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QUANTITATIVE ANALYSIS OF AMINO ACIDS IN CONIFER TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION OF THEIR 9-FLUORENYLMETHYL CHLOROFORMATE DERIVATIVES

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SUMMARY

Pre-column derivatization of amino acids in conifer extracts to form 9-fluorenylmethyl formate (FMOC) esters and the subsequent reversed-phase high-performance liquid chromatography (HPLC) analysis of these esters have been investigated. Qualitative analysis of putative FMOC-amino acid derivative HPLC peaks from a pine extract was performed by gas chromatography–mass spectrometry in order to confirm their identities. The precision, measured with ten replicate extracts, ranged from 2.2 to 15.6%. Theoretical responses from standards and extracts were compared with the actual response as an assay for systematic errors. The detector response was investigated as a function of the ratio amino acid/reagent and the range of linearity for each amino acid was established. The method is suitable for analysis of amino acids in crude extracts of small samples of Scots pine tissues, and for simultaneous determination of primary and secondary amino acids.

INTRODUCTION

Quantitative analysis of amino acids has traditionally been carried out with amino acid analysers, based on open-column ion-exchange chromatography followed by post-column derivatization, typically with ninhydrin. Although such procedures are used widely in many areas of experimental biology they have shortcomings such as long analysis times, low chromatographic efficiencies and inadequate levels of detection. Because of these limiting factors, alternative methods of analysis should be considered.

A variety of procedures for separation, derivatization and detection of amino acids based on high-performance liquid chromatography (HPLC) have been described[1]. Reversed-phase HPLC is used most extensively in conjunction with fluorescence detection of derivatives formed by pre-column reaction with *o*-phthalaldehyde (OPA)–mercaptoethanol[2–4]. OPA is a general derivatization agent which reacts with primary amino acids as well as with a range of other compounds such as di-

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peptides, amino sugars and primary amines[1]. The OPA technique is convenient since the reagent is non-fluorescent and the reaction is completed within 2–3 min and carried out in aqueous media. However, several OPA-amino acid derivatives, including those of Gly and Lys, are unstable and the fluorescence intensity falls off rapidly within minutes of derivatization. A further disadvantage is that OPA forms derivatives only with primary amino acids. As a consequence, the analysis of secondary amino acids, such as Pro, is dependent upon their oxidation with hypochlorite or chloramine-T prior to derivatization with OPA, *e.g.*, refs. 5, 6. This, however, is not practical if primary amino acids are also of interest since they are destroyed by the oxidative environment[7].

A derivatization procedure, using 9-fluorenylmethyl chloroformate (FMOC-Cl) to protect the amino group during peptide synthesis, has been described by Carpino and Han[8]. The same agent was proposed by Moye and Boning[9] for fluorescence labelling of both primary and secondary amines. Recently, Einarsson *et al.*[10] described an HPLC–fluorescence method based on the reversed-phase separation of 9-fluorenylmethyl formate (FMOC)-amino acid esters.

FMOC-Cl reacts with both primary and secondary amino acids forming highly fluorescent and stable derivatives. However, the derivatization agent is itself fluorescent and the excess of reagent has to be removed from the reaction mixture. This is usually achieved by partitioning the reagent into an organic solvent such as diethyl ether or pentane. If secondary amino acids are the primary target, an alternative method proposed by Einarsson[11] may be considered. In this case, primary amino acids are removed by derivatization with OPA–mercaptoethanol followed by labelling of the secondary amino acids with FMOC-Cl.

Our research on the nitrogen dynamics of Scots pine (*Pinus sylvestris* L.) trees is dependent on a detailed knowledge of the distribution pattern of amino acids. A wide range of amino acids has been found in conifers[12]. Conifers with a good nutrient status accumulate guanidino compounds, such as Arg[13] which frequently accounts for a large fraction of the nitrogen in the free amino acid pool. Other amino acids of quantitative importance in conifers are Gln, Asn, Asp, Glu, Ala and γ-aminobutyric acid (Gaba). During periods of drought, mineral deficiency or other stresses the imino acid Pro is also known to accumulate[14].

Analysis of trace substances in a complex matrix such as a conifer extract demands special care, during both the initial purification and the subsequent analysis. Conifer extracts are known to contain high levels of phenols and terpenoids, as well as a range of other substances that may influence the derivatization reaction and/or the subsequent HPLC separation. The adaptation of an analytical method for the quantitative analysis of amino acids in conifer tissues therefore necessitates a careful examination of the reproducibility of the technique.

The purpose of the present work was to apply the method described by Einarsson *et al.*[10] for analysis of amino acids as their FMOC derivatives in human fluids to conifer extracts. Quantitative analysis of FMOC-amino acid esters in pine extracts was examined in terms of accuracy and precision. The identity of the FMOC-amino acid HPLC peaks was confirmed by gas chromatography–mass spectrometry (GC–MS).

EXPERIMENTAL

Extraction of plant material

Extraction was performed by homogenization of the plant material in 80% aqueous ethanol. The homogenate was filtered and the residue washed two or three times with the same medium. Solutions were pooled and made up to a volume of *ca.* 100 ml/g (fresh weight tissue). Sub-samples of 1 ml were evaporated to dryness under a stream of nitrogen and redissolved in water. When necessary, samples were filtered through a 0.45-μm syringe filter prior to derivatization.

Derivatization

Volumes of 0.4 ml of either amino acid solutions or extracts were mixed with 0.1 ml 1 *M* borate buffer pH 6.3 in a 3-ml screw-cap vial, 0.5 ml of 15 m*M* FMOC-Cl in acetone were added and the reaction allowed to proceed for 30 s. The excess of reagent was removed by partitioning the sample three times against 1.5 ml pentane. The extracts and standard samples were diluted 5–50 times in the eluent prior to HPLC analysis.

High-performance liquid chromatography

Solvents were delivered at a flow-rate of 1 ml min^{-1} by a Waters liquid chromatograph system consisting of two M45 pumps, a dynamic solvent mixer and a Model M680 gradient master. Samples were introduced off-column via a Valco loop injector fitted with a 25-μl loop. Reversed-phase separations were carried out on a 250 mm × 4.6 mm I.D. ODS-Hypersil (5 μm) column eluted from 0 to 20 min with a 50–60% gradient of methanol in buffer (7 ml acetic acid and 1 ml triethylamine to 1 l water, adjusted to pH 4.2 with sodium hydroxide, followed by a second step of 60–100% methanol, from 20 to 40 min. The column effluent was directed to a Shimadzu Model RF 530 fluorescence detector (excitation 260 nm, emission 313 nm).

Calculations

Quantitative estimates of the endogenous amino acid content were based on internal standardization with Norleu or Norval. The precision of the final estimate was tested by dividing an extract into ten equal portions and processing each aliquot separately. The precision in this instance equals the standard deviation of the population of sub-samples.

GC–MS analysis

Putative FMOC-amino acid peaks from the reversed-phase HPLC of a Scots pine extract were collected, evaporated to dryness under a stream of nitrogen and dissolved in 200 μl 1 *M* sodium hydroxide. The extracts were hydrolyzed for 1 h at 25°C, acidified with 2 ml glacial acetic acid and evaporated to dryness. The samples were dissolved in 100 μl acetonitrile overnight and silylated by addition of 100 μl of bis(trimethylsilyl)trifluoroacetamide at 100°C. Mass spectrometric analysis on concentrated aliquots was performed with an HP 5890 gas chromatograph linked via a direct capillary inlet to an HP 5970B mass selective detector equipped with an HP 9000 computer system. Samples were introduced in the splitless mode (splitless time 2 min) at 225°C onto a 25 m × 0.31 mm I.D. cross-linked methyl silicone capillary

TABLE I

COMPARISON OF HOMOGENIZATION AND BOILING IN ETHANOL OR WATER OF AMINO ACIDS FROM SCOTS PINE NEEDLES

Values are averages of two separate extractions and analyses and shown as percentages of the yield in 80% ethanol homogenate.

Amino acid	*Homogenization*		*Boiling*	
	Ethanol	*Water*	*Ethanol*	*Water*
Asn	100	69	100	33
Gln	100	80	54	18
Asp	100	44	87	100
Ser	100	102	85	47
Glu	100	96	73	106
Arg	100	99	87	112
Gly	100	113	100	100
Thr	100	78	81	88
Ala	100	96	104	85
Gaba	100	140	136	111
Pro	100	113	119	111

column (film thickness 0.52 μm). The column temperature was initially held at 60°C for 3 min, then increased at 30°C min^{-1} to 130°C and at 7°C min^{-1} to 235°C. The interface temperature was maintained at 250°C. The retention time for air was 63 s.

RESULTS AND DISCUSSION

Extraction

Several techniques have been used for extraction of amino acids from plant material. In this study the recovery of amino acids from pine needles extracted with either water or 80% ethanol was compared. In addition, the efficiency of extraction based on homogenizing or boiling the samples was investigated (Table I).

Compared to homogenization in ethanol, homogenization in water yielded less Asn, Gln, Asp, Thr and Lys, equal amounts of Ser, Glu, Arg,Ala and Orn and greater quantities of Gly, Gaba and Pro. Similarly, boiling the samples in 80% ethanol yielded less of Gln, Asp, Ser, Glu, Arg, Thr, Orn and Lys, equal amounts of Asn, Gly and Ala and more of Gaba and Pro. Boiling in water resulted in lower yields of Asn, Gln, Ser, Thr, Ala, Orn and Lys; Asp, Glu and Gly were extracted with similar efficiency, while Arg, Gaba and Pro showed increased yields. The improved extraction of Arg in water is consistent with the results of Oland[15] However, considerable losses of Asn, Gln, Ser and Lys were apparent when samples were boiled in water. The losses of the amides could be due to hydrolysis of the amide group[16]. Glu may cyclize to give pyroglutamic acid when heated in water[16], and the amount of Glu detected may therefore be a result of its formation by hydrolysis of Gln and removal via cyclization to pyroglutamic acid. Except for Arg, Gaba and Pro, homogenization in 80% ethanol extracts more or equal amounts of all amino acids compared to the other extraction methods. Thus, with the exception of situations where Arg, Gaba or Pro are of special interest, homogenization in 80% ethanol seemed to be the most suitable of the methods investigated.

Derivatization

In essence, the derivatization procedure described by Einarsson *et al.*[10] was followed. The dependence of the derivatization reaction on time was studied for groups of five samples treated for 15, 30, 45, 60 and 120 s respectively. The reaction was almost instantaneous, and no difference in yield was observed. In addition, the effect of the pH of the reaction buffer on derivatization efficiency was studied (Fig. 1). Increased yields of all amino acids except Pro and Tyr was observed when the pH was increased from 6 to 7. Most amino acids showed maximum yield at pH 7 although some, especially Asp and Glu, were derivatized more effectively at pH > 7. Formation of the hydrolysis product FMOC-OH was also enhanced at pH > 7.

FMOC-Cl can react with primary and secondary amines[10] and amino groups in peptides[9] as well as free amino acids, and the reagent consumption in samples with high amounts of these substances can therefore restrict the yield of the amino acid derivatives. To evaluate the effect of increasing amounts of reagent on the yield of amino acid derivatives, samples of needle extracts and standards were treated with 15, 30, 60, 90 and 120 m*M* FMOC-Cl solutions, respectively. The results were similar for standards (data not shown) and samples of extracts (Fig. 2). Considerably less

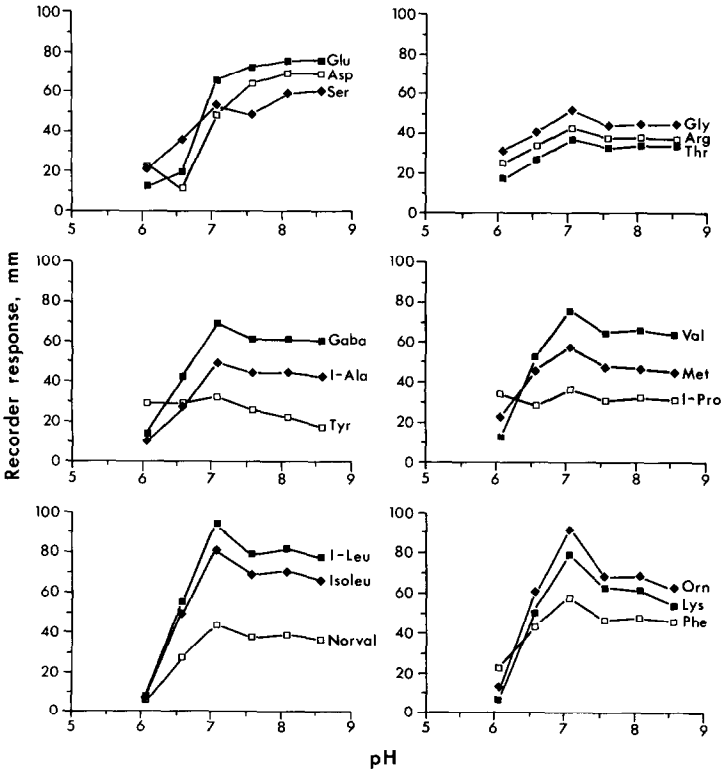

Fig. 1. pH dependence of the yield of amino acid derivatives. Values are the averages of two derivatizations at each pH. Derivatization was performed on a mixture of amino acids, each at a concentration of 50 μM. Injection of 50 μl after five-fold dilution.

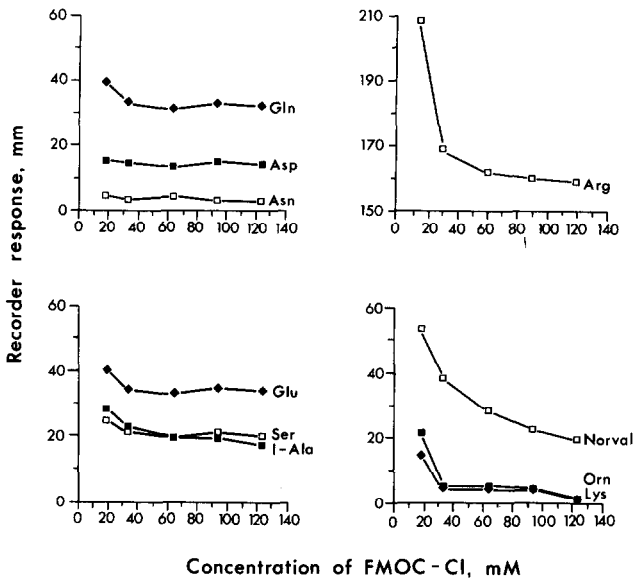

Fig. 2. Effect of increased amounts of reagent on the yield of amino acid derivatives from a pine needle extract. Reagent concentrations were 7.5, 15, 30, 45 and 60 μmol, respectively.

Arg, Norval, Orn and Lys was detected in extracts with a concentration of FMOC-Cl higher than 15 m*M* (Fig. 2).

In another experiment, increasing amounts of amino acids were derivatized in 7.5 μmol (15 m*M*) of reagent (Fig. 3). Asp and Glu showed decreased derivative formation when the total amount of amino acids exceeded 1 μmol, while, for the other amino acids, no such decrease could be detected at 4 μmol of total amino acid in the sample. It thus seems that the ratio of the reagent and the total amino acid content has to be greater then 8 for maximum labelling of Asp and Glu. Since increased amounts of reagent had a negative effect on the yield of amino acids such as Val and Norval (Fig. 2), samples with high concentrations of amino acids have to be diluted prior to derivatization and analysis.

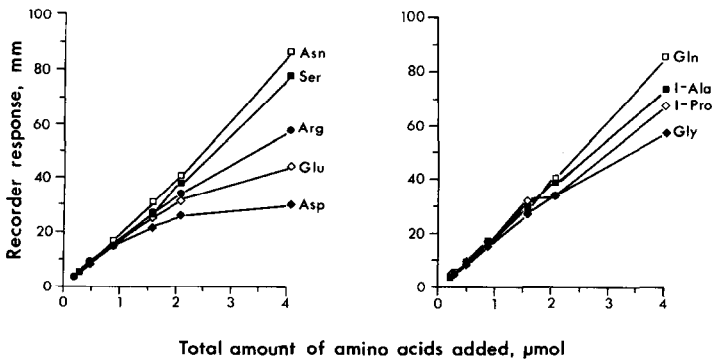

Fig. 3. Effect of increasing amounts of total amino acids on the yield of each amino acid derivative. Values are the averages of two separate derivatizations and HPLC analyses.

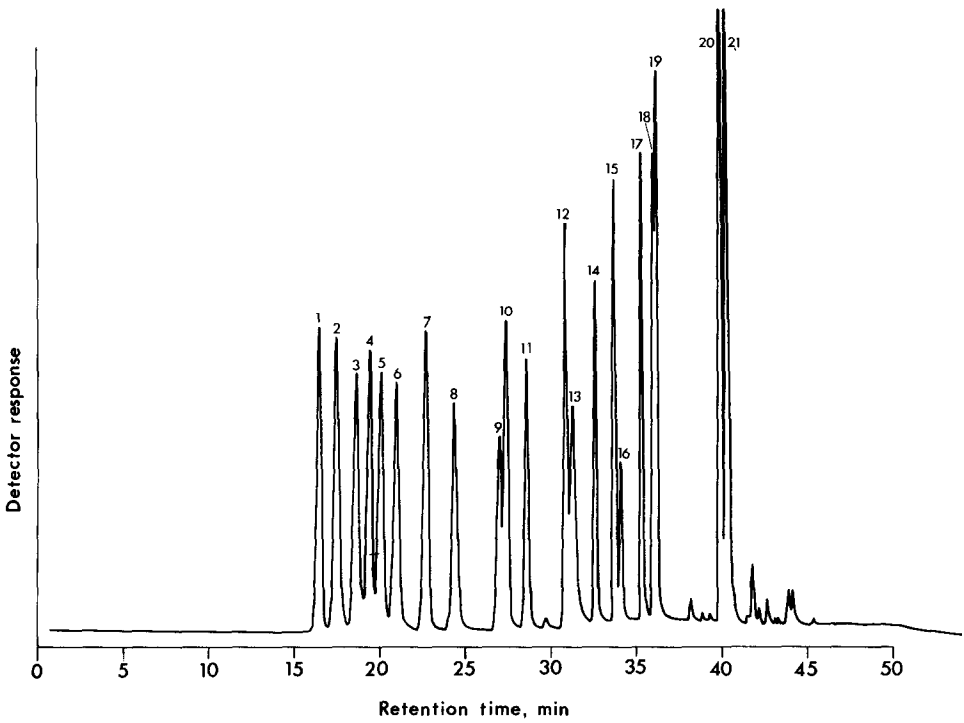

Fig. 4. Reversed-phase HPLC separation of a mixture of amino acid standard derivatives. Flow-rate: 1 ml min^{-1}. Gradient; 0–20 min: 50–60% methanol in buffer; 20–40 min: 60–100% methanol. Buffer concentration: 0.7% (v/v), pH 4.2. Peak identities: 1 = Asn; 2 = Gln; 3 = Asp; 4 = Ser; 5 = Glu; 6 = Arg; 7 = Gly; 8 = Thr; 9 = Tyr; 10 = Ala; 11 = FMOC-OH; 12 = Gaba; 13 = Pro; 14 = Met; 15 = Val; 16 = Norval; 17 = Phe; 18 = Isoleu; 19 = Leu; 20 = Orn; 21 = Lys.

HPLC separation

Eighteen standard amino acids were separated by reversed-phase HPLC (Fig. 4). Tyr and Ala, Gaba and Pro and Isoleu and Leu were eluted in closely associated pairs which made quantitative analysis difficult. The effects of pH and buffer concentration on the retention of the amino acid derivatives were investigated (data not shown). In agreement with the results of Einarsson *et al.*[10] a slight decrease in retention of most derivatives was observed with increasing buffer pH. Increased buffer concentration had a more complex effect on the retention of the derivatives. In all cases, except for Arg and Gaba, increasing the buffer concentration from 0.5 to 0.9% resulted in enhanced retentions, while a further increase to 1.5% resulted in Asp, Ser, Glu, Arg, Gaba and the hydrolysis product being less strongly retained.

GC–MS analysis of amino acids in a pine extract

Five quantitatively major amino acids from a pine needle extract were collected as their FMOC derivatives from the HPLC column for confirmation of their identities by GC–MS. First, the derivatives were hydrolyzed with sodium hydroxide. HPLC analyses of hydrolyzed and re-derivatized amino acids was used to confirm that the derivatives had been cleaved.

TABLE II

CHARACTERISTIC AND ABUNDANT IONS IN THE 70 eV ELECTRON IMPACT MASS SPECTRA OF ENDOGENOUS AMINO ACIDS IN PINE NEEDLES

Amino acid	M^+ *m/z (%)*	$M^+ - 15$ *m/z (%)*	*Base peak m/z*	*Other characteristic ions m/z (%)*	
Asp	349(1)	334(1)	232	218(19)	306(3)
Gln	–	–	73	156(75)	84(2)
Glu	363(1)	348(1)	246	156(8)	84(5)
Ser	–	306(1)	73	204(52)	218(25)
Orn	348(1)	–	73	142(69)	216(3)

The electron impact fragmentation MS pattern of N-trimethylsilyl amino acid trimethylsilyl esters has been studied by Iwase *et al.*[17]. In addition to M^+, characteristic ions were $M^+ - 117$, $M^+ - 43$ and $M^+ - 15$ corresponding to loss of –COO–TMS, $-COCH_3$ and $-CH_3$, respectively. Loss of the side chain (R) produces an *m/z* 218 fragment (TMS–NHCHCOO–TMS) which is characteristic for amino acids with branched side chains or aromatic rings such as Val, Isoleu, Ser, Thr, Phe and Tyr. In addition to these fragments, ions at *m/z* 73 [$Si(CH_3)_3$] and *m/z* 147 [$(CH_3)_3SiO–Si(CH_3)_2$] were also present in all amino acid fragmentation spectra.

The fragmentation patterns of five endogenous amino acids from pine needles are shown in Table II. The HPLC fraction corresponding to Asp produces a major GC peak at 12.29 min corresponding to $Asp(TMS)_3$ with characteristic ions at *m/z* 349(M^+), 334, 306, 232 and 218. The Gln HPLC peak gave rise to a GC peak at 11.89 min containing major fragments at *m/z* 73, 84 and 156, of which the last two are specific for Glu and Gln, respectively. The Glu HPLC fraction produced a GC peak at 13.73 min. Fragments from this peak occurred at *m/z* 363, 348 and 246. The abundant ion in the Gln fragmentation spectra, *m/z* 156, was also present in the Glu spectra although in proportionally lower amounts. The ion at *m/z* 84, in contrast, was more abundant in the Glu spectra which is also consistent with the findings of

TABLE III

COEFFICIENTS OF VARIATION FOR AMINO ACIDS

One sample extract was divided into ten subsamples and each derivatized separately.

Amino acid	*Coefficient of variation*
Asn	4.86
Gln	2.96
Asp	3.46
Ser	4.33
Glu	2.65
Arg	2.18
Gly	10.61
Thr	7.00
Ala	5.06
Gaba	15.74

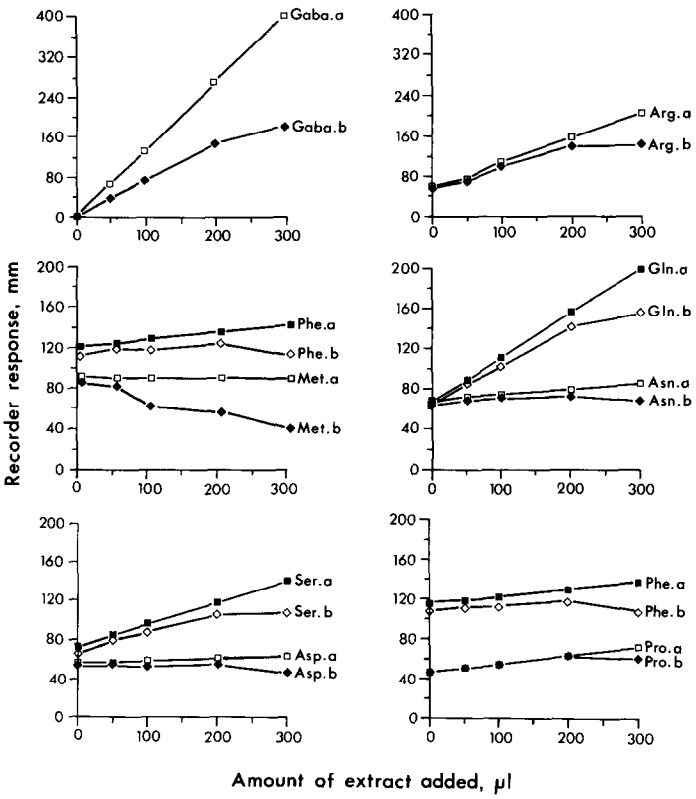

Fig. 5. Comparison of (a) detected and (b) theoretically estimated detector responses for ten amino acids. The determinations were made by comparing detector responses from mixed standards and extract solutions with the theoretical response obtained by summing responses from separately analysed standards and samples. The concentration of each standard amino acid was 25 μM at derivatization.

Iwase *et al.*[17]. The putative Ser HPLC fraction produced a GC peak at 9.82 min, the mass spectrum of which included ions at m/z 306, 204 and 218.

Analyses of putative Arg HPLC fractions and Arg standards via GC–MS did not reveal peaks with fragmentation spectra in accordance with the expected spectra for Arg. The main peaks in the GC chromatograms showed a pattern corresponding to Orn with three and four TMS groups attached. It therefore appears that the urea group of Arg is hydrolyzed during silylation yielding Orn with three or four TMS groups. These findings are consistent with the results of Bergström and Gürtler[18]. Supporting evidence is provided by a comparison of fragmentation patterns from Arg and Orn standards. The putative Arg fraction revealed a peak at 13.76 min in the GC system which corresponds to Orn$(TMS)_3$. Spectra of this peak contained characteristic ions at m/z 348, 142 and 216, all diagnostic for Orn[17].

Precision of HPLC-based analyses

The precision of quantitative estimates of the amino acid content of *Pinus* extracts based on HPLC-fluorescence analyses was investigated by comparing data

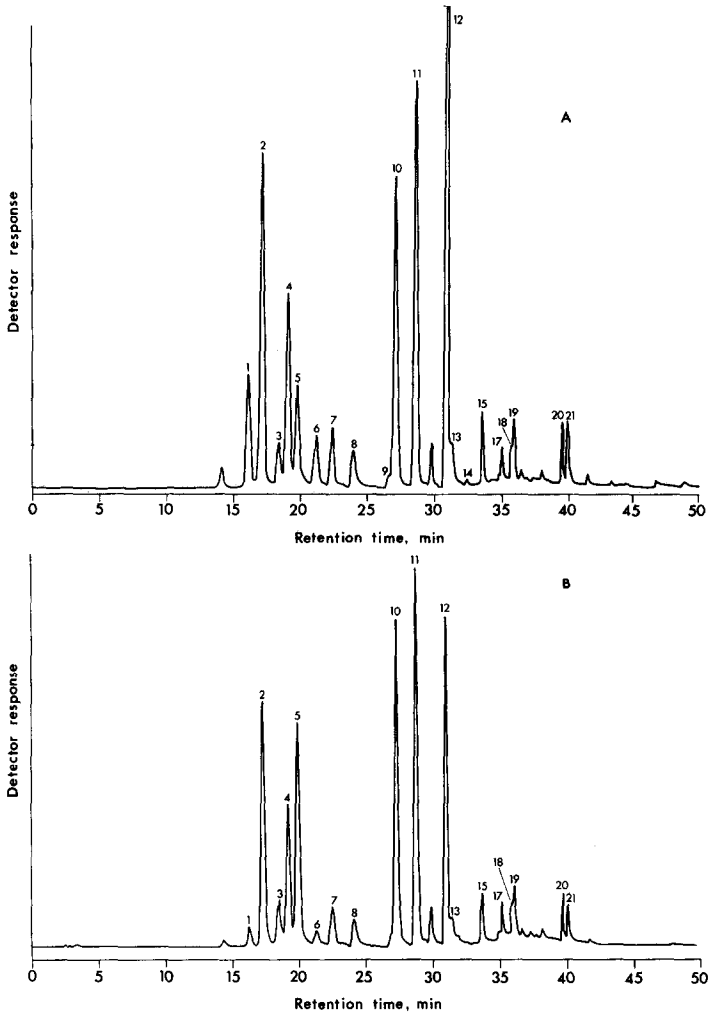

Fig. 6. Reversed-phased HPLC chromatograms of extracts from (A) primary and (B) secondary needles of Scots pine seedlings. Peak identities and conditions as in Fig. 4.

obtained with ten replicate samples (Table III). Highest precision was estimated for Gln, Glu and Arg, the dominant amino acids in the extract. The low precision for Gaba and Gly could be due to interference from Pro and Arg which were eluted in close proximity to Gaba and Gly, respectively. Improved precision can therefore probably be achieved by improving the HPLC separation.

A limited test on accuracy was made by comparing the detector response from mixed standards and extract solutions with the theoretical response obtained by summing responses from separately analysed standards and samples (Fig. 5). The amounts detected in the mixed solutions were generally lower than the theoretical estimates. This suggests a proportional non-random error component. For Met,

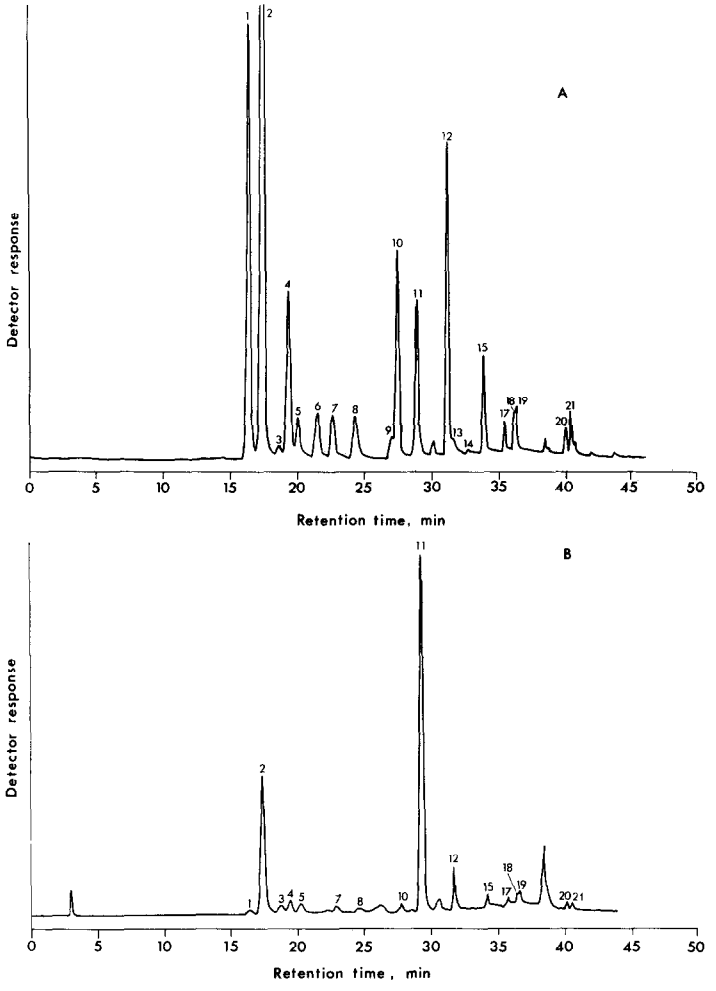

Fig. 7. HPLC chromatograms of extracts from roots (A) and xylem fluid (B) of Scots pine seedlings. Peak identities and conditions as in Fig. 4.

where the response seemed to be restricted by increased amounts of extract, the derivative was found to be unstable in a mixed standard solution. Thus, the results for Met probably only reflect the different times between derivatization and detection.

HPLC analyses of amino acids in shoots and xylem sap from *P. sylvestris* were carried out. Primary and secondary needles (Fig. 6A and B, respectively) contained relatively high amounts of Gaba, Ala, Gln, Ser and Glu while the root contains more of Asn, Gln and less Glu (Fig. 7A). Vacuum-extracted xylem fluid is clearly dominated by Gln and only traces of the other amino acids were detected (Fig. 7B).

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