oilbath at 100°C for 15 min. After evaporation to dryness at 60°C under vacuum, the water was again removed azeotropically with $CH_2Cl_2$.

Acylation

0.9 ml $CH_2Cl_2$ and 0.1 ml TFAA (note 4) were added to the flask and mixed in an ultrasonic bath and placed into two acylation vials to safeguard against loss through leakage of the vials. The rims of the vials were checked for unevenness and securely closed with Teflon liner in cap. A thick piece of rubber tubing was used to aid in tightening and to protect hands.

The vials were then placed in an oil bath at 150°C for 5 min. After cooling, the solution was evaporated just to dryness with $N_2$ and $CH_2Cl_2$ (note 4) added to the original volume.

Gas chromatography

5 µl of acylated solution was injected on each column, using the prescribed instrumental settings. The temperature program consisted of a 6 min isothermal period (95°C) after which the temperature was increased at a rate of 6°C per min until the last peak had appeared. For the EGA column, this occurred at approx. 200°C; the OV column ran to approx. 235°C. These

temperature values depend on the carrier gasflow, the type of sample being analyzed, and the performance of the column. With the equipment at hand, it was necessary to run each sample in triplicate.

The data produced by the integrator consist of the retention times of the peaks and a number of units representing the peak area.

To calculate the w/w% of the amino acids present in the sample, it is necessary to determine the Relative Weight Response of each amino acid to the Internal Standard in the calibration mixture

$$RWR_{a.a./IS(Std.\ mix)} = \frac{\text{Area of amino acid (Standard mixture)}}{\text{Area of Internal Standard (Standard mixture)}}$$

and in the sample

$$RWR_{a.a./IS\ (Sample)} = \frac{\text{Area of amino acid (Sample)}}{\text{Area of Internal Standard (Sample)}}$$

The w/w % of each amino acid is then calculated by substituting these values in the following formula.

$$\frac{\dfrac{\text{mg of amino acid (Std.mix.)}}{RWR_{a.a./IS\ (Std.mix.)}} \times RWR_{a.a./IS\ (Sample)} \times 100}{\text{Wt of sample in mg}} = \text{w/w \%}$$

$$\text{w/w \%} \times \frac{10^4}{\text{atomic weight in } \mu g} = \mu\text{mol/gm}$$

This formula is used when the same amount of Internal Standard is used in the sample as in the calibration mixture.

Advantages of this procedure are that no calibration curves need be prepared for the amino acids or the Internal Standard. The absolute quantity of the Internal Standard added need not be known as long as exactly the same amounts of Internal Standard are added to the calibration mixture and the sample.

As the RWR's of the amino acids remain constant, dilution, concentration, loss of material and size of injection become non-critical as long

as good size peaks are obtained.

Concluding remarks

In the Appendix are included a flow chart of the procedure as outlined, a table of molecular weights of the common amino acids (Table II) and a series of charts showing runs of a calibration mixture of amino acids and of extracts from grand fir bark and Douglas-fir needles. From these charts, it is evident that a good resolution was achieved with a reasonable baseline. It is also clear that in the extracts a few amino acids are present in very large amounts while most others represent only a small percentage of the total. Therefore the amount of Internal Standard has to be judiciously chosen. Table I gives results of a quantitative analysis of conifer needles.

The time required for extraction and ion exchange is the same for both t.l.c. and g.l.c. methods (about 4 hr for 4-8 samples). Preparation of the derivative for g.l.c. takes about 30 min (4-8 samples). On the gas chromatograph, 8-12 runs per day can be run compared to 3 runs per day using t.l.c.

The parameters as given in this report may have to be modified to suit other instruments and different tissues.

Through updating of our equipment and further streamlining of the procedure, accuracy, precision and speed of analyses are being improved and we hope to report on these developments in the near future.

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## APPENDIX

Notes

1. All glassware needs to be thoroughly washed and repeatedly rinsed with distilled water and dried. If it is treated with chromic acid, it requires rinsing with dilute HCl as traces of chromic acid can result in loss of methionine.
2. By using an internal standard, volumes do not have to be exact.
3. On the EGA column serine and γ-aminobutyric acid often overlap but they are well separated on the OV column.
4. Evaporating to dryness may result in loss of histidine (personal comm., J.P. Ussary, of Anal. Bio. Chem. Lab. Inc., who also suggests that acylation with 0.7 ml $CH_2Cl_2$ and 0.3 ml TFAA will bring the TFA peak closer to the solvent peak and away from the amino acid peaks. We were not able to confirm this under our conditions).
5. Adding acetone at full strength to the ion exchange resin dehydrates it and upsets the bed. Using a 1:1 mixture of acetone and water avoids this problem and results in a cleaner derivative.
6. Syringes are thoroughly cleaned after each injection with distilled water, acetone and methylene chloride and dried by vacuum.
7. Between uses, the evaporator should be rinsed to prevent formation of ammonium chloride.
8. After recycling the oven temperature to the initial temperature, allow enough time to equilibrate to ensure a reproducible starting procedure.
9. A preferred Internal Standard is tranexamic acid [4-(Aminomethyl)-cyclo hexanecarboxylic acid] (Aldrich Chemical Co.) because it can be added before the cleanup and thus improves the quantification of the analyses. We have not used it here as we were only recently able to obtain a supply.

TABLE I

Amino acid analysis of conifer needles (Douglas-fir)

by gas-liquid chromatography

| Amino acid | µmol/gm | | |
|---|---|---|---|
| | Run 1 | Run 2 | Average |
| Alanine | 2.560 | 2.545 | 2.533 |
| Valine | 0.199 | 0.199 | 0.199 |
| Glycine | 0.493 | 0.493 | 0.493 |
| Isoleucine | 0.155 | 0.138 | 0.147 |
| Leucine | 0.149 | 0.149 | 0.149 |
| Proline | 0.698 | 0.709 | 0.704 |
| Threonine | 0.325 | 0.320 | 0.323 |
| OH-Proline | 0.034 | 0.036 | 0.035 |
| Phenylalanine | 0.408 | 0.408 | 0.408 |
| Aspartic acid | 0.182 | 0.184 | 0.183 |
| Glutamic acid | 1.841 | 1.848 | 1.845 |
| Tyrosine | 0.300 | 0.291 | 0.296 |
| Ornithine | 0.145 | 0.143 | 0.144 |
| Serine | 1.057 | 1.102 | 1.080 |
| γ-aminobutyric a | 3.401 | 3.485 | 3.443 |
| Lysine | 0.057 | 0.067 | 0.062 |
| Arginine | 0.022 | 0.022 | 0.022 |

Average standard deviation of 2%.

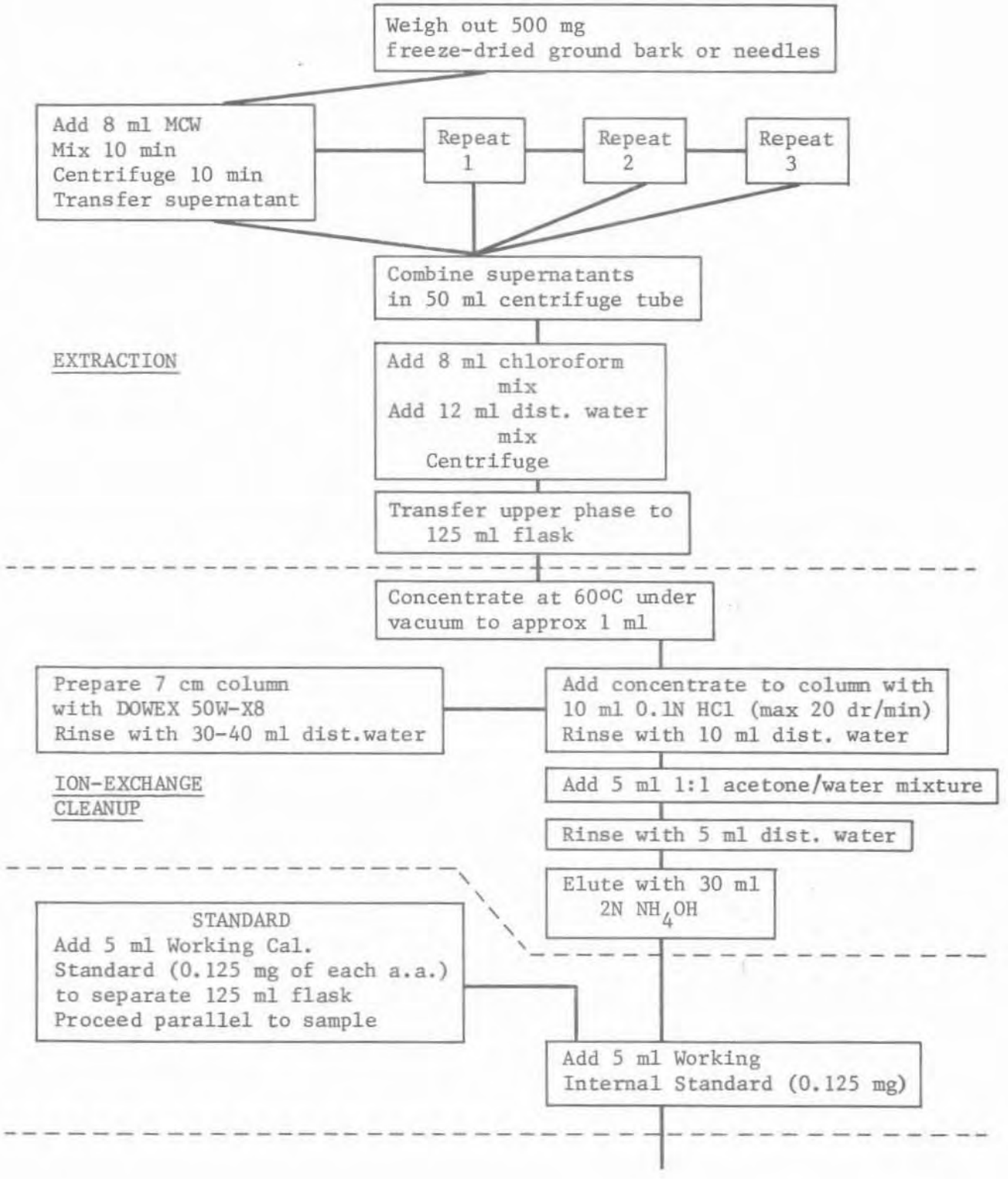
TABLE II

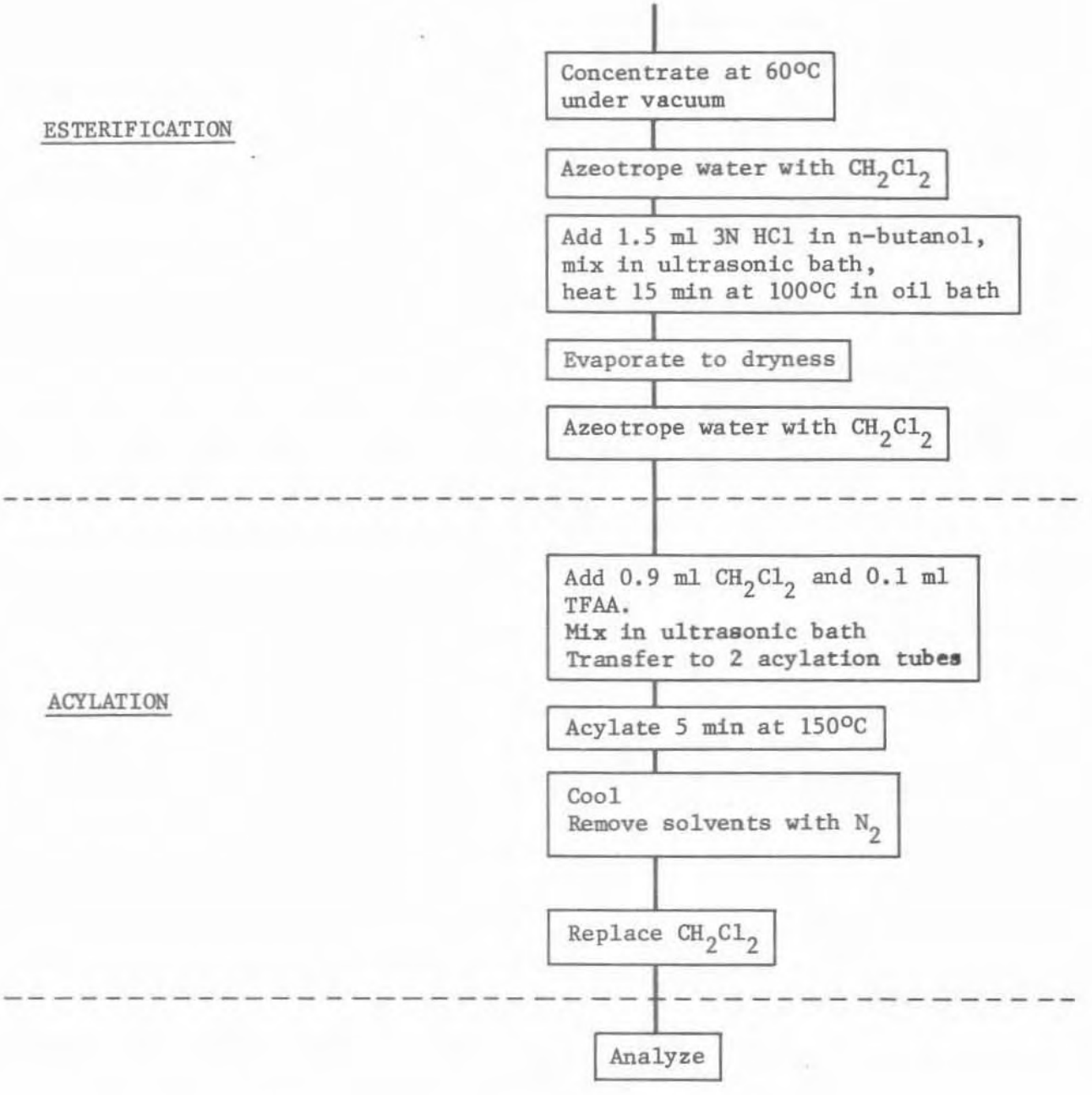
MOLECULAR WEIGHTS OF AMINO ACIDS (g)

| | |
|---|---|
| Alanine | 89.1 |
| Valine | 117.1 |
| Glycine | 75.1 |
| Isoleucine | 131.2 |
| Leucine | 131.2 |
| Proline | 115.1 |
| Threonine | 119.1 |
| Serine | 105.1 |
| γ Aminobutyric Acid | 103.1 |
| Cysteine | 121.2 |
| Methionine | 149.2 |
| Hydroxyproline | 131.1 |
| Phenylalanine | 165.2 |
| Aspartic Acid | 133.1 |
| Glutamic Acid | 147.1 |
| Tyrosine | 181.2 |
| Ornithine | 132.2 |
| Lysine | 146.2 |
| Tryptophan | 204.2 |
| Arginine | 174.2 |
| Histidine | 155.2 |
| Cystine | 240.3 |

FLOW CHART

Preparation of conifer bark or needle samples for amino acid analysis




ESTERIFICATION
Concentrate at 60°C under vacuum
Azeotrope water with $CH_2Cl_2$
Add 1.5 ml 3N HCl in n-butanol, mix in ultrasonic bath, heat 15 min at 100°C in oil bath
Evaporate to dryness
Azeotrope water with $CH_2Cl_2$
ACYLATION
Add 0.9 ml $CH_2Cl_2$ and 0.1 ml TFAA.
Mix in ultrasonic bath
Transfer to 2 acylation tubes
Acylate 5 min at 150°C
Cool
Remove solvents with $N_2$
Replace $CH_2Cl_2$
Analyze

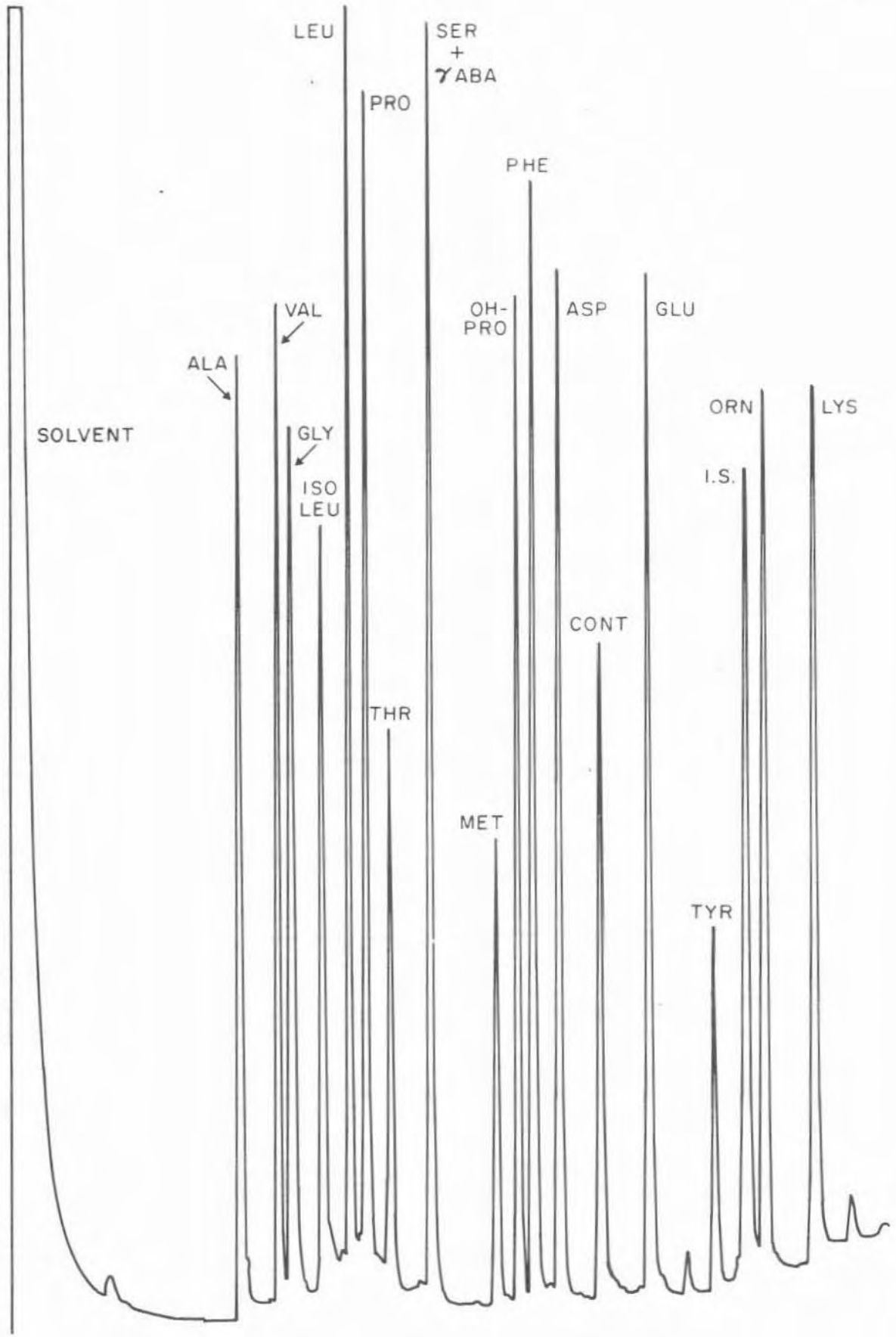



Fig. 1. Standard calibration mixture of amino acids on EGA. Note overlapping peaks of serine and γ-aminobutyric acid. I.S.=Internal Standard Butyl Stearate. CONT=non-amino acid contaminant.

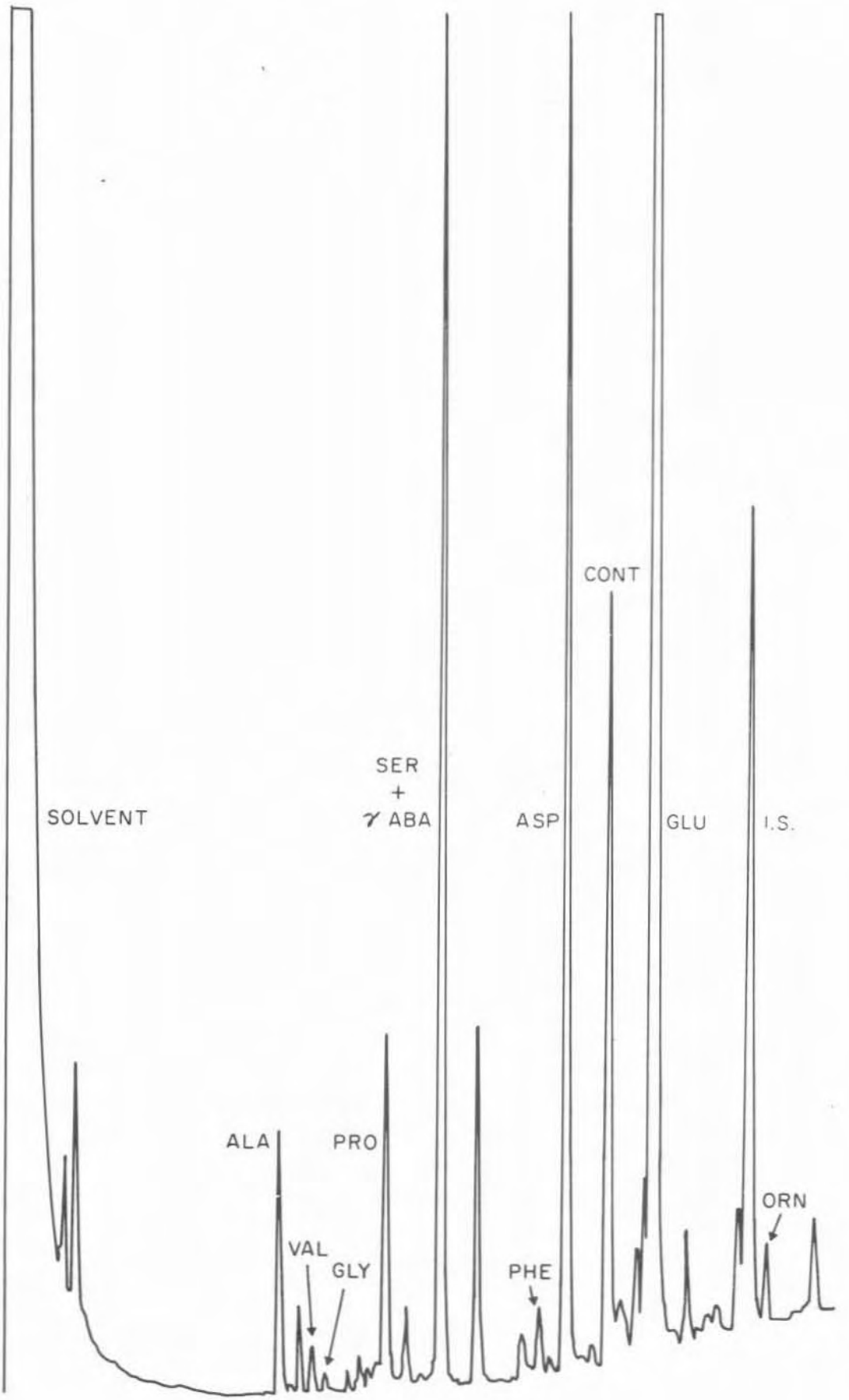



Fig. 2. Conifer bark extract on EGA. (grand fir)

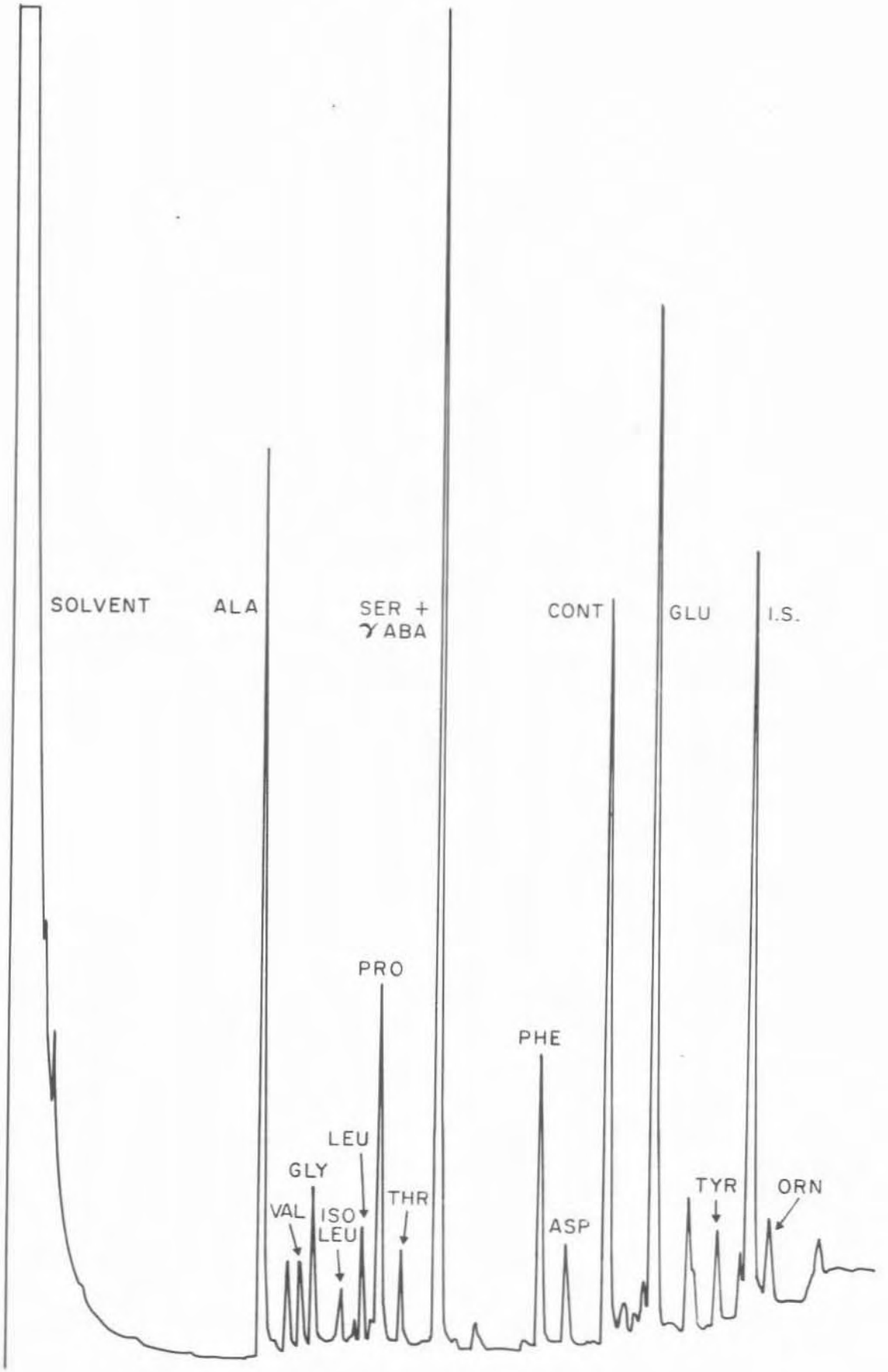



Fig. 3. Conifer needle extract on EGA. (Douglas-fir)

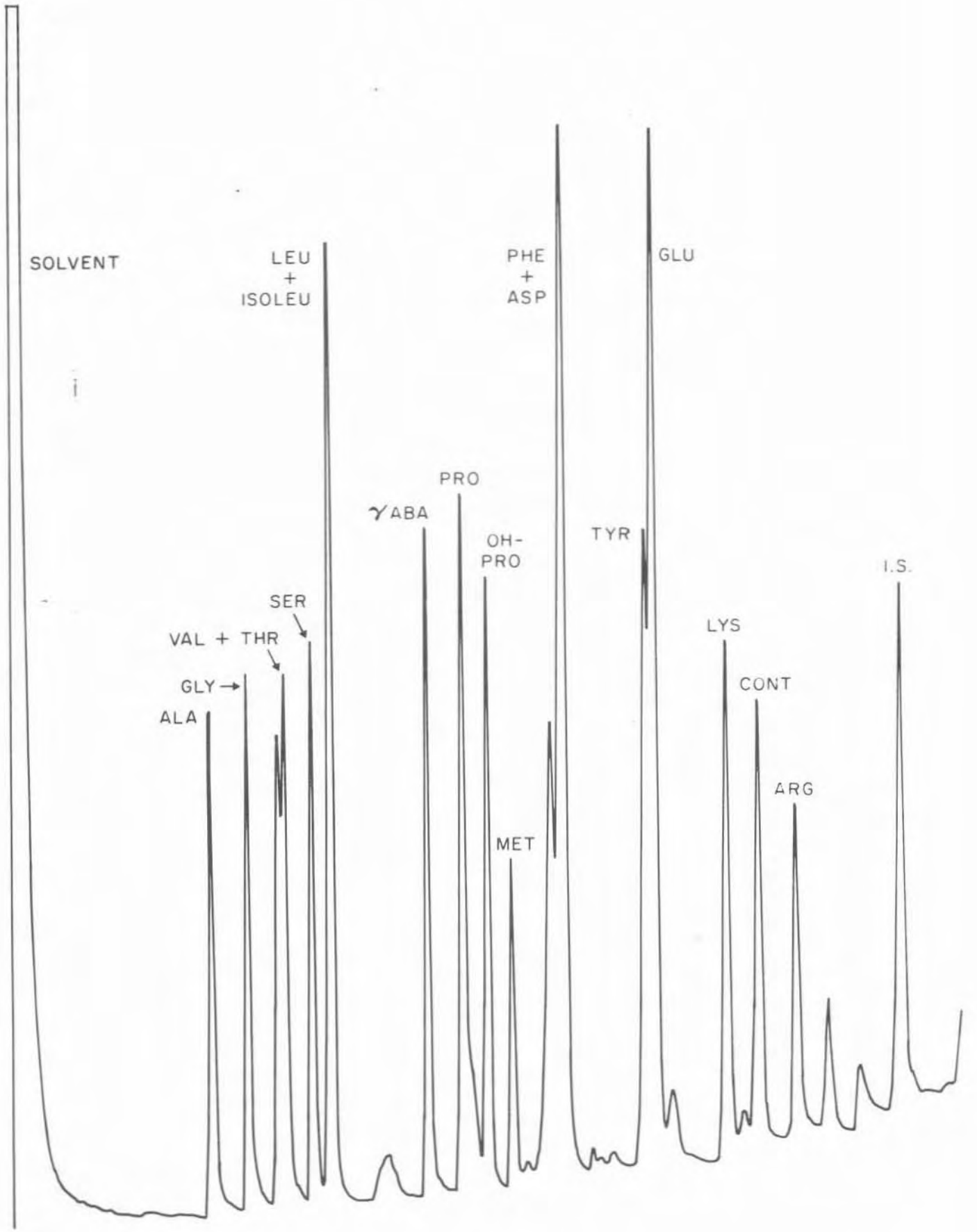



Fig. 4. Standard calibration mixture of amino acids on mixed OV-phase. Note separation of serine and γ-aminobutyric acid.

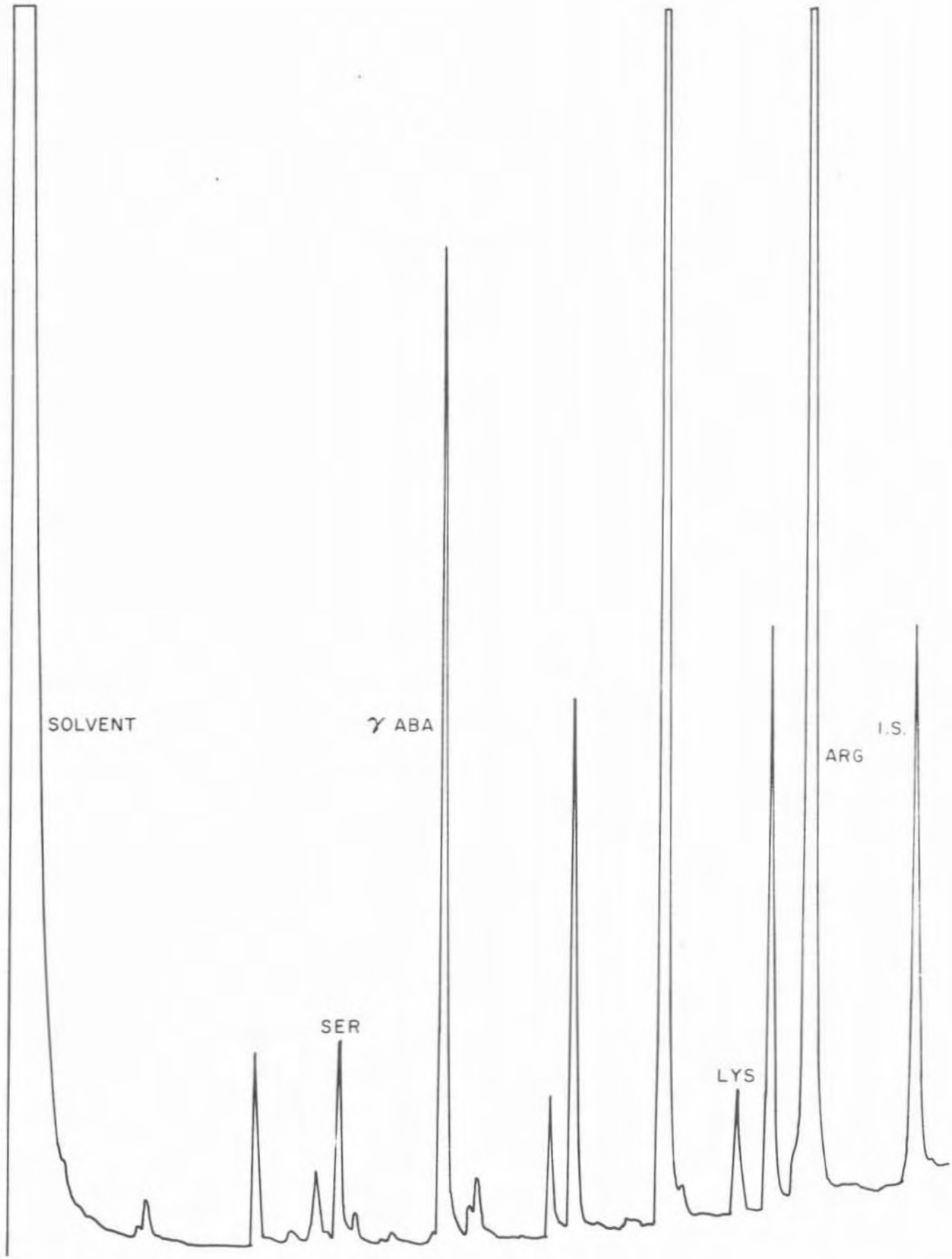



Fig. 5. Conifer bark extract on mixed OV-phase. (grand fir)

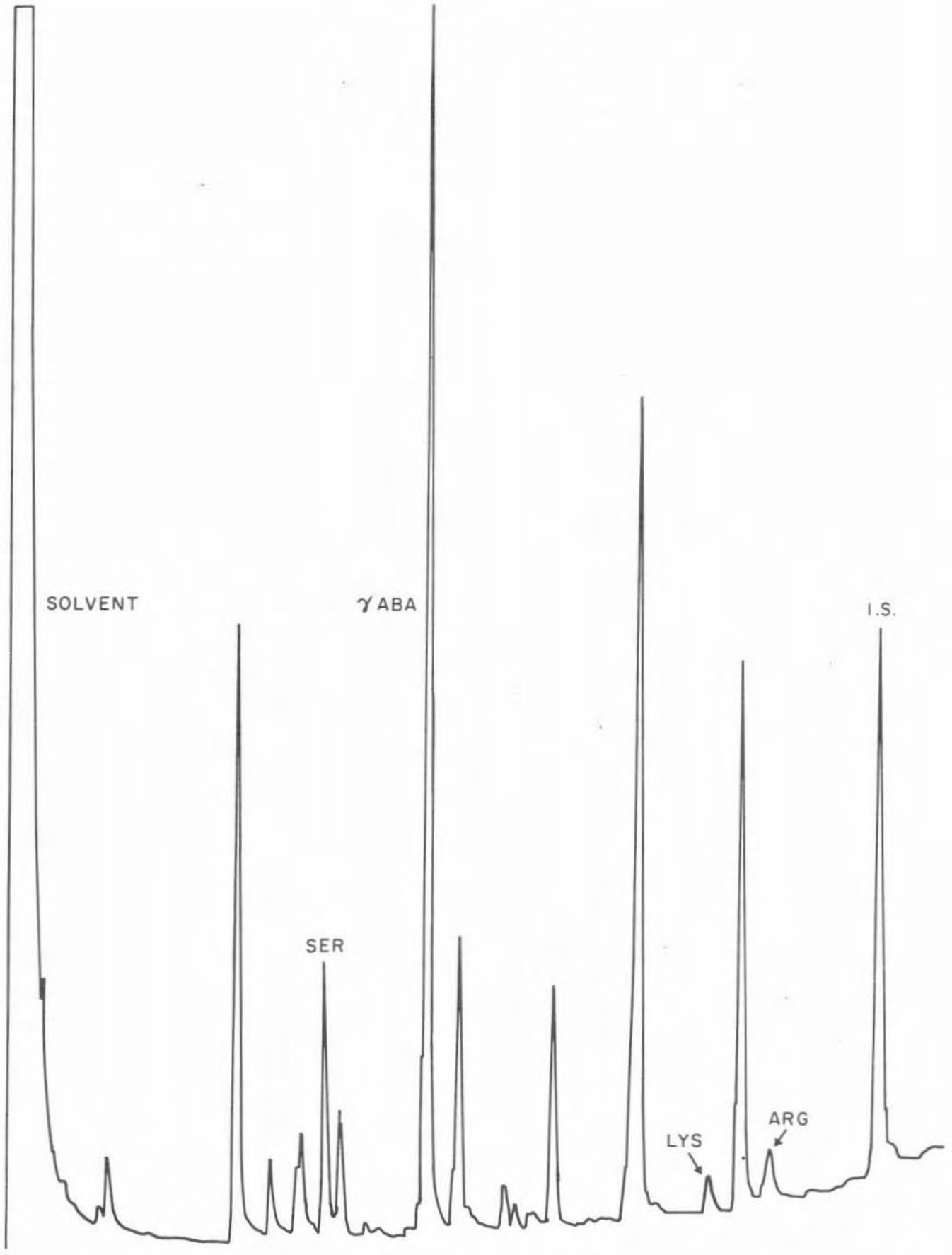



Fig. 6. Conifer needle extract on mixed OV-phase. (Douglas-fir)