3 days were noticeably higher, >200 ppm. Acetonitrile–water containing ethanolamine on C-8 and C-18 columns (Morris and Lee, 1981) gave adequate separation but was inconvenient to use because of the necessity of carefully adjusting the pH of the mobile phase. The system described in this paper gives good separation with the advantages of not having to adjust the pH of the mobile phase and a slightly lower background absorbance compared to ethanolamine. The selectivity of the system can be adjusted by changing the concentration of ammonium phosphate in the mobile phase.

Some work has been performed in this laboratory to evaluate the utility of the method for determining the glycoalkaloids that are present in other members of the Solanaceae. Slight changes in the strength of the wash and final elution solvents permitted the determination of the glycoalkaloids tomatine (present in green tomatoes) and soalsonine (present in eggplant). However, the minimum detectable quantity of tomatine is approximately one-tenth that of solanine and chaconine (detection range 2–200 $\mu g/g$) due to the lack of the unsaturated double bond at the Δ^5 position, which results in a lower molar absorptivity. Adjustments in the mobile phase composition would also be necessary for optimum separation if all four of the above glycoalkaloids were to be determined simultaneously. This would not likely be necessary as all four glycoalkaloids do not occur in any sizable amounts in any one of the aforementioned commodities. The method may also be applicable to the determination of other glycoalkaloids, including those that are not readily precipitated by base (Zitnak, 1968).

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Possible Misidentification of Pipecolic Acid in HPLC Analysis of Legume *Phaseoleae* Seed Oligosaccharides

Bernard Quemener,* Jean-Marc Brillouet, Marguerite Arendt, Daniel Tome, and Christian Diolez

This paper describes the purification, identification, and quantitation by high-pressure liquid chromatography (HPLC) of L-pipecolic acid, an unusual amino acid that was mainly found in common bean (*Phaseolus vulgaris*). Seeds from various species from *Phaseolus* and *Vigna* genera were examined for both their L-pipecolic acid and sugar contents by HPLC.

INTRODUCTION

Dry beans constitute a preponderant portion of traditional diet in many areas including India, Africa, and Central and North America. Apart major constituents (protein and starch) legume seeds contain free amino acids among which uncommon amino acids such as α- and γ-diaminobutyric acid may exhibit strong metabolic interactions in animals fed with legume-containing diets (Ressler et al., 1961). They contain also other minor components such as oligosaccharides, mainly α-D-galactosides, which have been also extensively investigated (Cerning-Beroard and Filiatre, 1976; Naivikul and d'Appolonia, 1978; Schweizer et al., 1978; Aman, 1979; Quemener and Mercier, 1980; Fleming, 1981; Sosulski et al., 1982; Sathe et al., 1983). High-performance liquid chromatography (HPLC) is the choicest method for analysis of oligosaccharides, detection being performed by differential refractometry. However, some noncarbohydrate compounds may interfere by yielding a refractive index response. Quemener and Brillouet (1983) have reported an unidentified compound in common bean (*P. vulgaris*) eluted before the succrose peak in HPLC analysis. We now report the purification, identification, and quantitation of

Institut National de la Recherche Agronomique, Centre de Recherches Agro-Alimentaires, Laboratoire de Biochimie et Technologie des Glucides, 44072 Nantes Cédex, France (B.Q., J.M.B., M.A.), Laboratoire de Technologie des Aliments pour Animaux, 44072 Nantes Cédex, France (D.T.), and Laboratoire Mativelle, 91000 Longjumeau, France (C.D).

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Table I. L-Pipecolic Acid and Sugar Distribution of Hull-Free Flours of Different Species from *Phaseoleae* (% Dry Matter)[a]

	L-pipecolic acid[c]	fructose[b]	glucose[b]	sucrose[c]	raffinose[c]	stachyose[c]	verbascose[c]
Phaseolus vulgaris	0.70	0.016	0.010	4.70	0.50	3.80	0.25
Phaseolus aureus	<0.1	0.008	0.003	1.30	0.20	1.20	3.30
Phaseolus lunatus	0.40	0.010	0.005	2.10	0.40	5.00	1.90
Phaseolus coccineus	0.60	0.020	0.010	5.40	0.50	2.90	0.05
Vigna unguiculata	<0.1	0.010	0.004	1.70	0.60	2.80	0.70

[a] Means of duplicate analyses. [b] Determined by enzymatic UV method (Boehringer, 1980). [c] Determined by HPLC.

this unknown compound that was ultimately found as L-pipecolic acid, an unusual amino acid. Seeds from various species from *Phaseolus* and *Vigna* genera were examined for both their L-pipecolic acid and oligosaccharide contents by HPLC.

EXPERIMENTAL SECTION

Plant Materials. The various species examined in this study were the common bean (*Phaseolus vulgaris* L., var. Michelet) from Algeria, the lima bean (*Phaseolus lunatus* L.) and cow pea (*Vigna unguiculata* L.) that were obtained from the Department of Crop Science, University of Saskatchewan, Saskatoon, Canada, and the green gram (*Phaseolus aureus*) and runner bean (*Phaseolus coccineus* L.) from commercial sources.

Extraction and Purification. Pipecolic acid was extracted from whole flour (250 g) of *P. vulgaris* seeds with 3 L of boiling 80% aqueous ethanol under reflux for 30 min. The extract was filtered, discolorized with activated charcoal, concentrated to 100 mL under vacuum 40 °C), and passed through a cation-exchange resin column (H^+ form) in order to separate pipecolic acid from simultaneously extracted oligosaccharides. Pipecolic acid was then eluted by 1 N HCl. The eluate was evaporated to dryness (40 °C) under vacuum, added with water, and reevaporated (×3). The residue was extracted with 100 mL of absolute ethanol, and the cleared extract was filtered and dried under vacuum. After redissolution in distilled water, the sample was applied to a (4 × 50 cm) preparative HPLC column (Jobin et Yvon, France), packed with 200 g of R Sil C18 (15–25 μm, Alltech Associates), and eluted with ultrapure water. Flux was monitored with both an Erma ERC 75-10 differential refractometer of a deflection type (for pipecolic acid) and a UV III detector from Laboratory Data Control (for substances absorbing at 280 nm that were present in plant extracts). Four successive chromatographic runs were required for a complete separation of pipecolic acid from other contaminating substances. After vacuum concentration, the pure sample was freeze-dried.

Identification. The purified compound was submitted to elemental analysis and to ^{13}C NMR with comparison with standard L-pipecolic acid (Fluka, Switzerland). ^{13}C NMR spectra were recorded in the pulsed Fourier transform mode on a Bruker WM 250 spectrometer operating at 62.896 MHz and equipped with a 10-mm probe. Tetramethylsilane (Me_4Si) was used as internal standard.

Quantitative Analysis. *Phaseolus* and *Vigna* seeds were hand dehulled and milled in an IK grinder (3 min) to pass through a 0.5-mm sieve. Cotyledon flour (2.5 g) was extracted twice with 40 mL of boiling 80% aqueous ethanol (30 min) (Cerning, 1970). Purification was performed by Carrez salts: 0.5 mL of Carrez solution I (23.8 g of zinc acetate trihydrate and 3 g of glacial acetic acid dissolved in 100 mL of water) and 0.5 mL of Carrez solution II (10.6 g of potassium ferricyanide dissolved in 100 mL of water) were added to the extract that was concentrated to 25 mL before HPLC analysis on a Waters liquid chromatograph equipped with a (0.46 × 25 cm) column packed with spherisorb NH_2 (5 μm, Harwell) (Quemener and Mercier, 1980). Solvent (acetonitrile/water, 70:30) was

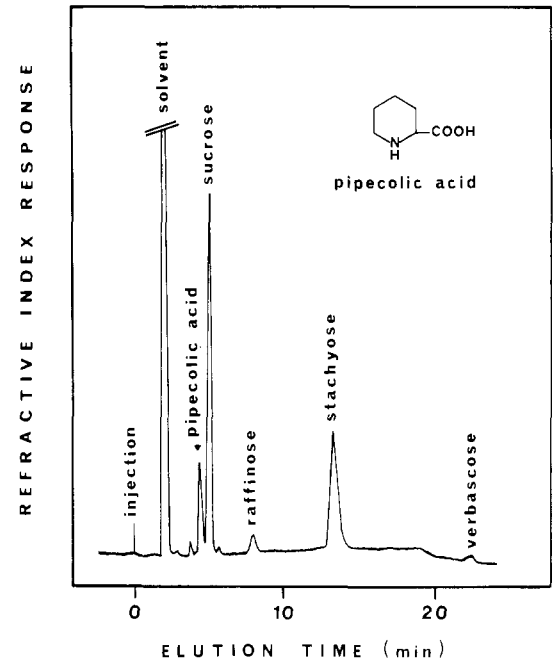

Figure 1. High-pressure chromatogram of alcoholic (80% ethanol) extract of *P. vulgaris* seed cotyledons.

delivered by a Waters 6000A pump at 2 mL/min. Detection was ensured by the same detectors than those used in preparative HPLC. The ethanolic extract from *P. vulgaris* was also analyzed for pipecolic acid content on a Kontron liquimat III autoanalyzer equipped with a (30 × 0.4 cm) column of cationic resin Durum DC 6A, using standard conditions. All the extracts were examined for glucose and fructose amounts by using the standard enzymatic UV method (Boehringer, 1980).

RESULTS AND DISCUSSION

The compound eluted just before sucrose (Figure 1) was previously reported as glucose according to its elution volume under the same analytical conditions as in our case, e.g., *P. vulgaris* extract, NH_2 column, acetonitrile/water 70:30, flow rate 2 mL/min (Conrad and Palmer, 1976). However, glucose enzymatic determinations (Table I) performed on various extracts from quoted seeds revealed only minute amounts of glucose that are undetectable by refractive index detection under conditions used by these authors (detection limit in 4× attenuation being about 1 μg of glucose, which corresponds to 0.1% dry matter basis). In addition, under more efficient separation conditions (acetonitrile/water, 85:15), standard glucose was well separated from the unknown compound. These results allowed us to conclude unequivocally that the compound eluted just before sucrose was not glucose in our case. Further tentative identifications by means of numerous techniques including comparison with standard mono- and disaccharides in HPLC and HPTLC (in association in this case with multiple spraying techniques for detection of sugars) and use of enzymes (β-fructosidase, α- and β-galactosidase, α- and β-glucosidase) failed to identify this compound. Preliminary attempts at purification showed

that it was firmly bound to cation-exchange resin (H^+ form) contrary to oligosaccharides that were not retained.

After extensive purification, including preparative HPLC chromatography on R Sil C18, which allowed separation from UV absorbing substances, elemental analysis of the purified unknown compound provided the following values: C, 55.5%; O, 24.5%; N, 10.5%; H, 8.5%. ^{13}C NMR spectrum showed six peaks at 174.7, 59.4, 44.0, 26.8, 22.0, and 21.8 ppm. They could be attributed to respectively C=O, C—H and CH_2 for the last four peaks. These values fit well with the elemental analysis (except N); such a structure was proposed that corresponded to pipecolic acid (Figure 1), an unusual amino acid known to be present in some legume seeds and typically in the *Phaseoleae* ones. The ^{13}C NMR spectrum of a standard L-pipecolic acid was fully identical with that of the purified compound. This standard was coeluted with our compound in both HPLC and automated amino acid analysis. In the second case, it was well separated and eluted just after valine. Pipecolic acid, which is the higher homologue of proline, exhibited a higher response at λ = 440 nm than at 570 nm when reacted with ninhydrin. An aminogram of the total alcoholic extract from *P. vulgaris* flour confirmed the presence of free pipecolic acid at a 0.7% level (dehulled dry matter basis), same amount being obtained by HPLC (Table I). We can infer, from this result, that no interfering substance is coeluted with pipecolic acid in HPLC. Several other free amino acids such as valine, leucine, isoleucine, tyrosine, phenylalanine, lysine, and arginine were present in trace levels (about 0.01%) except for glutamic acid (0.09%; alanine, 0.036%). Such very low concentrations are not expected to interfere in refractive index detection used in HPLC contrary to pipecolic acid, which is present in noticeable amounts. As a conclusion, it must be pointed out that great care must be taken for identification and quantitation of oligosaccharides by HPLC coupled to a refractometer since noncarbohydrate molecules can interfere and that the lowering of separation efficiency of an NH_2 column with age can even lead to cochromatography of pipecolic acid and sucrose, thus providing erroneous results (the number of theoretical plates, N, measured on the stachyose peak, decreasing from 2000 (Figure 1) down to about 500). This problem may be resolved by increasing the proportions of acetonitrile in elution solvent. In fact, association of a colorimetric detection procedure for sugars with refractive index measurement must be very helpful for prevention of wrong identifications and quantitations.

Taking advantage of possible coanalysis of both oligosaccharide and pipecolic acid by HPLC subsequent differential refractometry detection, we have investigated several species from two genera (*Phaseolus*, *Vigna*) belonging to the *Phaseoleae*. Results are indicated in Table I on a dehulled dry matter basis. The relative distribution of sucrose, raffinose, stachyose, and verbascose was very different from one to another seed. The richest in stachyose, an (DP 4) α-galactoside that is thought to be partly responsible of flatulence (Fleming, 1981) was *P. lunatus* (5%) while *P. aureus* showed the highest verbascose amount (3.3%). With regards to pipecolic acid content, other differences appeared. *P. lunatus*, *P. coccineus*, and *P. vulgaris* contained increasing amounts (0.4–0.7%), while levels of *P. aureus* and *V. unguiculata* are lower than 0.1%. The absence of pipecolic acid in *P. aureus*, *Phaseolus mungo* (Bell, 1966), *Phaseolus pilosus*, and *Phaseolus angularis* (Casimir and Le Marchand, 1966) led some taxonomists to suggest that these legumes without pipecolic acid are probably more closely related to the *Vigna* species, which are also free from pipecolic acid, than to the other *Phaseolus* ones.

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