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Papaver bracteatum Lindl.: Quantitative Extraction and Determination of Thebaine

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Abstract □ An interlaboratory procedure for the quantitative extraction and analysis of thebaine from different tissues of *Papaver bracteatum* Lindl. is presented. Each step was evaluated for the yield of thebaine by use of 1-^{3}H-thebaine and GLC. The method of drying and milling of tissue and the size of resultant particles were important factors in the quantitative recovery of thebaine.

Keyphrases □ *Papaver bracteatum*—various plant tissues, extraction and analysis of thebaine □ Thebaine—extraction and analysis from various plant tissues of *Papaver bracteatum* □ Alkaloids—thebaine, extraction and analysis from various plant tissues of *Papaver bracteatum* □ Narcotics—thebaine, extraction and analysis from various plant tissues of *Papaver bracteatum*

Papaver bracteatum Lindl. is being evaluated as a source of the alkaloid thebaine from which codeine may be derived (1, 2). Methods for the analysis of thebaine include quantitative TLC followed by spectrophotometry (3), colorimetry (4), GLC (5, 6), and high-speed liquid chromatography (7, 8).

A United Nations Working Group on *P. bracteatum* (9) recommended a general method for thebaine analysis. In this study, each step of the method is presented for the analysis of thebaine in roots, leaves, and capsules of *P. bracteatum*. The purposes of this investigation were to maximize the yield of thebaine and to provide a basis for comparison of interlaboratory results of analysis of thebaine in such tissues.

EXPERIMENTAL

Plant Culture—The seed source and plant culture were previously described[1] (6).

Preparation of Tritiated Thebaine—1-^{3}H-Thebaine was prepared by the acid-catalyzed tritiation of salutaridine, followed by borohydride reduction to the epimeric salutaridinols and subsequent conversion to thebaine. The entire sequence was first performed on the deuterated analogs, which permitted unambiguous assignment of the label to the 1-position by NMR and mass spectrometry. The specific activity was 6.17 μCi/mg.

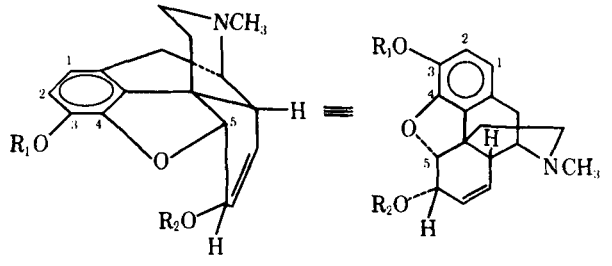
Sample Preparation—The effects on thebaine yield of oven drying (60°) *versus* freeze drying of plant tissue were compared. The effect of ball[2] or blade[3] milling was evaluated, as was the influence of particle size on thebaine recovery. The residual water content of samples before analyses was determined. Thebaine extractions with 5% aqueous acid and methanol–ammonium hydroxide (98:2) were compared. The efficiencies of other extracting solvents (5) are also discussed.

Assay—Thebaine was determined as previously described (6).

RESULTS AND DISCUSSION

(−)-Thebaine (I) (except for the position of the double bonds), (−)-codeine (II), and (−)-morphine (III) have the same skeletal structure and absolute configuration.

Apparently, thebaine was not stable at 60°. Thebaine, present at time of harvest, may have been transformed during heat drying into other compounds, as evidenced by the appearance of unidentified "alkaloidal" substances on thin-layer chromatograms following drying and by the appearance of several radioactive substances following addition of 1-^{3}H-thebaine to plant tissues before drying. The mechanism and nature of the additional substances derived from thebaine during oven drying have not been studied adequately; however, enzymatic and/or physical factors undoubtedly are involved in the transformation processes. No additional products from thebaine were observed when tissues were freeze dried, as measured by TLC of tissue extracts to which 1-^{3}H-thebaine had been added.



I: $R_1 = R_2 = CH_3$ (double bonds between C6—7 and C8—14)
II: $R_1 = CH_3$, $R_2 = H$
III: $R_1 = R_2 = H$

[1] The plant material was identified as *Papaver bracteatum* Lindl. by Dr. P. G. Vincent and M. L. Stiff, U.S. Department of Agriculture, Beltsville, Md. A voucher (preserved and living) specimen (see Ref. 6 for numbers) representing material collected for this investigation is available for inspection at the U.S. Department of Agriculture, Beltsville, Md.

[2] Dangoumau quantitative microgrinder; Prolabo, Microbroyeur Quantitatif Dangoumau, Appareil No. 07-449.02, France.

[3] Wiley.

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Table I—Distribution of Particle Sizes from Root Tissues of *P. bracteatum* Prepared by Ball Milling or by Blade Milling

Particle Size[a]	Ball Mill		Blade Mill	
	g	%	g	%
>40 (420 μm)	0.333	2.6	0.137	0.8
40–70 (420–210 μm)	0.533	4.2	2.647	16.1
70–140 (210–105 μm)	6.503	51.2	10.162	61.9
140–260 (105–56 μm)	2.713	21.4	1.772	10.8
<260 (56 μm)	2.618	20.6	1.707	10.4

[a] Particle size was determined by sieving after 2 min of ball milling and after passing 40- and 60-mesh sieves of blade milling.

Table II—Effect of Type of Milling on Recovery of Thebaine from Root and Capsule Tissues from *P. bracteatum*

Mill Type	Mean Thebaine[a], μg		
	Capsule	Root	Capsule[b]
Blade	2927	843	—
Ball	2730	1266	—
Total elution[c]	3497	1579	3385

[a] Triplicate GLC analyses from three replicate 250-mg samples of milled tissue. Samples were adjusted for residual water content. [b] Capsule tissue spiked with 1-^{3}H-thebaine. [c] Total elution refers to 250-mg tissue samples placed on a glass chromatographic column with glass wool at the bottom and 1 liter of 5% aqueous acetic acid eluted over the sample during 12 hr. Tissue was ball milled.

Drying of Plant Tissue—Oven drying of plant tissue at 60° and freeze drying at −55° were compared. In all tissue types extracted (*i.e.*, root, aboveground parts, stem, and capsule), 13–17% less thebaine was extracted from the oven-dried tissues than from freeze-dried tissues. Root tissue from identical sources prepared in a manner differing only in the method of drying gave thebaine values of 5.91 μg/mg of tissue *versus* 7.14 μg/mg of tissue for oven-dried and freeze-dried tissue, respectively. Fairbairn and Helliwell (10) reported that drying at 105° rather than 60° led to no loss of thebaine, but they did not evaluate freeze drying.

Extraction Solvent—The efficacy of acetic acid (5% aqueous) and methanol–ammonium hydroxide (98:2) as extraction solvents was evaluated since these two systems are widely used. Other solvent systems and various extraction conditions were studied previously (5). Numerous analyses of all types of plant tissues consistently showed that the aqueous acetic acid (5%) extraction was 22–25% more efficient in extracting thebaine than methanol–ammonium hydroxide (98:2). The acetic acid extraction may have been more efficient because thebaine probably exists as a quaternary salt in the plant and protonation of the molecule under dilute acid conditions in an aqueous system favors "release" of the alkaloid.

One problem that may exist with tissue extraction under mild acid conditions is that acid-catalyzed reactions may result from protonation of the nitrogen atom and produce reactive salts. Acid-catalyzed reaction products were detected and will be the subject of a future study.

Purification Protocol—The procedure for extraction and purification of plant tissues is as follows.

Harvest the plant, freeze dry the plant parts (24-hr minimum), and grind the material (2 min by ball milling). Weigh a 100–500-mg sample and place it in a flask with 30 ml of extraction solvent [5% aqueous acetic acid (v/v)]. Weigh a sample for dry weight determination and determine the dry weight after 12 hr at 100°.

Agitate the sample in the extraction solvent for 1 hr. With a Büchner funnel, filter[4] the sample. Wash the flask three times with 10-ml portions of extracting solvent and also wash the sides of the funnel. Rinse out a 500-ml evaporating flask and a funnel with 10 ml of extracting solvent. Pour the filtered sample through the funnel into the evaporating flask and wash the flask with 10 ml of extracting solvent.

Combine the extracts. Wash the extracts three times with 25 ml of ether when pigments, *i.e.*, from stems and leaf tissue, are to be removed. Adjust the aqueous phase to pH 9.5 with concentrated ammonium hydroxide and add it to a separator. Wash the beaker with 10 ml of chloroform. Then add 20 ml of redistilled chloroform, shake, and release gas three times.

Tap off the chloroform phase containing the alkaloids (bottom phase) and keep it. Add 30 ml of chloroform to the water phase (top phase) and shake. Repeat the procedure two more times. Backwash the chloroform phase with 30 ml of 0.1 *N* NH_4OH and discard the water phase (top phase).

Collect the chloroform phase in an evaporating flask and evaporate it to dryness *in vacuo* (50°) under nitrogen. (Residual water, if present, may be removed under a stream of dry nitrogen gas.) Add 1 ml of ethanol containing an internal standard[5]. Then inject a 1–2-μl aliquot into the chromatograph and compare the peaks to the standards.

The GLC columns were glass, packed with 2% OV-17 on 100–120-mesh Gas Chrom Q.

Sample Preparation—Particle size and weight correction were important in the determination of thebaine from tissues of *P. bracteatum* prepared by ball milling or blade milling (Table I). Data showed a greater percentage of fines; *i.e.*, more 105–56-μm particles were produced by ball milling than by blade milling. A finer particle size offered a greater surface area for solvent extraction. A similar percent distribution of particle sizes was found when leaf, stem, or capsule tissues were ball or blade milled. Blade milling of capsule tissue was difficult because tissues gummed behind the blades; gumming resulted from the high lipid content even though seeds were removed before processing.

Table II shows the effect of milling type on the recovery of thebaine from root and capsule tissue of *P. bracteatum*. The thebaine content of capsule tissue did not differ between the two milling types, but large differences were evident for root tissue, *i.e.*, 33% less in blade-milled than in ball-milled tissue. To determine the maximum thebaine recoverable, 1 g of capsule tissue was placed in a 5-cm diameter glass column and eluted with 5% aqueous acetic acid. One liter of the acid was allowed to pass over the tissue for 12 hr, and the eluant was collected in a flask containing ammonium hydroxide. In some experiments, 1-^{3}H-thebaine was added to the capsule tissue before elution.

For total elution of unspiked and spiked thebaine tissue, an average of 3442 ± 80 μg of thebaine was recovered. Total elution (Table II) with 5% aqueous acetic acid of capsule tissue yielded ~18% more thebaine than tissue incubated in the same solvent 1 hr before shake extraction, regardless of the type of milling. When 1-^{3}H-thebaine was added to any of the particle classes of tissues and extracted with aqueous acid for 1 hr, about 99% of the radioactivity was in the extract and supernate. About 0.06% of the radioactivity remained in the marc. For root tissue, 17 and 45% less thebaine were recovered after ball and blade milling, respectively, than after total elution. In root and capsule tissue, 1% of the isotope was recovered from the marc. No isotope exchange or degradation of the 1-^{3}H-thebaine was observed.

Particle size and, hence, effective surface area affected thebaine yield. On a total dry weight basis, the fines yielded the greatest quantity of thebaine, although there were few differences between the particle-size classes on a concentration basis. In the formation of particle classes, some cell-type segregation may occur; *i.e.*, lacticifers are much finer cell types than sclerenchyma. Physical processes are probably also involved such as surface area phenomena, speed of diffusion from the particle, irreversible absorption or adsorption to the particle, "bound" thebaine (10), and other complex interactions.

These data suggested that there were inherent difficulties in comparison of thebaine content in tissues milled by different techniques. Thus, milling devices should be standardized for interlaboratory comparisons. Thebaine yield apparently was higher and more consistent when ball milling was used. Therefore, the effect of time of ball milling of *P. bracteatum* capsule tissue on the distribution of particle size and thebaine content was studied (Table III).

In each of the four time periods, thebaine yields were consistently the highest in the >260-mesh fraction and lowest in the <40-mesh fraction. A milling time of 2 min gave the highest average yield of thebaine. Thebaine yields decreased as milling time was increased beyond 2 min. This decrease may have resulted, in part, from heat generated from friction by the high velocity movement of the balls. The heat produced may have acted as a catalyst in the presence of natural plant acids for the decomposition of thebaine.

Sample Size—A wide weight range (5 mg–1 g) of sample sizes of the plant tissues was analyzed for thebaine. Reproducibility was greatest in samples with weights of 250 mg because of tissue availability (<2% deviation between replicates), although 100-mg samples were adequate providing they were taken from a homogeneous source. Particle segregation during storage gave samples for extraction and GLC analysis that were highly variable. Therefore, stored milled tissues were mixed thoroughly before subsampling for thebaine analysis.

GLC Determination of Thebaine—Thebaine was determined quantitatively by GLC, and the results were corroborated by TLC and spectrophotometry at 285 nm (6). Working limits for linear detector re-

[4] Whatman No. 3 paper.

[5] Internal standards were cholesterol acetate and tetrahydrothebaine (1 mg/ml).

Table III—Effect of Time of Ball Milling of *P. bracteatum* Capsule Tissues on Distribution of Particle Size and Thebaine Content [a]

Milling Time, min	Mesh Size	Sieve Weight g	Sieve Weight %	Thebaine Weight, %	$\bar{x}$	Δ%[b]
1.0	>40	4.77	27	1.12		
	40–70	4.62	26	1.20		
	70–140	6.41	36	1.31	1.264	−1.7
	140–260	1.19	6	1.60		
	<260	0.95	5	1.64		
2.0	>40	0.92	4	0.99		
	40–70	4.14	17	1.06		
	70–140	11.63	47	1.21	1.286	—
	140–260	4.59	19	1.51		
	<260	3.26	13	1.62		
3.0	>40	0.22	2	0.95		
	40–70	1.18	8	0.96		
	70–140	7.30	48	1.07	1.229	−4.4
	140–260	1.90	13	1.35		
	<260	4.30	29	1.53		
4.5	>40	0.09	1	0.83		
	40–70	0.33	2	0.89		
	70–140	7.12	47	1.06	1.227	−4.6
	140–260	3.10	20	1.31		
	<260	4.57	30	1.47		

[a] Capsules were deseeded, crushed, freeze dried for 24 hr, and ball milled. [b] Percent difference from 2 min of milling time thebaine yields.

sponse were 0.1–1.0 μg of thebaine/sample. Detector and/or integrator saturation occurred when the thebaine concentration per GLC injection exceeded ~2 μg. Reproducibility between triplicate analyses at each concentration of alkaloid was ±1%. Integrator responses to samples yielding $<10^5$ μv/sec were reanalyzed to give counts $>10^5$ either by adjustment of the injection volume or by increased electrometer sensitivity.

Linear detector responses for isothebaine, papaverine, alpinigenine, laudanosine, salutaridine, reticuline, and several additional alkaloids from *Papaver* except morphine were similar. The detector response was not linear with morphine because it severely tailed under GLC conditions, possibly because of adsorption onto the column. Morphine was calibrated as its *N,O*-bis(trimethylsilyl)acetamide derivative for samples suspected of containing this alkaloid but was not usually included in standard calibration mixtures.

GLC flame-ionization responses to varying concentrations of thebaine *versus* a constant level of cholesterol acetate were linear throughout the range analyzed. Linear ratio detector responses also were obtained when tetrahydrothebaine was used as an internal standard. The disadvantage of tetrahydrothebaine in routine analyses, alone or in combination with cholesterol acetate, was its relatively short retention time, especially at high column temperatures. Tetrahydrothebaine usually eluted from the GLC column as a rider to the solvent peak, which may have complicated manual peak area determinations. Laudanosine, antipyrine, and tetrahydropalmatine were not suitable for use as internal standards in GLC analyses, although they were recommended by the Third Working Group on *P. bracteatum* (9).

A small quantity of thebaine (<1%) always adsorbed to new OV-17–Gas Chrom Q columns. Saturating the columns with an alkaloid standard containing thebaine (~0.2–0.4 μg) by injection of standard mixtures obviated this problem. Calibration solutions consisted of 1 μg of cholesterol acetate plus 0.2–1 μg each of thebaine, isothebaine, and codeine in 1 ml of absolute ethanol.

By carefully standardizing and checking each step of the procedure for loss of thebaine, it was determined that this procedure can be used for comparison of intra- and interlaboratory analyses.

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^{13}C-NMR Spectra of Strychnos Alkaloids: Brucine and Strychnine

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Abstract □ The natural abundance ^{13}C-NMR spectra of brucine and strychnine were obtained using the pulse Fourier transform technique. The chemical shifts of various carbon resonances were assigned on the basis of substituent effects on benzene shifts, intensities of signals, multiplicities generated in single-frequency off-resonance-decoupled spectra, and comparisons with the chemical shifts of structurally related compounds.

Keyphrases □ Brucine—^{13}C-NMR spectrometry, chemical shifts assigned □ Strychnine—^{13}C-NMR spectrometry, chemical shifts assigned □ ^{13}C-NMR spectrometry—brucine and strychnine, chemical shifts assigned

Several literature reports show the correlation of the structure of the various classes of alkaloids with their ^{13}C-NMR spectra (1–6). The structure and stereochemistry of strychnos alkaloids, brucine (I) and strychnine (II), were characterized (7, 8), but no reports are available concerning the assignments of their carbon resonances.