The free acid melted at 172–173°.

6,7-Diethoxy-1-(3′,4′-diethoxybenzyl)-isoquinoline (perparine) was obtained by decarboxylation of the preceding acid in methylnaphthalene as described above. It melted at 93–95° (lit.[6] 95–96°).

Anal. Calcd. for $C_{24}H_{29}O_4N$: C, 72.88; H, 7.35; N, 3.54. Found: C, 73.10; H, 7.78; N, 3.80.

126 BUCKINGHAM RD., YONKERS, N. Y.

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[CONTRIBUTION FROM THE BUREAU OF ENTOMOLOGY AND PLANT QUARANTINE, AGRICULTURAL RESEARCH ADMINISTRATION, U. S. DEPARTMENT OF AGRICULTURE]

Alkaloids from *Tripterygium wilfordii* Hook.—Wilforine and Wilfordine[1,2]

BY MORTON BEROZA

Wilfordine has been shown by countercurrent distribution to be a mixture of alkaloids. Two very similar alkaloids, designated wilforine and wilfordine, were isolated from the mixture by partition chromatography and proved pure by countercurrent distribution. The compounds are insecticidally active ester alkaloids which, upon saponification, yield 1 mole of benzoic acid, 5 moles of acetic acid, and 2 moles of steam-non-volatile acid per mole of compound.

Acree and Haller[3] have recently reported the isolation of wilfordine, an insecticidal alkaloid from the roots of *Tripterygium wilfordii* Hook. They found wilfordine to be an ester alkaloid consisting of a polyhydroxy nucleus esterified with 5 moles of acetic acid, 1 mole of benzoic acid, and 1 mole of a nitrogen-containing dicarboxylic acid; however, they reported that the formula for the sum of the component parts of wilfordine, $C_{43}H_{49}O_{18}N$, did not agree with the molecular formula, $C_{42}H_{47}O_{19}N$, calculated for the entire alkaloid. An investigation of the discrepancy was undertaken.

Although very little fresh root was available at the outset of this investigation, a large quantity of 9-year old root remained from previous work in this Bureau. Preparations of wilfordine, isolated from both fresh and old root, were compared with a sample kindly supplied by Fred Acree, Jr. The melting points checked within a few degrees and mixed melting points showed no depression. The ultraviolet spectra of the preparations were almost identical, and the crystalline structures likewise seemed identical. The three preparations exhibited the same order of toxicity to newly hatched larvae of the European corn borer.[4] Finally, carbon, hydrogen, nitrogen, molecular weight, saponification equivalent and volatile acids data were in agreement with the results of Acree and Haller.

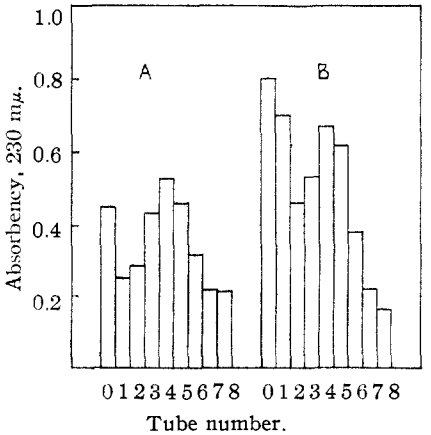

Fig. 1.—Countercurrent distribution of wilfordine (methanol-insoluble fraction), benzene-5% HCl: A, Acree's sample; B, from fresh root.

Wilfordine did not melt sharply but rather changed from a white solid to a clear resin at different temperatures between 167° and 174° for the various lots prepared. This behavior indicated that wilfordine might not be a single compound. Purity studies by chromatography were therefore undertaken, but the results were unsatisfactory. Countercurrent distribution[5,6] proved more successful.

After it had been determined that wilfordine from old root would have a partition coefficient close to 1 when distributed between benzene and 5% hydrochloric acid, a 21-plate countercurrent distribution was performed. The results of this distribution (Fig. 1 in reference 7) show at once that wilfordine is impure and indicated the presence of two principal alkaloids plus smaller amounts of other alkaloids. The two alkaloids were also demonstrated to be present in Acree's sample (Fig. 1,A) and in a sample of wilfordine isolated from fresh root (Fig. 1,B). The latter preparation was recrystallized only once, since additional recrystallizations might remove more of one component than of the others.

The two alkaloids were separated by partition chromatography of the Martin and Synge type.[8] Two main zones were eluted (Fig. 2 in reference 7). The first main compound (C-1) eluted from the column will be called wilforine, while the name, wilfordine, will be retained for the second main compound (C-2). Acree and Haller's wilfordine will henceforth be referred to as the methanol-insoluble fraction.

By countercurrent distribution it was shown that both compounds were not pure. Each zone was then rechromatographed and the alkaloids were isolated in pure form by selecting those zone fractions whose absorbency ratios at 270 and 255 mμ approached a constant value and rechromatographing these combined fractions until a constant ratio was obtained.[7]

(1) Report of a study made under the Research and Marketing Act of 1946. Article not copyrighted.

(2) Part of Ph.D. thesis submitted by M. Beroza to Georgetown University.

(3) Acree and Haller, THIS JOURNAL, **72**, 1608 (1950).

(4) Tests to determine the insecticidal activity of these samples were carried out by D. D. Questel and R. V. Connin of this Bureau.

(5) Craig, *J. Biol. Chem.*, **155**, 519 (1944).

(6) Craig, *Federation Proc.*, **7**, 469 (1948).

(7) Beroza, *Anal. Chem.*, **22**, 1507 (1950).

(8) Martin and Synge, *Biochem. J.*, **35**, 1358 (1941).

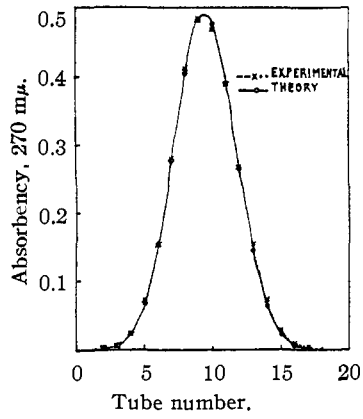

Fig. 2.—Countercurrent distribution of wilfordine, benzene–4% HCl.

The countercurrent distribution pattern of wilfordine (Fig. 2) shows excellent agreement with the theoretical.[9] Wilfordine showed some decomposition when distributed between benzene and 10% hydrochloric acid. When distributed between benzene–hexane and 2% hydrochloric acid (Fig. 3), the experimental results are in good agreement with the theoretical, except that the experimental curve deviates to the right for the lower concentrations and a deviation from a linear partition isotherm is indicated. By determination of the partition ratios at several concentrations, a shift in partition ratio for this system was verified.

The two compounds are therefore pure on the basis of homogeneity by partition chromatography with all fractions having a constant ultraviolet absorbency ratio, and, on the basis of their countercurrent distribution patterns.

The formulas of wilforine and wilfordine have been calculated from molecular weight, carbon, hydrogen and nitrogen determinations to be $C_{43}H_{49}O_{18}N$, and $C_{43}H_{49}O_{19}N$, respectively. Alkimide determinations were negative.

Both compounds were found to be ester alkaloids that upon saponification yielded 8 acid equivalents, of which 6 were steam volatile. The volatile acids were composed of 1 mole of benzoic acid and 5 moles of acetic acid per mole of compound.

Investigations on this problem are being continued.

Experimental[10]

Sources of Plant Material.[11]—Two lots of fresh root were collected in Wellborn, Texas, early in 1949 and 1950. The main source of material was 100 kg. of root that was collected in 1940 at Glenn Dale, Maryland, and had been stored in metal cans in the attic of the laboratory for nine years prior to processing. When tested it was found to be very toxic to the European corn borer,[4] an indication that the insecticidal principles are much more stable than the Chinese believe.[12]

(9) Williamson and Craig, *J. Biol. Chem.*, **168**, 687 (1947).

(10) Elementary and alkimide determinations by the Clark Microanalytical Laboratory, Urbana, Ill., and the Oakwold Laboratory, Alexandria, Va. All ultraviolet spectrophotometric measurements were made on a Beckman model DU quartz spectrophotometer. M.p. determinations were made with a Fisher–Johns apparatus. All m.p.'s are corrected.

(11) Whole roots of plant were furnished by the U. S. Bureau of Plant Industry, Soils and Agricultural Engineering.

(12) Swingle, Haller, Siegler and Swingle, *Science*, **93**, 60 (1941).

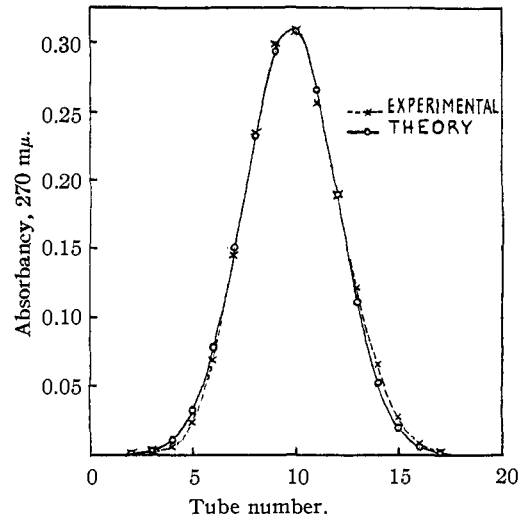

Fig. 3.—Countercurrent distribution of wilforine, 36.7% benzene in hexane–2% HCl.

Isolation of Crude-Alkaloid Fraction.—In small-scale extractions the crude-alkaloid fraction was isolated as described by Acree and Haller.[3] In large-scale extractions the following procedure was used to overcome the troublesome emulsions encountered with Acree and Haller's method. In addition the extraction solvent is not inflammable and large batches may be conveniently processed.

Five kg. of finely ground,[13] air-dried root was moistened with 2 liters of 10% ammonium hydroxide, thoroughly mixed, and allowed to stand in a closed vessel for several hours. The root was then exhaustively extracted with ethylene dichloride. The solvent was removed below 46° under reduced pressure. Alcohol was added several times to facilitate its removal. The residue was dissolved in a minimum volume of ether and thoroughly mixed with an equal volume of 5% hydrochloric acid. Upon removal of the ether and alcohol at 50° under reduced pressure (water-pump) a viscous, tarry residue remained with the hydrochloric acid. The acid was removed by decantation through a Buchner funnel. This process of extraction of the tarry residue with ether and hydrochloric acid was repeated (5 ×) until a silicotungstic acid test on the acid extract was very weak. The acid extract was cooled to about 5°, filtered, and treated with concentrated ammonia until alkaline to litmus, the temperature of the solution being maintained below 10°. An hour later the precipitated crude-alkaloid fraction was filtered off, washed with distilled water, and dried. Approximately 9.55 and 11.5 g. of material were isolated from old and fresh root, respectively.

Isolation of the Methanol-insoluble Fraction.—The dried crude-alkaloid fraction was dissolved in sufficient absolute methanol to form a heavy sirup and then seeded. The product, which crystallized in an ice-box overnight, was filtered off and then washed with a minimum of cold absolute methanol. The filtrate was evaporated to dryness and set aside for future study. For recrystallization, the product was dissolved in a minimum of acetone and diluted with absolute methanol. After several recrystallizations the melting point remained fairly constant for any one lot, although the melting points of different lots varied between 167° and 174°. A gram of crude-alkaloid fraction yielded 0.11–0.14 g. of methanol-insoluble fraction. The product melting at 174° was analyzed. The results are in good agreement with those obtained by Acree and Haller.

Anal. C, 58.37, 58.37; H, 5.48, 5.58; N, 1.69, 1.76; mol. wt.,[14] 840.

Saponification of Methanol-insoluble Fraction.—Because Acree and Haller believed they obtained incomplete saponification with alcoholic alkali, the alkaloid in 30- to 50-mg. lots was saponified according to a modified method of Rede-

(13) Kindly ground by Combustion Engineering-Superheater, Inc., Raymond Pulverizer Division, Chicago 22, Ill.

(14) Method of Clark, *Ind. Eng. Chem., Anal. Ed.*, **13**, 820 (1941).

mann and Lucas.[15,16] This method is known to saponify refractory esters and thus eliminates the possibility of obtaining the half-ester, which Acree and Haller believed they obtained. The saponification equivalent was determined to be 109–110 (4 anal.) which is equivalent to 7.7 equivalents of acid per mole.

The titrated solution of the saponified material was made acid to congo red with sulfuric acid, and steam distilled exhaustively with diminishing volume. The distillate contained an average of 5.75 equivalents (3 anal.) of steam-volatile acid per mole. By difference there were 1.95 equivalents of steam-non-volatile acid per mole.

The titrated volatile acids were analyzed according to the method of Ramsey and Patterson,[17,18] using a 5-g. silica gel column impregnated with a water solution of the R–NH_4 indicator. Two zones formed, plus a third, indistinct zone which at first was just below the acetic zone as the chromatogram was developed. The indistinct zone was never found in chromatographing the known pure acids and it was not propionic acid. When this zone was isolated, it never titrated more than a very small fraction of an equivalent. The ratio of the acetic acid to benzoic acid was determined to be 4.94, 4.83 (av. 4.88).

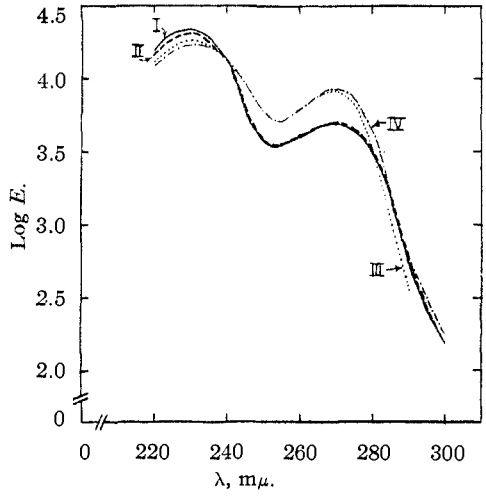

Fig. 4.—Ultraviolet absorption curves: I, wilforine in absolute ethanol; II, wilfordine in absolute ethanol; III, wilforine in 1% HCl; IV, wilfordine in 1% HCl.

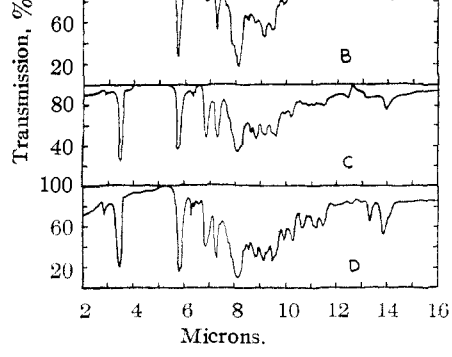

Fig. 5.—Infrared absorption curves of wilforine and wilfordine: A, wilforine in CCl_4, 25 mg. per ml.; B, wilfordine in CCl_4, 25 mg. per ml.; C, wilforine as Nujol mull; D, wilfordine as Nujol mull. Curves determined with Baird spectrophotometer.

Isolation of Wilforine and Wilfordine by Partition Chromatography.—The methanol-insoluble fraction was chromatographed in a water-jacketed column, maintained at 15° by means of a steady flow of tap water. The immobile solvent, dilute hydrochloric acid, was equilibrated with the mobile solvent, ether,[19] at the column temperature. The following conditions have resulted in the best separations.

In a mortar 50 g. of silicic acid[20] and 29 ml. of 0.6% hydrochloric acid was thoroughly mixed. The fine slurry prepared by stirring in ether into the mixture was added to the tube which was then tapped to settle the gel. Pressure was applied until the solvent sank into the gel. One-half gram of alkaloid dissolved in about 30 ml. of ether was added and washed into the gel with several small portions of ether. The column was filled with ether and the pressure adjusted to give an effluent rate of 300–400 ml. per hour. The column was never permitted to run dry and the solvent added was at the same temperature as the column. Fifty-ml. fractions of the effluent were collected, the last 3 ml. of each fraction directly in the cuvette. The absorbency of each fraction was measured at 270 and 255 mμ. The first main zone leaving the column (0.188 g.) was impure wilforine and the second (0.210 g.), impure wilfordine.[21] The wilforine zone was rechromatographed with 1.75% hydrochloric acid as the immobile solvent and the fractions possessing the most constant absorbency ratios weighed 0.106 g. After another chromatographing 0.089 g. of pure wilforine was obtained.

Only 0.150 g. of the wilfordine zone had a constant absorbency ratio. This material was chromatographed with 0.6% hydrochloric acid as the immobile solvent and 0.111 g. was separated. After another chromatographing 0.100 g. of pure wilfordine was obtained. The impure fractions of both zones were set aside and added to the next batches of alkaloids to be resolved. The compounds were dried over boiling acetone under high vacuum (oil-pump).

Wilforine: *Anal.* Calcd. for $C_{43}H_{49}O_{18}N$: C, 59.6; H, 5.69; N, 1.6; mol. wt., 867.8. Found: C, 59.77, 59.75; H, 5.53, 5.40; N, 1.6, 1.8; mol. wt.,[14] 862.

Wilfordine: *Anal.* Calcd. for $C_{43}H_{49}O_{19}N$: C, 58.43; H, 5.58; N, 1.6; mol. wt., 883.8. Found: C (av. 4 anal.), 58.66; H, 5.70; N (av. 3 anal.), 1.8; mol. wt.,[14] 891.

Characterization of Wilforine and Wilfordine.—Wilforine melts at 169–170°. Wilfordine melts at 175–176°. The compounds actually do not melt, but collapse from a white crystalline solid to a clear resin. Wilfordine crystallizes from acetone–methanol solutions as equilateral triangular plates, whereas wilforine crystallizes as triangular plates with one corner cut off. The compounds are optically active. In acetone $[\alpha]^{25}D$ +30 for wilforine and $[\alpha]^{25}D$ +12 for wilfordine.

The compounds have almost identical ultraviolet absorption spectra in absolute ethanol and in dilute hydrochloric acid (Fig. 4). Their infrared absorption spectra[22] (Fig. 5) in carbon tetrachloride are likewise strikingly similar. Because the reliability of the spectra at those wave lengths where carbon tetrachloride absorbs strongly (12.1–13.6 microns) is questionable, the infrared absorption spectra as Nujol mulls were determined. The absorption peaks at 2.83 microns indicate the presence of hydroxyl groups in both compounds. Preliminary data on active hydrogen confirm the presence of these groups, wilfordine probably having one more hydroxyl group than wilforine. Both compounds are soluble in chloroform, acetone, carbon tetrachloride, benzene and hydrochloric acid; less soluble in ether, carbon disulfide, methyl alcohol and ethyl alcohol; and practically insoluble in petroleum ether and water.

Countercurrent Distribution.—The distributions were carried out in the following manner. Ten ml. of each phase was used. From 1 to 2 mg. of alkaloid was employed for an 8-plate distribution and from 3 to 5 mg. for a 20-plate dis-

(15) Redemann and Lucas, *Ind. Eng. Chem., Anal. Ed.*, **9**, 521 (1937).

(16) Schneider, "Qualitative Organic Microanalysis," John Wiley and Sons, Inc., New York, N. Y., 1946, p. 161.

(17) Ramsey and Patterson, *J. Assoc. Offic. Agr. Chemists*, **28**, 644 (1945).

(18) While the method is meant for analysis of C_1–C_4 acids, the authors state that benzoic acid is eluted from the column with butyric acid so that the method can be used to separate acetic and benzoic acids.

(19) U. S. P. XIII ether treated for removal of peroxides and distilled.

(20) Mallinckrodt 2847 analytical reagent grade.

(21) C-1 and C-2, respectively, in Fig. 2 of reference 7.

(22) Kindly run by S. P. Sadtler and Son, Inc., Philadelphia 3, Pa.

tribution. The distributions were performed manually in bottles with a special transfer syringe[23] in the same manner as described by Craig, *et al.*,[24] for use with separatory funnels.

The amount of alkaloid in each bottle was determined by cooling the contents to about 5° and then making it slightly alkaline with concentrated ammonia. After thorough shaking the alkaloid was shifted completely into the benzene layer. The benzene solution was washed once with water and then allowed to come to room temperature. Two-ml. aliquots of the benzene layer were evaporated to dryness on a 60° water-bath with a current of dry air and the benzene removed completely under a high vacuum (oil-pump) at room temperature. The residue was taken up in absolute ethanol or 1% hydrochloric acid for absorbency measurements. Corrections were made for blank determinations and for volumes of the solutions. The absorbencies of the alkaloids obey Beer's law at the wave lengths used.

The methanol-insoluble fraction was distributed between benzene and 5% hydrochloric acid. Benzene and 4% hydrochloric acid were used to distribute wilfordine (Fig. 2). When wilforine was distributed between benzene and 10% hydrochloric acid,[25] it seemed to contain an impurity that could not be removed by rechromatographing. It was subsequently demonstrated that the compound was being decomposed by prolonged exposure to the 10% hydrochloric acid during the manual 20-plate distribution (duration 6 hours). An 8-plate distribution (duration 1 hour) of wilforine (Fig. 6,A) was compared with a similar distribution of the same material that had been shaken with benzene and 10% hydrochloric acid for 4 hours prior to distribution (Fig. 6,B). The appearance of appreciably larger amounts of alkaloid in tubes 7 and 8 of Fig. 6,B clearly shows that the 10% hydrochloric acid does slowly decompose wilforine. To overcome this destruction a countercurrent distribution of wilforine was carried out, using a 36.7% benzene solution in hexane (by volume) and 2% hydrochloric acid as the solvents (Fig. 3).

TABLE I

SAPONIFICATION AND VOLATILE ACIDS DATA ON WILFORINE AND WILFORDINE

	Wilforine		Wilfordine	
Saponification equivalent	109.3	108.8	110.8	110.3
Acid equivalents	7.94	7.98	7.99	8.03
Steam-volatile acid equivalents	6.01	6.00	5.98	6.02
Benzoic acid equivalents	1.00	1.015	0.98	1.00
Acetic acid equivalents	5.01	4.98	5.00	5.02
Steam-non-volatile acid equivalents (by diff.)	1.93	1.98	2.01	2.01

(23) Beroza, *Anal. Chem.* (in press).

(24) Craig, Golumbic, Mighton and Titus, *J. Biol. Chem.*, **161**, 321 (1945).

(25) Figure 7 of ref. 7.

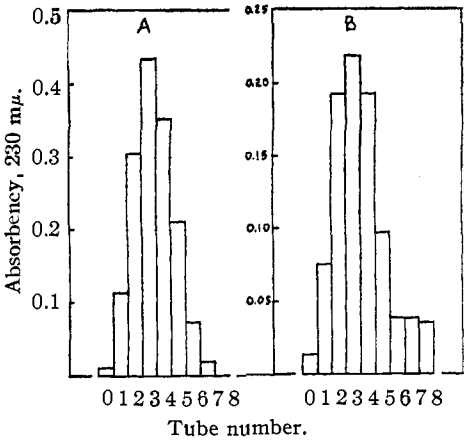

Fig. 6.—Countercurrent distribution of wilforine, benzene–10% HCl: A, without treatment; B, after being shaken with 10% HCl and benzene for 4 hours.

Saponification of Wilforine and Wilfordine.—The compounds were saponified and the volatile acids were determined in the same manner as described for the methanol-insoluble fraction, except that in using Ramsey and Patterson's method[17] acetic acid was determined by difference. While the recovery of benzoic acid from known mixtures was quantitative, that of acetic acid was always low. Elsden's[26] recommendations for the determination of acetic acid by difference was therefore adopted. The data on the saponification and analysis of the volatile acids in the compounds are given in Table I.

Identification of Volatile Acids.—The benzoic acid zone from each compound was rechromatographed on silicic acid as above, except that no indicator was used on the column. The acid was detected by following the absorbency of the eluate at 270 mμ. The acid was 95% neutralized (no indicator added) with sodium hydroxide, the chloroform was removed, and the solution evaporated to dryness in a test-tube. About 0.5 ml. of water was added plus a drop of dilute sulfuric acid. A precipitate formed, which was filtered off and washed with cold water. The material isolated from each alkaloid melted at 119–121° and showed no depression in admixture with benzoic acid. The behavior on silica gel (threshold volume) checked that of benzoic acid, and the ultraviolet spectrum in absolute ethanol was identical with that of benzoic acid.

Acetic acid was identified by Duclaux numbers and by its behavior on silica gel. Duclaux numbers: known acetic acid 6.7, 7.3, 7.45; acetic acid from wilforine 6.8, 7.3, 7.55; acetic acid from wilfordine 6.9, 7.3, 7.4.

BELTSVILLE, MD. RECEIVED JANUARY 4, 1951

(26) Elsden, *Biochem. J.*, **40**, 252 (1946).