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A RAPID QUANTITATIVE METHOD FOR THE DETERMINATION OF THEBAINE IN *PAPAVER BRACTEATUM*

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Key Word Index—*Papaver bracteatum*; Papaveraceae; TLC; thebaine analysis; alkaloid.

Abstract—A rapid method enabling a quantitative analysis of thebaine in capsules and latex of *Papaver bracteatum* has been devised, based on a TLC technique. This method was compared to the commonly used GLC procedure, and highly significant correlation coefficient ($r = 0.90$) and linear regression were found between the two methods. Values for concentrations to the nearest $\pm 0.25\%$ of the standard spots can be reached by this simple and rapid thebaine determination.

INTRODUCTION

Intensive studies at the chemical, physiological, genetical and agrotechnical level have been conducted in laboratories in different countries in order to develop the species *Papaver bracteatum* Lindl. as a commercial source for thebaine. This alkaloid can be converted into codeine, an analgesic widely used in medicine [1], as well as for the preparation of antagonist drugs for the treating of addicts [2].

A large chemical variation in thebaine content of the capsules has been reported [3, 4] and observed in our studies [5]. In order to exploit this variation for genetical and breeding purposes, the need for a rapid and reliable method of thebaine determination emerged quite early in our studies. The existing methods of thebaine analysis were found to be laborious, slow and therefore of limited value for a large scale screening of populations of this species. A rapid and reliable method described in this study has been developed for this purpose.

RESULTS

Description of the method

The method is based on a standardization of TLC involving the comparison of spot sizes and intensities of the tested samples appearing on fluorescent chromatoplates with calibrated standard spots of known concentrations. The standard concentrations were prepared in C_6H_6 solutions as *equivalents* of thebaine in the actual tested dried material, e.g. a solution used as a standard for 1% thebaine in the sample contained 1 mg of alkaloid in 1 ml C_6H_6 and so forth. Accordingly, the samples were prepared by using 100 mg dry powdered samples in 1 ml of C_6H_6. Aliquots of 5 μl drawn both from the various standards and the samples were carefully applied on the chromatoplates to produce the smallest possible spots. Following development, the sample spots' sizes and intensities were visually evaluated in UV light, and compared to the standard spots.

Since the standard analytical procedure is tedious and time consuming, a simplified way of extraction was also worked out and adapted to the method. This method simplified the extraction procedure and reduced markedly the operation time (see Experimental).

Evaluation of the method

The accuracy of the method was first determined on a number of known samples and found satisfactory. Subsequently, in order to compare more precisely this method to the standard and commonly used procedure of GLC, a large number of samples were analysed by both methods.

Samples of capsules from 31 different plants were selected to be determined by both ways: the TLC and the GLC methods, the latter using the more thorough extraction procedure used for such measurements. The results are presented in Table 1. In the TLC procedure, the reference spots were at concentrations equivalent to 1, 2 and 3‰, enabling an estimation of concentrations of

Table 1. Comparison of the data obtained for the concentration of thebaine in capsules (in %), using the rapid TLC method and the GLC accurate measurement

No.	Sample No.	% Thebaine TLC*	% Thebaine GLC	No.	Sample No.	% Thebaine TLC*	% Thebaine GLC
1	257	2−	1.75	18	221	2+	1.80
2	522	3−	2.61	19	225	3−	2.31
3	523	2	2.42	20	266	2	1.47
4	524	3−	2.25	21	507	3.5	3.96
5	526	2	2.34	22	508	3	2.88
6	527	2−	1.64	23	511	3.5	3.87
7	528	1.5	1.76	24	512	2+	2.10
8	529	2	2.06	25	78	1+	1.51
9	530	1.5	1.66	26	97	1.5	1.48
10	531	3−	2.49	27	100	1.5	1.47
11	532	2	2.04	28	106	2−	1.61
12	533	1+	1.39	29	104	1	0.94
13	534	1.5	1.47	30	103	2−	1.64
14	535	2−	1.47	31	101	2−	1.75
15	536	2−	1.60				
16	218	2.5	2.02				
17	220	2	2.21				

* The (+) and (−) following the number refers to concentrations above or below that number by 0.25%.

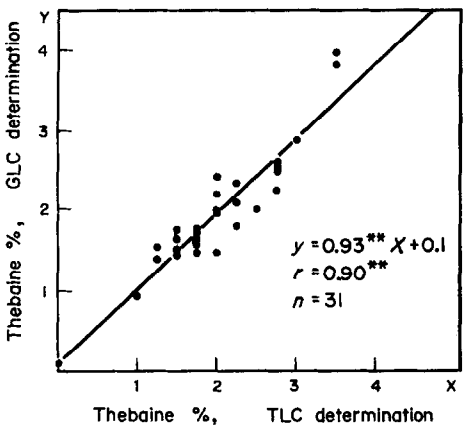

Fig. 1. Correlation coefficient and linear regression between TLC and GLC methods. ** Significant at 1% level.

thebaine of 0.5% between two values. However, in the samples in which the concentration was estimated to be between a reference concentration and the nearly 0.5% value, the sample was given the value followed by a (+) or a (−) indicating therefore that such concentrations were within 0.25% above or below the reference. Thereby, a value of 3− was considered for statistical analysis as 2.75 and 3+ as 3.25.

The accuracy of the method was determined by calculating the correlation coefficient and the linear regression between the concentrations of thebaine determined by the TLC and GLC methods. A highly significant correlation ($r = 0.90$) and linear regression ($y = 0.93x + 0.1$) were found between the two methods, thus indicating the high accuracy of the TLC when compared to the GLC method (see Fig. 1).

Sensitivity of the method

Although the reference spots are whole numbers, the results obtained show that the resolving power of this rapid method enables the determination of the thebaine content in samples with values to the nearest ±0.25% of the standard spot. In additional experiments, reference concentrations covering also half values (0.5; 1.0; 1.5; 2.0; 2.5 and 3.0) were run and afforded more accurate measurements. However, the use of so many reference spots obviously limits the space left for the samples to be analysed.

DISCUSSION

A number of methods for the determination of thebaine using various systems of chromatography [6–8] and colorimetry [9] have been described; most strive to high accuracy and are tedious, requiring appropriate instrumentation, some are proposed as semi-quantitative but actually might be considered as qualitative rather than quantitative, and no correlation to recognised accurate methods is provided [10, 11].

One of the limiting factors in the genetic improvement and breeding of medicinal plants is the lack of rapid and reliable analytical methods for the determination of the desired component. In plants for which appropriate rapid methods have been developed, as the radioimmunoassay technique [12], the advancement of the breeding was relatively fast. In the case of *Papaver bracteatum* an urgent need for such a method was felt; the more so, as the time between the harvest of the capsules (July), and the time of the sowing in the fall (August–September) is very short. For breeding purposes it is crucial for the selection of the high-thebaine-containing plants to have in hand the chemical data within the above short span of time for progeny tests during the following season. Moreover, with the standard methods used, the number of samples, and therefore the plant population size which can be handled chemically, is limited; accordingly, the expected progress in selection is very slow.

The method presented here was found to be rapid, easy to handle and enabling series of tests to be performed on the row. Its accuracy following appropriate training, is almost as good as that of the standard GLC method. Six analyses of one sample by the GLC method gave a mean of 24.7 mg thebaine per g dry capsule, and a standard deviation of 3.2 mg/g. These figures were 22.9 and 4.6 mg/g for the TLC method.

This method was developed and used mainly for capsule analysis, however, it was also extended to analyses of latex samples from unripe capsules. In this case the reference standard solutions had to be made for equivalents of 10 to 25% thebaine in benzene, the actual concentration in latex. One of the factors of the efficiency of this method has to be ascribed to the fact that in *P. bracteatum* the predominant alkaloid is thebaine (up to 98%) [13].

EXPERIMENTAL

For the chromatoplates, fluorescent TLC aluminium sheets, Si gel 60 F254 (precoated, thickness 0.25 mm Merck Darmstadt), were used. For quantitative application of the solns, an Eppendorf microliter pipette (B 315-E) with fixed stroke of 5 μl and disposable tips was used. The solns were carefully applied on the chromatoplates to produce the smallest possible spots. The developing solvent was toluene–Me_2CO–EtOH–6 N NH_4OH (80:80:15:4). For the GLC measurements, a Packard 839 instrument with flow ionization detection and programming of the temp. with glass columns was used. Cholesteryl acetate was the internal standard. The method has been previously described in detail [5, 6].

Preparation of standard solutions for reference spots. A set of 5 concns of thebaine in C_6H_6 soln was prepared. These concns were made as equivalents of 1, 1.5, 2, 2.5 and 3% thebaine in the sample, i.e. a soln used as *a standard for 1% thebaine in the plant* was prepared by dissolving 10 mg of thebaine in 10 ml C_6H_6. The other concns were scaled up accordingly. Following the application of the spots and their development, five clear spots of different sizes and intensities could be observed in UV light.

Determination of thebaine in ripe capsules. In small capped sample tubes, 100 mg of powdered capsules were soaked with a few drops of an NH_3 soln (2.5%) for 0.5 hr, and then exactly 1 ml C_6H_6 was added, the tubes were closed with their caps and shaken for several min. After standing for *ca* 5 hr, 5 μl were drawn from the supernatant solns using the microliter pipette. These 5 μl were carefully applied on the base line of the plate between a predetermined number of standard spots, and developed as above. The standards were selected according to the requirements of the experiment and spread among the samples, preferably at the center of the plate. Determination was done by comparing sample spots to the standards and recording the results as described.

Determination of thebaine in the latex. The method was adapted for the determination of latex drawn in the field on capsules when still green. In this case the standard solns involved

concn equivalents from 10 to 25% thebaine in C_6H_6. For 10 μl latex samples, for example, 1–2.5 mg thebaine quantities were dissolved in 1 ml C_6H_6 each according to the required scale. Latex sampling was done by making the cuts on green capsules, and 10 μl drawn using the same piston pipette; the sampling was done as carefully as possible under field conditions. The latex was introduced into capped tubes having some C_6H_6, and the tips flushed with the same solvent. Following a number of attempts with different solvents, C_6H_6 was found to be the best for this latex testing, efficiently flushing the tips. Back in the laboratory, the solvent was evapd from the sample tubes *in vacuo* at 60°, using a rotatory evaporator with a specially adapted tube. To the sample tubes, following drying, exactly 1 ml C_6H_6 and a few drops of 2.5% NH_3 were added. A thin needle rod is used to mix the latex into the solvent. The tubes are then capped, well shaken and left at room temp. for 30 min. Then 5 μl of the solns were applied on the base line of the chromatoplates as described above, with standard solutions of required concns dispersed between the sample spots at the base line. Comparisons of the readings were done as above.

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