ever it is not safe to rely on this index since under some conditions, such as prolonged heating of milk at high temperatures, the measured amount of free amino groups increased while the quality of the protein was reduced.

While the measurement of soluble nitrogen has served as an excellent index of heat treatment for soybean oil meal, it was not satisfactory when applied to soymilks, possibly because the protein changes occurring in milk follow different patterns than those seen when dry beans are heated. For example, after an initial sharp drop in nitrogen solubility, further heating does not decrease and may even increase the per cent of soluble nitrogen in soymilk. As a result, soluble nitrogen levels of between 20 and 40% can be found in milks given a wide range of heating conditions and a variety of biological values.

A knowledge of the history of a dried soymilk facilitates the use of protein indices in evaluating possible protein damage. The relation between indices and PER was dependent to some extent on whether the heat was applied to dry beans or to liquid milks. Free amino groups were particularly sensitive to the method of heating. The most reliable indices were available lysine and Hunter "L" values.

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NUTRIENTS IN NUT KERNELS

Free Amino Acids in English Walnut (*Juglans regia*) Kernels

LOUIS B. ROCKLAND and BENLEY NOBE

Fruit and Vegetable Chemistry Laboratory, Pasadena, Calif.

THE SOLUBLE nitrogenous constituents in walnut kernels have not been characterized previously. It has been proposed (16) that nitrogen compounds may react with carbonyl compounds evolved from the degradation of sugars (1, 5, 11) or lipids (14, 24, 28) to form rancid flavors and odors. Recent studies on the mechanisms of rancidity in various protein foods (20) support this proposition. Two-directional filter paper chromatographic studies by the present authors of extracts of fresh and rancid walnut kernels suggested that the development of rancidity involved changes among the ninhydrin-positive constituents (unpublished data). The presence in walnuts of reducing and nonreducing sugars, as well as large amounts of unsaturated lipids, has been established. Characterization of the free

amino acids and related nitrogenous constituents is a prerequisite to investigations on reactions between these compounds and the sugars, lipids, and their degradation products, and the influence of their products on walnut quality.

Attempts to identify the major nitrogenous constituents in kernels by filter paper chromatographic techniques only partially succeeded. Unequivocal identification of many spots was impossible because of insufficient material for chemical studies, the unavailability or nonspecificity of distinctive color reactions applicable to paper chromatograms, or diffuse spots which suggested concurrence of a number of compounds having similar chromatographic properties. Therefore, gradient elution ion exchange fractionation was employed for preliminary separation and isolation

of the major constituents in sufficiently pure form to permit a study of their chemical, physical, and chromatographic properties.

Experimental

Paper Chromatography. Paper chromatography was employed for the analyses of ion exchange column eluates, purified fractions, and crystalline products. The techniques were procedures described previously (18, 19) modified (17) to improve sensitivity and reproducibility.

Solvent I. A stable mixture of *tert*-butyl alcohol : water : 85% formic acid in a ratio of 69.5:4.0:26.5 by volume at 24.0° C. (14).

Solvent II. Phenol : water : ammonia (19).

Water-soluble nitrogenous constituents have been examined in an extract of defatted walnuts. The aqueous extract obtained from 976 grams of hexane- and chloroform-extracted kernels was fractionated on a large Dowex-50 ion exchange column, using gradient elution and ammonium formate buffer. Alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, phenylalanine, tyrosine, and valine were isolated in crystalline form from the column eluate and characterized by their infrared spectra, chromatographic behavior, and color reactions. Asparagine, histidine, hydroxyproline, isoleucine, methionine, proline, serine, and threonine were not completely resolved by the ion exchange fractionation. These compounds were identified in the column eluate by specific color reactions and precision R_f values on paper chromatograms. Methionine sulfoxide, lysine, γ-aminobutyric acid, pipecolic acid, citrulline, and sarcosine were identified tentatively by R_f values and color reactions on paper chromatograms prepared from ion exchange column eluates. Thirty-nine additional ninhydrin-positive compounds were distinguished at lower concentrations. These compounds were characterized chromatographically.

Solvent III. A mixture of 85% tert-butyl alcohol : 15% water by volume at 24° C. This solvent system was equivalent to the tert-amyl alcohol system employed for ascending chromatography by Miettinen and Virtanen (12), which required 2 weeks' irrigation to separate the leucines. Small-scale ascending chromatography (26) on S & S 507 or 589 Blue Ribbon filter paper with four $^3/_4$- X 5-inch strips of Eaton-Dikeman 625-78 filter paper stapled to both sides at the top to absorb the solvent front, separated the leucines within 50 hours. Under the same conditions, valine, methionine, and phenylalanine were separated from the leucines and from each other.

WASHING CHROMATOGRAMS. Color tests on paper chromatograms were improved by extracting the completed, dry but unstained chromatograms with hot chloroform. The azide-iodine-starch and silver nitrate tests were previously not applicable to chromatograms irrigated with phenol. After washing the phenol-irrigated papers with hot chloroform, sulfhydryl and other reducing compounds were readily visible. Sensitivity of Ehrlich, Sakaguchi, and diazotized sulfanilic acid reagents also increased. The 5- X 5-inch chromatograms were washed with hot chloroform in a large Soxhlet extractor. The chromatograms were rolled into a cylinder and placed in a 90- X 200-mm. Soxhlet extraction thimble. Blotting paper was placed over the thimble to absorb water droplets emerging from the reflux condenser to prevent them from wetting and distorting the chromatographic patterns. Approximately 10 to 12 washing cycles took place in 4 hours without any detectable loss of nitrogenous constituents.

REAGENTS FOR COLOR TESTS ON PAPER CHROMATOGRAMS. No. 1. Ninhydrin (27).

No. 2. Isatin (19).

No. 3. Cupric Nitrate–Ninhydrin, (13).

No. 4. Pyridoxal-Ninhydrin, a modification of the technique suggested by Kalyankar and Snell (9). Chromatograms were dipped into a solution of 0.25 gram of pyridoxal hydrochloride in 100 ml. of 95% ethyl alcohol, dried in air, heated for 10 minutes at 90° C., cooled, and dipped into ninhydrin reagent (No. 1).

No. 5. Ehrlich Reagent (15).

No. 6. Proline-Hydroxyproline Reagent. Chromatograms were dipped into isatin reagent (No. 2) and dried. Proline and hydroxyproline gave blue-green against a yellow background. A number of other amino acids gave indiscriminate colors at high concentrations. Washing the chromatograms with 1N hydrochloric acid removed the background coloration as well as colors formed by components other than proline or hydroxyproline. Proline and hydroxyproline were both "fixed" blue against a white background.

No. 7. Diazotized Sulfanilic Acid. Referred to by Block et al. (3) after Baldridge and Lewis (2). The reagent was prepared by dissolving 1 gram of sulfanilic acid in a solution of 8 ml. of concentrated hydrochloric acid in 100 ml. of water and combining an equal portion of the solution with 0.69% sodium nitrite solution. Chromatograms were sprayed with a freshly prepared diazotized sulfanilic acid reagent, dried at 25° C., and sprayed with 10% sodium carbonate.

No. 8. Periodate-Acetylacetone (Serine Reagent). A modification of the method described by Schwartz (27) was employed. The chromatograms were dipped into the periodic acid–collidine reagent, drained, and immersed in the acetylacetone solution while still moist. The specific yellow color for serine appeared within 30 minutes. The color test was estimated to be about one fifth as sensitive as ninhydrin reagent.

No. 9. Periodate-Nitroprusside-Piperidine (Threonine Reagent). The reagents described by Schwartz (27) were employed. The chromatograms were dipped rather than sprayed with both the periodate-collidine and nitroprusside-piperidine reagents. It was necessary to dip the chromatograms in the nitroprusside-collidine reagent almost immediately after applying the periodic acid reagent to obtain a satisfactory color reaction for threonine. The sensitivity of this reaction was estimated to be about one fifth that of the ninhydrin reagent.

No. 10. Azide-Iodine-Starch. Chromatograms irrigated with phenol were washed with hot chloroform as described previously. The reagent was prepared by dissolving 0.75 gram of sodium azide, 1.0 gram of potassium iodide, and 0.01 gram of iodine in 13 ml. of distilled, deionized water and adding 150 ml. of redistilled 95% ethyl alcohol. The chromatograms were dipped into the azide-reagent for 5 seconds or less, drained, blotted at the lower edge to remove excess reagent, and allowed to stand at ambient temperature until the background faded to a pale yellow color. The chromatograms were sprayed uniformly on both sides with a 1% aqueous solution of soluble starch. Methionine, cysteine, and other reactive sulfur compounds bleached a white area against an intense blue background. Under optimum conditions, the sensitivity of this reagent is somewhat greater than that of the ninhydrin.

No. 11. Iodoplatinate (25).

No. 12. Silver Nitrate. Chromatograms irrigated with phenol were washed with hot chloroform and dipped into a saturated solution of silver nitrate in 95% ethyl alcohol. Strong reducing compounds including ascorbic acid and

some reducing sugars reduced the silver immediately. Reactions with the amino acids required ammonia fumes and varying degrees of exposure to light. Chromatograms were "fixed" by immersion in dilute acetic acid-bisulfite or commercial "hypo."

Infrared Spectra. Infrared spectra of pure standard compounds and unknown compounds were observed by the KBr pressed-disk procedure (10), except that 0.5% rather than 1.0% concentrations of standards or unknowns were used for window preparations.

Preparation of Walnut Extract. Light-colored kernels of the Placentia Perfection variety were employed. They contained 4.03% moisture, 2.72% nitrogen, and 71.1% lipid on a dry-weight basis. A 976-gram portion of sliced kernels was extracted with hexane for 6 hours in a Soxhlet extractor. The walnut meal was dried for 24 hours at ambient temperature, ground to a powder, and triturated with chloroform. The denser seed coat particles (pellicle) settled in an excess of chloroform. The meal, which floated near the surface or remained suspended, was decanted and filtered. The triture, flotation, decantation, and filtration processes were repeated three times. The filtered meal, almost free of pellicle, was dried at ambient temperature. Removal of the pellicle was necessary to minimize the co-extraction of tannins from the pellicle during the extraction of the nitrogenous constituents with water. The dry weight of defatted meal was 282 grams. The meal was suspended in 1000 ml. of water, allowed to stand overnight at 5° C., filtered, and washed with water. The extraction was repeated five times, and the filtrates and washings were combined. Polymeric carbohydrates and proteins were precipitated by an equal volume of

acetone, and washed with aqueous acetone. The combined extract and washings were evaporated to about 2 liters under reduced pressure.

Ion Exchange Separations. The brown extract was placed on a 54- × 400-mm. column of Dowex-50 × 8, minus 400 mesh, pre-equilibrated with 0.5N formic acid. The column was washed with 0.5N formic acid to elute the sugars which emerged close to the solvent front. A trace of ninhydrin-reactive material was eluted during washing of the column to remove the sugars. The nitrogenous constituents were eluted with 2 liters of 1N ammonium hydroxide. The eluate was lyophilized, made up to 100 ml. with water, adjusted to pH 3.5 with concentrated formic acid, and placed on a fresh, essentially identical Dowex-50 × 8 ion exchange column. Just as the upper surface of the solution penetrated the resin, 0.5N formic acid at pH 2.0 was admitted from the mixing chamber. During gradient elution, which required 14 days, the following buffer solutions were placed successively in the reserve buffer chamber: pH 2.0, 250 ml.; pH 2.3, 800 ml.; pH 2.6, 2700 ml.; pH 2.9, 3000 ml.; pH 3.1, 2900 ml.; pH 3.2, 2900 ml.; pH 3.4, 2900 ml.; pH 3.6, 2800 ml.; pH 4.0,

3000 ml.; pH 4.6, 2400 ml.; pH 5.0, 2750 ml.

Finally, 4 liters of 1N ammonium hydroxide were percolated through the column starting after fraction 1180 was obtained. The initial column flow rate was 78 ml. per hour, increasing to as high as 115 ml. per hour before regressing to about 75 ml. per hour at the end of the process. The fraction collector removed fractions ranging from 20 to 25 ml. The pH gradient of the column eluate is shown in Figure 1. The total of 1450 fractions was about 28 liters. Nitrogenous constituents in the eluate were detected by placing a drop of solution from every fifth fraction on a strip of S & S 507 filter paper, drying it and dipping it in ninhydrin reagent (No. 1). Ninhydrin-reactive fractions were retained while the rest were discarded.

Aliquots from every other tube were chromatographed in one direction. Multiple spots were observed for many fractions. Those fractions which apparently contained the same compounds were combined and evaporated to dryness using a freeze-dryer. Residual ammonium formate was sublimed at 50° C. under vacuum (1 mm. or less). Small two-directional chromatograms were prepared for each of the combined residues
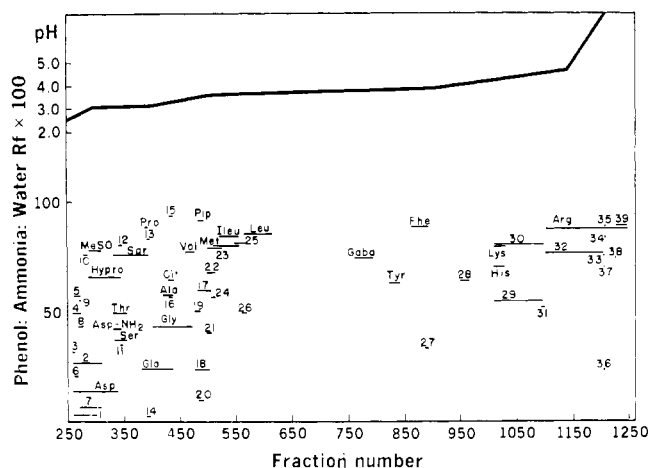

Figure 1. Gradient elution ion exchange fractionation of nitrogenous constituents in defatted walnut kernel extracts

Upper curve represents the pH gradient; horizontal lines or dots indicate fractions which contained compounds designated by letters or numbers; position of lines on the vertical axis indicates the R_f values observed for the compounds in phenol:ammonia:water solvent mixture
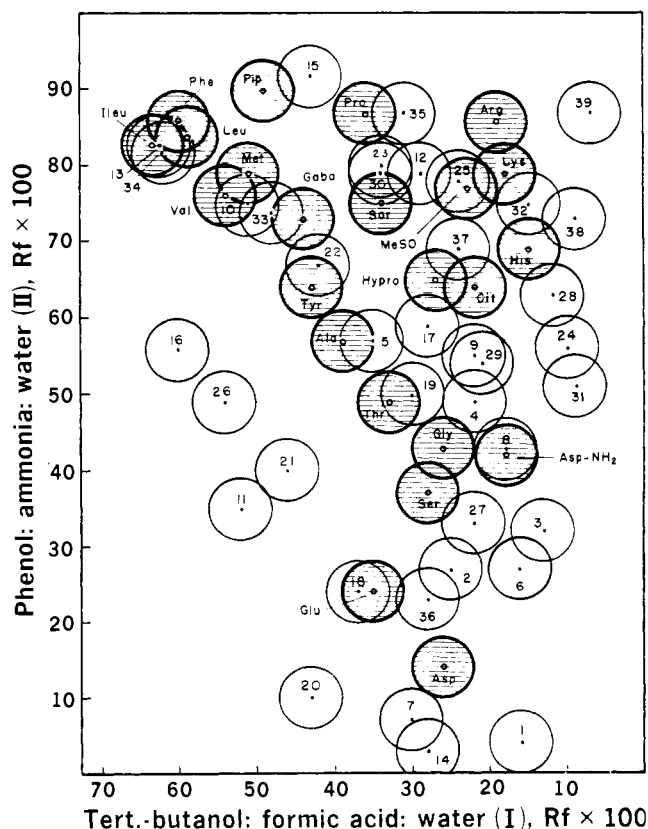

Figure 2. Diagrammatic chromatographic map of nitrogenous constituents observed in defatted walnut kernel extract

Shaded circles indicate compounds which have been characterized; abbreviations and numbers are same as those in Tables I and II

Table I. Ion Exchange and Paper Chromatography Characterization of Major Nitrogenous Constituents in Walnut Kernel Extracts

Compound	Ion-Exchange Fraction	$R_f{}^a$ (× 100)	Color Reactions on Paper Chromatograms with Various Reagents[b]				
			No. 1	No. 2	No. 3	No. 4	No. 5
Aspartic acid	standard	23/15	B	G-B	Y or B-G	Rust or P	Y (late)
(Asp)	258 to 342	26/14	B	G-B	B .	Rust	Y (late)
Methionine sulfoxide	standard	23/79	P	P	Y	P	Y (late)
(MeSO)	285 to 312	23/77	P	P	Y	O or P	Y (late)
Hydroxyproline	standard	26/65	Y	G-B	Y	Y	
(Hypro)	285 to 348	27/65	Y	G-B	Y	Y	
Sarcosine	standard	31/75	P (late)	P	Light P (late)	B-P (late)	Neg.
(Sar)	329 to 398	32/75	P (late)	P	Light P (late)	B-P (late)	Neg.
Threonine	standard	30/49	P	B-P	B-P	O or P	
(Thr)	329 to 398	33/49	P	P	P	Rust O or P	
Serine	standard	24/36	P	P	Y to P	P	
(Ser)	332 to 359	28/37	P	P	Bn to P	P	
Asparagine	standard	16/42	Y to Bn to P	B-P	O or Rust	O	
(Asp-NH₂)	332 to 348	16/42	Y to Bn to P	B-P	O	O	
Glutamic acid	standard	32/23	P	G or B-P	P	O or Rust	Y (late)
(Glu)	382 to 442	35/24	P				
Proline	standard	35/87	Y	B-G	Y	Y	
(Pro)	392 to 396	36/87	Y	B-G			
Glycine	standard	25/42	P	P	Y-O or Rust	B-P	
(Gly)	401 to 476	26/43	P				
Alanine	standard	36/57	P		P	Rust or P	Y (late)
(Ala)	422 to 442	39/57	P				
Citrulline	standard	22/64	P		P	Rust or P	Y (fast)
(Cit)	432 to 442	22/64	P				Y (fast)
Valine	standard	51/77	P	Gn or P	P	O or Rust	Y (late)
(Val)	462 to 480	54/76	P			O	
Pipecolic acid	standard	44/90	R-P (late)			B-P (late)	
(Pip)	484 to 496	48/90	R-P (late)			B-P (late)	
Methionine	standard	51/80	P	B-P	P or P-Bn	P	Y
(Met)	511 to 560	51/79	P			P	Y
Isoleucine	standard	64/84	P	B-G or P	P	O or Rust	Y (late)
(Ileu)	532 to 560	63/83	P	P		O or Rust	
Leucine	standard	63/85	P	P	Bn-P	O-Rust or P	Y (late)
(Leu)	564 to 620	65/84	P			Rust or P	
γ-Aminobutyric acid	standard	41/75	P (slow)		Y to Bn to P	B-P	Pink
(Gaba)	762 to 800	43/73	P (slow)		Y to Bn to P	P	Pink
Tyrosine	standard	41/63	P	G	Neg.		Y (late)
(Tyr)	824 to 847	43/64	P				
Phenylalanine	standard	54/87	P	G or B-G to B-Gy	Y or Rust	O or Rust	Y (late)
(Phe)	865 to 896	60/86	P	B-G			Y (late)
Histidine	standard	14/70	Gy-P	B-P	Gy-B to Gy-P	O or P	O or Pk
(His)	1010 to 1032	15/69	Gy-P	B-P	Gy-P	O or P	O or Pk
Lysine	standard	16/79	P	P-Gy to Gn-P	O or Rust or P	O or P	Y or Pk
(Lys)	1010 to 1032	18/79	P	P-Gy to Gn-P	P	P	Y or Pk
Arginine	standard	16/89	P	P	P	P	Y or O
(Arg)	1300 to 1450	19/86	P	P	P	P	O

[a] Average of at least 3 replicate two-directional paper chromatograms irrigated with Solvents I and II, respectively, designated $(R_f$ in $I)/(R_f$ in II).

[b] Color designations: B blue; G green; Y yellow; O orange; R red; P purple; Bn brown; Gy grey; Pk pink. Two colors separated by a hyphen indicate an intermediate color. Example: B-G indicates blue-green. Two colors separated by "to" indicate a gradual color transition over a period of up to 24 hours. Example: Y to Bn is a transition from yellow to brown. Two colors separated by "or" indicates a variable color reaction depending upon concentration of test material or other anomalous variables. Example: O or Rust indicates that both colors have been observed. The relative rates of reactions with various reagents are indicated by: (fast), instantaneous reaction. (slow), full color development required up to 24 hours, (late), color formation required 24 hours or longer.

using improved precision techniques (17). At least three and generally six replicate chromatograms were prepared from each fraction.

The elution pattern obtained from the walnut extract is shown in Figure 1. The column fraction number is plotted against the R_f value observed in Solvent II. Many fractions contained multiple components, which were distinguished on two-directional paper chromatograms. A few and sometimes single fractions contained a pure or essentially pure compound. However, in most cases, the single tube fractions did not contain sufficient material to permit isolation, purification, and characterization of the compound. To obtain enough material for purification and characterization of a compound, tubes containing a major proportion of this compound were combined even though small amounts of minor components, eluted concurrently, may have been included. Fractions which appeared to contain one major constituent, identified tentatively by chromatography and color tests, were evaporated and the dried residue was purified (6).

The following criteria were considered evidence for the characterization of constituents: elution sequence from ion exchange column; precision R_f values on two-directional paper chromato-

grams; distinctive color tests at appropriate loci on paper chromatograms; infrared spectra of those compounds obtained in crystalline form; color tests on purified fractions, and NMR spectra.

Results

Table I summarizes the characteristics of 23 major compounds found. A similar compilation of the properties of 39 additional unknown compounds is shown in Table II. A two-directional chromatographic map, showing the loci of all compounds that could be distinguished, is presented in Figure 2.

Aspartic Acid. The dry solid obtained from fractions 314 to 318

Table II. Ion Exchange and Paper Chromatography Characterization of Unknown Constituents in Walnut Kernel Extracts

No.	Fractions	$R_f{}^a$ (× 100)	Color Reactions on Paper Chromatograms with Various Reagents[b]				
			No. 1	No. 2	No. 3	No. 4	No. 5
1	258 to 306	16/4	P	P	Y or O		
2	258 to 312	25/27	P	P		O or P	
3	258 to 260	13/32	P				P
4	258 to 274	22/49	P		Bn-P	O	
5	262 to 274	35/57	P	B-P or P-Gy	Pk-P	O or P	
6	262 to 268	16/21	P				
7	270 to 312	30/7	P	B-G or B-Gy	Y	O or Rust or P	Y (late)
8	270 to 280	18/43	P		Bn or P-Bn		
9	270 to 274	22/55	P		Bn or P-Bn	P	
10	278 to 286	51/75	P	P		O or P	
11	344 to 348	52/35	P	Neg.	Neg.	Pk	Y (late)
12	344 to 348	29/79	P	G	Neg.	Pk	Neg.
13	392 to 398	62/82	P		Neg.	Pk or Rust	
14	392 to 398	28/3	P		Neg.	Neg.	
15	432 to 442	43/92	Y	Neg.	Y	Neg.	Neg.
16	432 to 442	60/56	P	Neg.	P	Rust-P to P	Y
17	484 to 510	28/59	P	Neg.	Y	Y or B-P	Neg.
18	478 to 506	37/24	P	Neg.	Y	B-P or P	Neg.
19	478 to 490	30/50	P			Gy	
20	484 to 496	43/10	P		Y or Pk or Bn	O or P or B-P	Pk
21	501 to 510	46/40	P	Neg.	Y or Rust	B-P	
22	501 to 516	42/67	P	Neg.	Y	B-P	
23	501 to 516	24/78	B-P	Neg.	Y or P	Rust or B-P	
24	508 to 516	10/56	P	Gy-P	Bn-P	O or Rust	
25	550 to 572	34/80	Y	Neg.	Y	O or Y	
26	564 to 572	54/49	P				
27	888 to 896	22/33	P				Neg.
28	952 to 969	12/63	P	P	P	O or Pk or P	Pk
29	1010 to 1101	21/54	P	Gy-B or B	Bn to R-P	O or Rust or P	Neg.
30	1010 to 1102	34/79	P	Neg.	Bn or Rust	P	Neg.
31	1096 to 1101	9/51	P				
32	1102 to 1248	15/75	P	B-Gy	Y	Pk or Rust or P	
33	1247	48/74	P	P	Neg.		Neg.
34	1247	62/82	P	P	Neg.		Neg.
35	1247	31/87	P	G	Neg.	Gy	Neg.
36	1247	28/23	P	P	P	Pk or Rust or P	Neg.
37	1247 to 1248	24/69	P	P	P	P	
38	1250 to 1300	9/73	P	B-P	P		
39	1250 to 1450	7/87	P	P	P	O to Rust	O

[a] Average of at least 3 replicate two-directional paper chromatograms irrigated with Solvents I and II.
[b] Color and other designations are the same as those presented in the footnotes to Table I.

appeared homogeneous on paper chromatograms. It was recrystallized from water and alcohol and finally from hot water. The compound corresponded with L-aspartic acid in respect to its R_f values and color reactions with reagents Nos. 1 to 5 at the same locus on filter paper chromatograms. The purified material had an infrared spectrum similar to authentic L-aspartic acid with the exception of a shoulder at 3.0 microns and a small peak at 12.5 microns. The extra peaks are presumably from a trace of serine, since an ad hoc mixture of 87% L-aspartic acid and 13% serine had a spectrum nearly identical with the recrystallized material. The NMR spectrum of the crystalline material was identical with that of authentic L-aspartic acid.

Methionine Sulfoxide. Fractions 285 to 312 contained a compound which corresponded to authentic methionine sulfoxide in respect to R_f values and color tests Nos. 1 to 5 at the appropriate loci on two-directional paper chromatograms. Since methionine also appears in walnut extracts, possibly all or a portion of the methionine sulfoxide

may have been formed during extraction or subsequent passage through the ion exchange column.

Hydroxyproline. Fractions 285 to 312 contained a compound which turned yellow with ninhydrin at the locus of hydroxyproline on two-directional chromatograms. The major portion of the compound appeared in fractions 332-342. It corresponded to L-hydroxyproline in respect to R_f values and color tests Nos. 1 to 4. The blue-green formed on paper chromatograms by the unknown and authentic hydroxyproline after treatment with isatin reagent (No. 2) was converted to a light blue by washing with $1N$ hydrochloric acid. Of the known, naturally-occurring amino acids only proline and hydroxyproline respond in this manner. All other nitrogenous constituents that yield light, indiscriminate colors with isatin reagent were removed by the acid wash.

Sarcosine. A compound believed to be sarcosine was observed in fractions 329 through 398. It was eluted from the ion exchange column as a broad band, encompassing the successive elu-

tion of concurrent zones of serine, asparagine, hydroxyproline, threonine, and other constituents. Insufficient pure material was obtained to complete its characterization. The compound corresponded with authentic sarcosine in respect to R_f values and color reactions with reagents 1, 2, 4, and 5 on two-directional paper chromatograms. Although sarcosine has not been observed frequently in natural products, it has been reported in cow's milk (22), rat urine (8), peanuts (7), actinomycin (4), and other materials cited in these references.

Threonine. Fractions 329 to 359 contained a compound which corresponded to L-threonine in respect to its R_f values and color reactions at the same loci with reagent Nos. 1 to 4 on two-directional paper chromatograms. The compound responded identically to authentic threonine on paper chromatograms treated with Schwartz's (21) highly specific threonine reagent (No. 9).

Serine. Fractions 332 to 359 also contained a compound which gave R_f values and color reactions of paper chromatograms with reagent Nos. 1 to 4

that corresponded with authentic L-serine. Identical color reactions were obtained for the compound and serine on paper chromatograms using the highly specific color reagent (No. 8) described by Schwartz (27).

Asparagine. Fractions 344 to 348 contained the highest concentrations of a compound that gave the yellow-to-brown-to-purple color sequence on paper chromatograms stained with ninhydrin reagent (No. 1) that is characteristic of asparagine at the observed locus. The compound corresponded to authentic L-asparagine in respect to R_f values and color reactions with reagent Nos. 2, 3, and 4 on two-directional paper chromatograms. An aliquot of the same fractions was refluxed with 6N hydrochloric acid for 30 minutes, neutralized, and chromatographed on paper in the usual manner. The absence of a spot at the asparagine locus and a slight increase in ninhydrin color at the aspartic acid locus was further evidence of asparagine.

Glutamic Acid. Fractions 382 to 388 gave only a single purple spot at the locus of L-glutamic acid on two-directional paper chromatograms stained with ninhydrin reagent (No. 1). About 75 mg. of crude, dry material was obtained. It was recrystallized twice from hot water and washed with 80% methyl alcohol. About 6 mg. of pure, crystalline material was obtained. The infrared and NMR spectra of this material were identical with those of authentic L-glutamic acid.

Proline. Two-directional paper chromatograms of fractions 393 to 398 indicated the presence of at least six ninhydrin-positive compounds including a compound which gave a brilliant yellow spot that dominated the chromatogram. This compound was eluted sharply from the column, in only five tubes. The compound corresponded to authentic L-proline in respect to R_f values and color reactions with reagent Nos. 1, 2, and 4. The blue-green at the proline locus on two-directional paper chromatograms stained with isatin (No. 2) reagent was converted to deep blue by washing with 1N hydrochloric acid. Of the known naturally-occurring amino acids, only proline and hydroxyproline respond in this manner.

Glycine. Only one major spot was observed on paper chromatograms prepared from fractions 408 to 418. Approximately 100 mg. of crude, light-brown material was left after removal of solvent and buffer salts. This material was recrystallized twice from water and ethyl alcohol. The purified compound gave a single spot at the locus of authentic glycine on ninhydrin-stained, two-directional paper chromatograms. The infrared spectrum of this material was identical with that of purified glycine except that absorption peaks at 9.7 and

11.0 μ were about 0.1 μ lower and the peak at 14.4 μ was about 0.2 μ lower than from authentic glycine. However, the glycine samples were identical after each was dissolved in a small amount of water and lyophilized prior to the preparation of the KBr windows. The NMR spectrum was identical with that of authentic glycine.

Alanine. Chromatograms of fractions 422 to 430 suggested the presence of alanine. After removal of the solvent and buffer salts, the crude solid was dissolved in a minimum of hot water and made up to 75% ethyl alcohol. Precipitation of the alanine was forced by addition of acetone and cooling at 5° C. R_f values of the purified material corresponded to L-alanine on two-directional paper chromatograms stained with ninhydrin reagent (No. 1). The infrared and NMR spectra of the isolated material were identical with those of authentic L-alanine.

Citrulline. Fractions 432 to 442 contained a compound identified tentatively as citrulline. It gave an immediate brilliant yellow color with Ehrlich's reagent (No. 5) corresponding to authentic citrulline at identical loci on two-directional paper chromatograms. At high concentrations, alanine, glutamic acid, and a number of other nitrogenous compounds gave a latent yellow color with Ehrlich's reagent. However, none of the other constituents produced a color reaction at the level required to obtain a strong reaction with the unknown.

Rinderknecht (15) presented evidence for the presence of citrulline in dates. However, this compound has been observed only rarely in natural products (29).

Valine. Fractions 462 to 468 appeared to contain only a single component which corresponded to L-valine in respect to its R_f values and color reactions with reagent Nos. 1 and 4 on two-directional paper chromatograms. The residue obtained after removal of the solvent and sublimation of the buffer was recrystallized from hot water and ethyl alcohol. The infrared spectrum of the purified crystalline material was identical with authentic L-valine.

Pipecolic Acid. Fractions 484 to 496 contained a compound presumed to be pipecolic acid. This compound corresponded to authentic pipecolic acid in respect to its R_f values and distinctive latent formation of red-purple with ninhydrin reagent (No. 1), as well as latent development of a blue-purple with pyridoxal-ninhydrin reagent (No. 4) on two-directional paper chromatograms.

Methionine. Two-directional paper chromatograms of fractions 526 to 531 contained a compound whose R_f values and color reactions were essentially identical with those of authentic L-

methionine. In addition to the close correspondence between the unknown and methionine in respect to their reactions with ninhydrin (No. 1), pyridoxal-ninhydrin (No. 4), and Ehrlich's (No. 5) reagents, identical reactions were obtained at the same loci with azide-iodine-starch (No. 10) and iodoplatinic acid (No. 11) on two-directional paper chromatograms.

Isoleucine. Fractions 550 to 560 contained a compound which had the same R_f values and color reactions with reagent Nos. 1, 2, and 4 on two-directional paper chromatograms as authentic L-isoleucine. The compound migrated at a rate identical with isoleucine on a one-directional paper chromatogram irrigated with Solvent III.

Leucine. Fractions 564 to 620 contained a compound which corresponded to either leucine or isoleucine in respect to R_f values and color reactions with reagent Nos. 1 and 4 on two-directional paper chromatograms. However, isoleucine was observed only in fractions 532 to 560 and four blank tubes (about 80 ml.) were obtained before this compound emerged from the ion-exchange column. Fractions 580 to 582 produced only a single spot on two-directional chromatograms irrigated with Solvents I and II, as well as on a one-directional chromatogram irrigated with Solvent III. In the latter system, the compound migrated at a rate identical with leucine and distinctly different from isoleucine or any other compound known to have a locus near leucine on a two-directional paper chromatogram. Fractions 564 to 580 contained a major proportion of the compound. The infrared spectrum of the crude, dry solids obtained from these fractions was identical to that of pure L-leucine.

γ-Aminobutyric Acid. Fractions 762 to 800 contained a single nitrogenous constituent which corresponded to authentic γ-aminobutyric acid in R_f values and color reactions with reagent Nos. 1, 3, 4, and 5 on two-directional paper chromatograms. It is of interest that the compound was brown with silver nitrate (No. 12) but pink with Ehrlich's reagent (No. 5). Of the known naturally-occurring amino acids, only γ-aminobutyric acid, lysine, and histidine produce a pink color with Ehrlich's reagent under the same conditions. The residue after removal of solvent and buffer salts, was too small to permit further work.

Tyrosine. Fractions 842 to 847 produced only a single ninhydrin-positive component on two-directional paper chromatograms. The R_f values of the compound corresponded with that of L-tyrosine. The crude, dry material, obtained by removal of solvent and sublimation of buffer salts was recrystallized from boiling water and yielded long, thin white needles, characteristic

of tyrosine. They turned red with Millon's reagent and had an infrared spectrum identical with that of authentic L-tyrosine.

Phenylalanine. Two - directional paper chromatograms of fractions 865 to 876 contained only a single spot which corresponded with L-phenylalanine in respect to R_f values and color reactions with ninhydrin (No. 1) and isatin (No. 2). A small amount of crude, solid material remained after removal of solvent and buffer salts. It was dissolved in a minimum of boiling water, and ethyl alcohol was added to 90%. Acetone and cooling at 5° C. for 24 hours forced precipitation of solids. Approximately 5 mg. of a light-colored solid was obtained. The infrared spectrum of this material was identical with that of authentic L-phenylalanine.

Histidine. Two - directional chromatograms of fractions 1010 to 1032 indicated a compound which corresponded with L-histidine in respect to R_f values and color reactions with reagent Nos. 1 to 5 and sulfanilic acid (No. 7). The red obtained with the latter reagent is distinctive at the histidine locus on two-directional chromatograms. The orange produced by the unknown and authentic histidine on one-directional chromatograms treated with Ehrlich's reagent (No. 5) further distinguished this compound from lysine which lies near the same locus but turns yellow. Unknown No. 30 also produced red with diazotized sulfanilic acid (No. 7) reagent at a different locus from that occupied by histidine on two-directional chromatograms.

Lysine. Fractions 1010 to 1032 contained a compound presumed to be lysine on the basis of R_f values and color reactions obtained with reagent Nos. 1 to 5 on two-directional chromatograms. The lack of a distinctive test for lysine or of sufficient material for further characterization permitted only tentative identification of this compound which appeared just above the histidine locus on two-directional chromatograms. A major component with an elution volume identical with that of lysine was observed during the analysis of walnut extracts using a Spackman, Stein, and Moore (23) automatic amino acid analyzer.

Arginine. Only faint traces of unrecoverable nitrogen compounds were eluted from the ion exchange column between fractions 1032 and 1200. Therefore, the gradient elution process was abandoned and the column was eluted with 1N ammonium hydroxide. Fractions 1300 to 1450 contained the major portion of a compound which corresponded to arginine in respect to R_f values and color reactions with reagent Nos. 1 to 5. In addition, the compound gave a positive reaction with Sakaguchi

reagent (27). The combined fractions 1300 to 1450 were reduced to a dry solid and taken up in 5 ml. of warm water. After a small amount of flavianic acid was added to the warmed extract, chilling brought out a yellow-orange precipitate. The precipitate was removed and several successive crops of material were obtained by further successive additions of flavianic acid. When the orange-yellow precipitate was dissolved in dilute ammonium hydroxide and chromatographed on strips of filter paper irrigated with Solvent I, a yellow spot, characterized as free flavianic acid, and a ninhydrin- and Sakaguchi-positive spot corresponding to L-arginine developed. The infrared spectrum of the flavianate derivative was identical to that of authentic L-arginine flavianate.

As succeeding crops of arginine flavianate were removed by gradual additions of flavianic acid, the filtrate became enriched with unknown No. 39. This compound also gave a positive Sakaguchi reaction. However, there were only traces and it could not be separated from the large amounts of arginine.

Characterization of Unknown Constituents. At least 39 unidentified ninhydrin-positive compounds were detected in the eluates from the gradient elution fractionation of walnut kernel extracts. These compounds could not be characterized completely or identified because of insufficient material or gross contamination with other compounds eluted simultaneously. A summary of the chromatographic properties and reactivities of these compounds on paper chromatograms treated with reagent Nos. 1 to 5 is presented in Table II. Figure 2 shows the loci of the unidentified compounds in relationship to those which have been characterized more completely. Each of these compounds was distinguished from each other according to the criteria discussed earlier. Precision paper chromatography techniques (17) were particularly useful in distinguishing compounds which were eluted in close succession and which responded similarly to the color tests. In general, at least three replicate R_f values were averaged. If the average value for the unknown was outside the range of a reference standard established at the 99% confidence level, the compound was considered a separate moiety. Minimum average differences of 0.05 and 0.04 R_f unit were required using Solvents I and II, respectively.

Discussion

Evidence has been presented for the presence in walnut kernels of at least 62 nitrogenous constituents. Of these, 17 have been identified and six more have been identified tentatively. These 23

compounds, a major proportion of the nitrogenous constituents, have been observed before in other plant and animal tissues. The 39 minor constituents which have been characterized chromatographically had been obscured in the past by the superimposition of the major constituents on paper chromatograms prepared from unfractionated walnut extracts. Routine ion exchange analyses of walnut extracts had suggested the presence of few minor constitutents.

Preliminary paper chromatography studies of unfractionated extracts of defatted walnuts permitted tentative identifications of 16 of the 23 compounds characterized in the present study. The earlier studies suggested the presence of glutamine in walnut extract. However, this compound was not observed in the ion exchange column eluate, presumably because of its conversion to pyrrolidone-carboxylic acid, which does not give a color reaction with ninhydrin on paper chromatograms. The seven major compounds which were not detected by paper chromatography prior to ion exchange fractionation included the cyclic amino acids tyrosine, phenylalanine, and hydroxyproline as well as presumed sarcosine, pipecolic acid, citrulline, and methionine sulfoxide. The last compound may have been formed from methionine during ion exchange fractionation.

Many of the unidentified compounds have loci on two-directional paper chromatograms almost identical with those of some of the common amino acids which are distributed ubiquitously in biological systems. The extent to which minor nitrogenous constituents occur in other plant and animal extracts cannot be estimated. However, the major free amino acids would obscure observation of many of the minor constituents on paper chromatograms of unfractionated material. It is also possible that some of the unidentified components could be mistaken for the more common amino acids on the basis of identifications made from paper chromatography R_f values, and general color reactions. However, ion-exchange fractionation, paper chromatography separations, specific or distinctive color tests performed on paper chromatograms, and the use of microanalytical techniques such as infrared and NMR spectroscopy circumvent preparation and analyses of derivatives in many instances, especially when authentic reference compounds are available for direct comparisons.

It would appear important to complete the identification of the minor constituents and to determine their chemical and biological significance. Work is in progress on the preparation of larger amounts of material for the identification of minor constituents and an evaluation of their chemical and biological significance.

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CLOVER CONSTITUENTS

Isolation of Daphnoretin from Ladino Clover

A. L. LIVINGSTON, E. M. BICKOFF, and LEONARD JURD

Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany, Calif.

The isolation of the novel coumarin, daphnoretin, from ladino clover is described. The clover extract was purified by solvent distribution followed by column chromatography. Identity was confirmed by the preparation of derivatives and by comparison of their mixed chromatograms, melting points, and ultraviolet absorption spectra with those of authentic samples.

IN THE COURSE of isolating saponin from ladino clover (*8*), an ether extract was prepared from which a crystalline monomethoxyphenol, $C_{19}H_{12}O_7$, has now been isolated. The formation of monoacyl and monoalkyl derivatives indicated the presence of only one phenolic hydroxyl group. The fluorescence of the compound (blue in ultraviolet light) and its λ_{max} (343 mμ) suggested a coumarin structure. The possible presence of two lactone groupings, as in dicoumarol, was indicated by an unusually wide band at 1710 cm.$^{-1}$ in the infrared spectrum of the phenol.

While this investigation was in progress, Tschesche *et al.* (*7*) reported the isolation of a novel dicoumarin from daphne, daphnoretin(I).
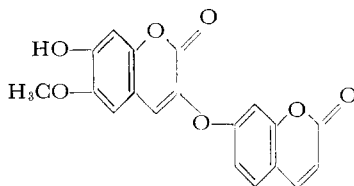
The ultraviolet spectra of daphnoretin and its acetate were identical with that of the ladino clover phenol (Figure 1), and the melting points of the phenols and their derivatives were in close agreement. Direct chromatographic, mixed melting point, and infrared comparisons of the two phenols confirmed their identities.

The final step in the biosynthesis of this compound may well involve the formation of the dicoumarin structure by means of the oxygen bridge. It is of considerable interest that two plants of such widely different species should contain this unusual compound.

Most coumarins have been shown to have a number of physiological effects on animals. Dicoumarol was found to be the hemorrhagic factor in sweet clover by Campbell and Link (*4*). Bose and Sen (*3*) found that ayapin and ayapinin were the hemostatic principles in the leaves of *Enpatorium ayapana*, and Iida (*5*) found that fraxin caused par-