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THE ALKALOIDS OF HEMLOCK (*CONIUM MACULATUM* L.).—II

EVIDENCE FOR A RAPID TURNOVER OF THE MAJOR ALKALOIDS

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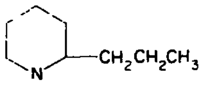
Abstract—Previous work, based on analyses of samples of developing fruits collected at weekly intervals, has been confirmed. Samples were also collected at 4-hourly and 2-hourly intervals and analyses showed that remarkably rapid changes in the alkaloidal picture took place at short intervals during 24 hr. Furthermore, as the coniine (I) content increased, the γ-coniceine (II) content decreased and vice versa. This interrelationship was particularly marked during the critical stages of pericarp development, but was not obvious during the development of the vegetative parts, where γ-coniceine and conhydrine (III) were the only known alkaloids detected. Since coniine and γ-coniceine differ only by two hydrogen atoms, it is suggested that these two alkaloids are involved in oxidation–reduction processes in the developing pericarp. The occurrence of the minor alkaloids conhydrine and N-methyl coniine (IV) and the presence of some unknown alkaloids are also reported and commented on.

INTRODUCTION

In a previous communication[1] it was shown that the total alkaloidal content and the proportions of individual alkaloids in hemlock fruit varied with the development of the fruit and with the season. It was suggested that γ-coniceine (II) was the precursor of the saturated alkaloids, such as coniine (I), and that this change, which appeared to be reversible, is associated with active growth. These conclusions were based mainly on the examination of samples collected at weekly intervals; we have repeated the work over a further two seasons and have also examined samples collected at daily, 4-hourly and 2-hourly intervals. Much of the earlier work has been confirmed but interesting new facts, based on the samples taken at short intervals, have come to light. Sampling and analytical methods are referred to at the end of this paper.



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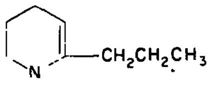

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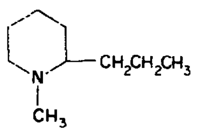

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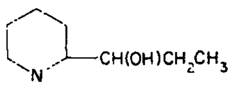

III

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[1] J. W. Fairbairn and S. B. Challen, *Biochem. J.*, **72**, 556 (1959).

RESULTS

Weekly samples. Samples were collected at noon on the same day each week and Fig. 1 shows the analytical results for the two major alkaloids in 1958 (a wet season) and 1959 (a dry season). In both seasons a peak of γ-coniceine content preceded a peak of coniine content, and during the first few weeks the increase in coniine content kept pace with the increase in dry weight. The year 1959 was particularly dry; the fruits matured prematurely and dehisced at week 5, in contrast to the three previous seasons when they remained on the plants until week 8.

Daily samples. Samples were collected at noon on each of the 14 days between week 2 and week 4 in 1958. Comparative paper chromatograms of the samples showed no marked changes in the alkaloidal picture, but rather a gradual increase consistent with that shown by the weekly samples in Fig. 1. Quantitative analyses of these samples were not therefore carried out at this stage.

Four-hourly samples. In 1958 samples were collected at 4-hourly intervals between 8 a.m. and 8 p.m., a few days after the week 3 samples had been collected. Chromatograms,

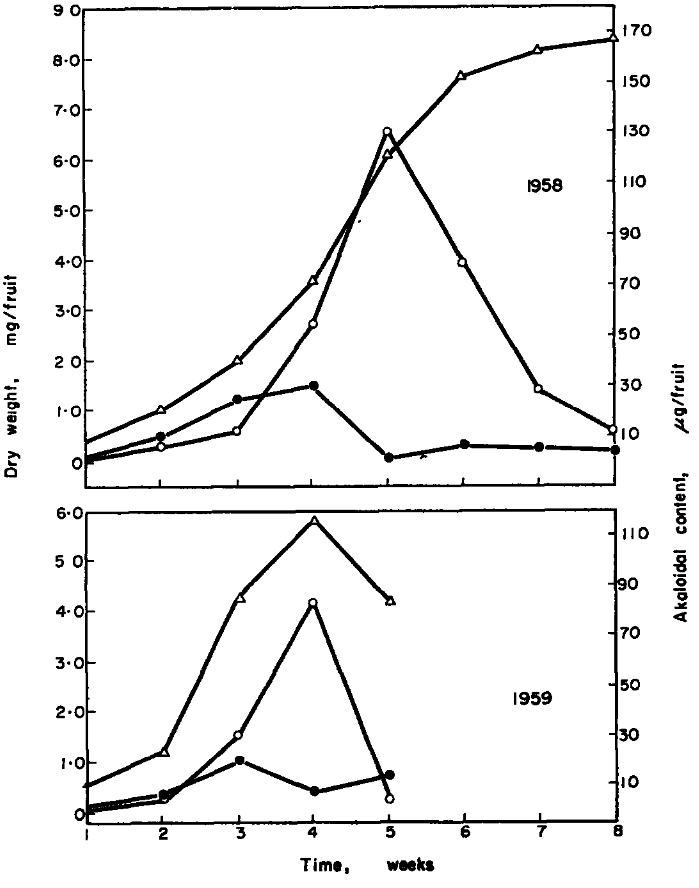

FIG. 1. ALKALOIDAL CONTENT AND DRY WEIGHT OF WEEKLY SAMPLES IN 1958 AND 1959.

Dry weight —Δ—Δ—
Coniine —○—○—
γ-Coniceine —●—●—

prepared from equal numbers of fruits, indicated that considerable variations in the alkaloidal content occurred at short intervals. Consequently 4-hourly samples were collected over a period of 24 hr a few days after the week 4 samples had been collected and a few days after the week 5 samples had been collected. Comparative paper chromatograms (an example of which is given in Fig. 4) again indicated marked changes in the amounts of alkaloid in each sample. Spectrophotometric analyses of all the samples were therefore carried out and the results are shown in Table 1. It will be noted that the amounts of coniine (I) and γ-coniceine (II) vary considerably during the day and that increase in one corresponds to decrease in the other.

Table 1. Analyses of four-hourly samples taken during a period of 24 hr in week 4 and in week 5, 1958

Time	Week 4 (μg/fruit)		Week 5 (μg/fruit)	
	Coniine	γ-Coniceine	Coniine	γ-Coniceine
4 a.m.	90	1	226	0
8 a.m.	2	6	130	2
12, midday	4	15	174	9
4 p.m.	120	1	8	21
8 p.m.	38	10	200	0
12, midnight	132	0	213	0

Two-hourly samples. In 1959 2-hourly samples over a period of 24 hr were collected on the same day as the week 3 and week 4 samples were collected and the results of spectrophotometric analyses are shown graphically in Figs. 2 and 3. In these figures the variations

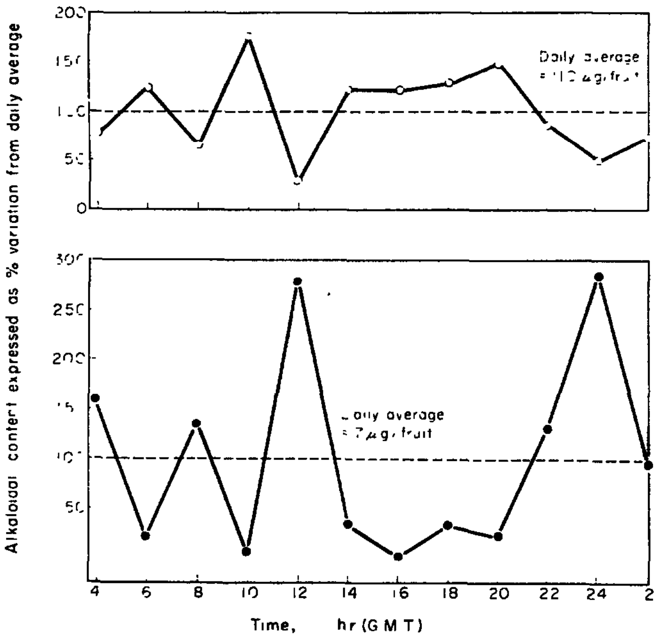

Fig. 2. Alkaloidal changes during 24 hr; week 3, 1959.
Coniine —O—O—. Average content for 24 hr period = 110 μg/fruit.
γ-Coniceine —●—●—. Average content for 24 hr period = 7 μg/fruit.

in the two major alkaloids are emphasized by representing the variations as percentages of the average content for that particular day, based on analyses of all the samples. Placing both curves on the same diagram makes it clear how they are interrelated, although the average absolute amount of coniine present was generally about 10–20 times that of γ-coniceine.* Four-hourly and 2-hourly samples over a period of 24 hr were also attempted

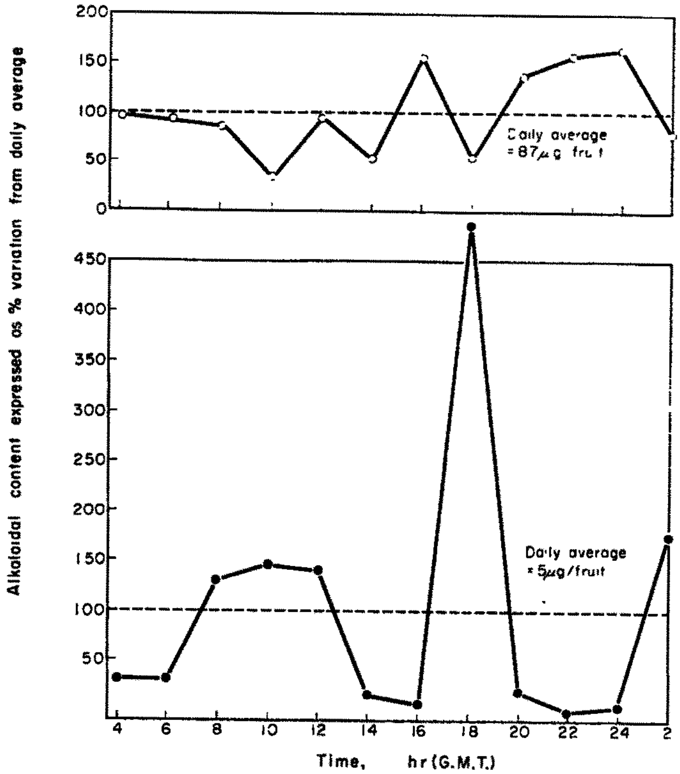

FIG. 3. ALKALOIDAL CHANGES DURING 24 HR IN WEEK 4, 1959.
Coniine —O—O—. Average content for 24 hr period = 87 µg/fruit.
γ-Coniceine —●—●—. Average content for 24 hr period = 5 µg/fruit.

in weeks 1, 2 and 5. In week 1, (flowering stage), the amounts of alkaloid present were too small for quantitative analyses; inspection of chromatograms however showed that γ-coniceine was the predominant alkaloid and indicated close interrelationship between the changes in γ-coniceine and coniine contents. In week 2, more alkaloids were present and quantitative examination was possible for three-quarters of the samples; the results once more indicated close interrelationship between the changes in γ-coniceine and coniine content; the average amount of coniine (23 µg/fruit) however exceeded the average amount of γ-coniceine (4·5 µg/fruit). In neither weeks were the changes so marked as in weeks 3 and 4. In week 5 many of the fruits had dehisced, but seven 2-hourly samples (from

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* The values for alkaloidal content given in Fig. 1 are based on midday samples only and do not necessarily correspond to those based on daily averages given in Figs. 2 and 3. Results so far, however, confirm that the trend shown in Fig. 1, and in previous years, would also be shown if figures based on daily averages were used, except that in 1959 maximum coniine content occurs in week 3. This is not surprising in view of the short time required for fruit development in 1959.

8 a.m.–8 p.m.) were collected. The results of quantitative analyses are shown in Table 2 and indicate that little change took place in the alkaloidal picture except for the sample collected at midday.

TABLE 2. ANALYSES OF TWO-HOURLY SAMPLES TAKEN DURING A PERIOD OF 14 HR IN WEEK 5, 1959

Time	Coniine (μg/fruit)	γ-Coniceine (μg/fruit)
8 a.m.	52	traces
10 a.m.	42	traces
12 midday	0	14
2 p.m.	43	traces
4 p.m.	55	0
6 p.m.	60	0
8 p.m.	49	0

Alkaloids other than Coniine and γ-Coniceine

Fig. 4 represents comparative chromatograms prepared from the 2-hourly samples collected in week 3, 1959. This chromatogram shows the same changes presented graphically in Fig. 2. Additional information on some of the minor alkaloids, however, is also given, and has been confirmed by examining similar chromatograms prepared from samples taken on other occasions. When γ-coniceine content is at a maximum small quantities of conhydrine (III) occur; when coniine is at a maximum conhydrine is absent. N-methyl coniine (IV) does occur occasionally in this "Chelsea variety" of hemlock, but only in small quantities and mainly when coniine is at a maximum.

Unknown Alkaloids

An alkaloid of high R_f value (0·73) occurred in four of the samples shown in Fig. 4, but it was not identified nor was it discovered in any other samples examined. In contrast, an alkaloidal spot of low R_f value (0·08) occurred in all samples of fruit, leaf and root examined. Preliminary examination showed that the spot contained several amino-acids and two alkaloid-like substances. These last two gave a red colour with Dragendorff's reagent, a brown colour with iodine in light petroleum, but no red colour with alkaline sodium nitroprusside (distinction from piperideines). They differed from most alkaloids in that they cannot be extracted from alkaline solution with chloroform, and from the normal hemlock alkaloids in that they are sparingly volatile in steam.

Four-hourly Samples of Seedlings

Young seedlings were grown in the open in 1959 and two weeks after the first foliage leaves opened, when they were about 7 cm high, samples of five seedlings were collected at four-hourly intervals during a period of 24 hr, washed rapidly and extracts prepared as for the fruits. Paper chromatographic examination of the extracts showed the presence of γ-coniceine and traces of conhydrine in all samples, but there was no indication of any change in the quantities. Coniine and N-methyl coniine were not detected. Similar experiments were performed on samples from the same crop of seedlings a week later, when they were about 12 cm high. Separate analyses were made on the roots and the aerial parts. No alkaloids were found in the roots; in the aerial parts γ-coniceine alone was detected and the quantities did not appear to vary markedly during the 24-hr period. As already stated the compound of low R_f value always occurred in all parts of the plant.

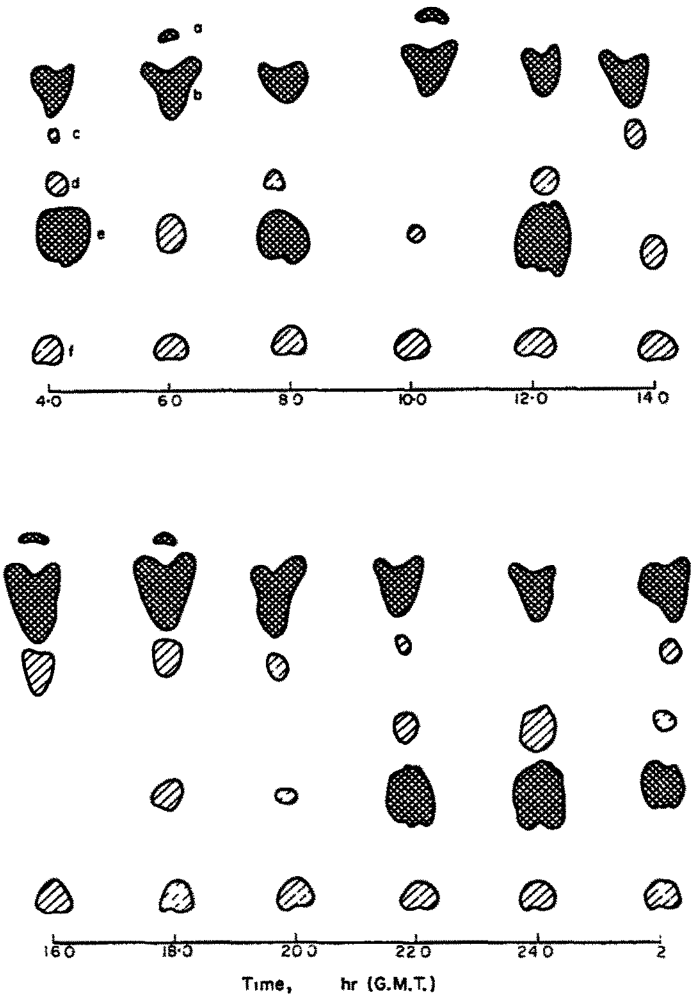

FIG. 4. PAPER CHROMATOGRAMS OF 2-HOURLY SAMPLES EACH REPRESENTING THE SAME NUMBER OF FRUITS; WEEK 3, 1959.

(*a*) Unknown alkaloid of $R_f = 0{\cdot}73$. (*b*) Coniine. (*c*) N-methyl coniine. (*d*) Conhydrine. (*e*) γ-Coniceine. (*f*) Unknown alkaloid $R_f = 0{\cdot}08$. Depth of shading and area of the spots roughly proportional to amount of alkaloid present.

Second Year Plants

A well-developed plant was removed in March 1958 before signs of spring growth were showing; analysis showed that γ-coniceine alone occurred, both in the aerial parts and in the roots. A similar experiment was carried out in June 1958 when growth was vigorous; γ-coniceine was the only alkaloid detectable in the aerial parts, but no alkaloids were detected in the roots, apart from the low R_f value substance.

DISCUSSION

The results based on weekly and daily samples collected during 1958 and 1959 have confirmed some of the conclusions reached by earlier workers.[1,2] Thus, γ-coniceine is the predominant alkaloid in the vegetative parts and in the very early stages of development from flower to fruit. The overall change from γ-coniceine to coniine is associated with rapid development of the fruits. At the later stages of fruit maturation the coniine content falls and there is a slight increase in the γ-coniceine content, indicating a possible re-conversion of coniine into γ-coniceine (see Fig. 1).

The results of all the work on hemlock alkaloids up to this point would be consistent with a slow accumulation of alkaloids as minor end products of metabolism together with gradual changes in their composition. Our work on 4-hourly and 2-hourly samples has however shown that remarkably rapid changes in the composition and amounts of alkaloid occurred over short periods of time. As far as we know such dynamic changes in the alkaloidal picture have not been demonstrated so clearly before. James[3,4] points out that only in certain seedlings and senescent leaves is there evidence of the return of alkaloidal materials to metabolic circulation and the quantities involved are always extremely small. In a discussion on the physiology of alkaloids, Mothes[5] states: "If, therefore, the alkaloid content, during the course of a year, fluctuates with the changing growth pattern, we may raise the question whether this phenomenon obtains during the course of a day." He then refers to the difficulties involved in answering this question. Hemberg and Flück[6] determined the total alkaloids in samples of *Datura* leaf and root collected at 4-hourly intervals during 24 hr. There was a gradual increase in the amount of total alkaloids during the day and a fall at night, but no information is given on the relative proportions of individual alkaloids. Somewhat similar results were obtained with *Datura* and *Atropa* by Kozlova[7] although the quantities of alkaloids were determined by histochemical means. Miram and Pfeifer[8] determined individual alkaloids in poppy plants at 6-hr intervals, but since the results were expressed as mg per cent dry weight it is not clear whether the absolute amounts of alkaloid present varied. Our results indicate that the change from γ-coniceine to coniine is geared to some active metabolic process; and since only the addition of two hydrogen atoms to γ-coniceine is involved it is possible that the latter acts as a hydrogen acceptor in some oxidation–reduction process. These changes however appear to be restricted to the developing fruit only and are most marked during the weeks when pericarp development is at a maximum.

Quantitative Aspects

Apart from the rapid changes in the two major alkaloids, the following related facts should be noted.

Careful examination of the results given in Figs. 2 and 3 shows that the interchange between γ-coniceine and coniine is not a simple reversible change *quantitatively*. For example, from Fig. 2 it will be seen that between 10 a.m. and 12 noon the coniine content

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[2] B. T. CROMWELL, *Biochem. J.*, 64, 259 (1956).

[3] W. O. JAMES, *The Alkaloids*. Edited by R. H. F. MANSKE and H. L. HOLMES. Vol. I, p. 82. Academic Press, New York (1950).

[4] W. O. JAMES, *Endeavour*, 12, 76 (1953).

[5] K. MOTHES, *Ann. Rev. Plant. Physiol.*, 6, 412 (1955).

[6] T. HEMBERG and H. FLÜCK, *Pharm. Acta Helv.* 28, 74 (1953).

[7] N. A. KOZLOVA, *Izvest. Akad. Nauk. Latv. SSR.*, 6, (70), 65 (1953).

[8] R. MIRAM and S. PFEIFER, *Sci. Pharm.* 27, 34 (1959).

fell from 175 per cent to 28 per cent of the daily average (110 μg/fruit); this represents a loss of 162 μg/fruit. During the same period the γ-coniceine content increased from almost zero to 280 per cent of the daily average (7 μg/fruit); this represents an increase of only 19 μg/fruit. This means that during a period of 2 hr 143 μg of coniine have disappeared and have not been accounted for by the formation of γ-coniceine or any other alkaloid. This loss corresponds to about 21 mg/umbel. As the samples were collected on a dry day this relatively large loss cannot be accounted for by rain washing. Neither is it likely to be due entirely to volatilization as there must have been at least 1000 active umbels in the row of plants being visited every 2 hr and such a large loss by volatilization (over 20 g alkaloid) would produce a very marked odour. No very strong odour was noticed on any occasion. Furthermore, it is well known that much of the coniine is stored in the inner layers of the pericarp, in the endocarp or "coniine layer". The question as to what happens to the excess coniine naturally arises; the use of isotopically labelled alkaloids will obviously greatly assist in solving this problem.

Relation to the Development of the Pericarp

During the first few weeks of fruit development there is a rapid increase in the average alkaloidal content. During this period the pericarp, to which the alkaloids are restricted, develops rapidly and marked anatomical changes take place.[1] The increasing "demands" of the developing pericarp for alkaloids may therefore be closely related to the increasing number of living cells. This would be consistent with the view already expressed, that the alkaloids play a significant part in the intracellular metabolism of the pericarp.

Source of the Alkaloids

The fruit does not develop in isolation so that at least three possible sources for its alkaloids exist. (*a*) γ-Coniceine is the precursor of all the alkaloids and enters the fruit by the vascular bundles and schizogenous ducts as suggested previously.[1] If this is so the incoming γ-coniceine must be rapidly converted to coniine, since only small quantities of γ-coniceine occur; at times this conversion is so rapid that the γ-coniceine content falls to zero. This continuous supply of γ-coniceine must result in a considerable daily production of coniine which, in turn, is converted into a non-alkaloidal substance. (*b*) Coniine is formed from incoming γ-coniceine and at certain times combines reversibly with another molecule to form a non-alkaloidal substance; at later stages coniine is released from this loose compound. A small proportion of the coniine may also be re-converted to γ-coniceine. In these circumstances the daily production of coniine may not be large, but would show a gradual increase with increase in size of the pericarp. (*c*) The alkaloids are mainly synthesized in the fruit itself and the incoming γ-coniceine may play only a minor part in the total coniine production.

Minor Alkaloids

The appearance and disappearance of the minor alkaloid conhydrine runs parallel with the increase and decrease of γ-coniceine. This is consistent with the fact that both alkaloids are oxidation products of coniine and, conversely, could be formed into coniine by reduction. On the other hand, the occasional appearance of N-methylconiine and the unknown alkaloid of $R_f = 0{\cdot}73$ is associated with a maximum of coniine and it is not so clear how N-methylation would be connected with a reduction process.

The universal occurrence of the compounds of low R_f value (0·08) and some of their properties might at first sight indicate that they are the precursors of the normal alkaloids, but we found no evidence of the marked changes in quantity shown by the main alkaloids. Cromwell[2] does not mention this compound, but some of his chromatograms show a very small spot of R_f value 0·1. As he used steam distillates for his assay work it is unlikely that much of this compound would be present, as we found it was only sparingly volatile in steam.

EXPERIMENTAL

The "Chelsea variety" of *Conium maculatum* described by Fairbairn and Challen[1] was used throughout. The plants were grown at Myddelton House, Enfield, Middlesex, and the same sampling techniques were used as described previously,[1] week 1 collection being made when the flowers were fully expanded. In view of the large number of samples required, only four compound umbels were collected at a time. These yielded about 250 fruits for chemical analyses and about 250 fruits for dry weight determination. The chromatographic and analytical techniques of these authors were also used except for the following modifications.

Paper chromatograms. An improved method of determining the quantities of alkaloids based on measuring the areas of spots on paper chromatograms was devised.[9] Whatman No. 20 paper, which had been washed with N HCl in a chromatographic tank (descending technique) for 18 hr, followed by a similar washing with distilled water, was used. The solvent system was *tert*-pentanol/*tert*-butanol/N HCl (9 : 3 : 2), development time 15 hr at 20° (ascending technique). After drying, the alkaloidal spots were revealed by spraying with bismuth iodide reagent.[10]

Spectrophotometric methods. The yellow colour referred to previously,[1] which was produced by the interaction of bromethymol blue and the conium alkaloids, was measured in 1 cm cuvettes in a Hilger Uvispek spectrophotometer. The peak of the absorption curve was 402 μ; log ε_{402} for coniine was 4·313 and for γ-coniceine, 4·327. For convenience the alkaloidal contents are expressed as hydrochlorides throughout.

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[9] J. W. FAIRBAIRN and P. N. SUWAL, *Pharm. Acta Helv.* **34**, 561 (1959).

[10] R. MUNIER and M. MACHEBOEUF, *Bull. Soc. Chim. Biol. Paris* 33, 846 (1951).