Studies on the Orthophosphoric Acid Method for the Pyrethrum Assay Naomichi Baba, Mitsunori Kirihata and Minoru Ohno (Institute for Chemical Research, Kyoto University), Takenosuke Takano and Keiko Kawano (Japanese Institute for Pyrethrum Research, Uji) Received August 20 (1972). *Botyu-Kagaku*, 37, 155, 1972.

23. ピレトリンの分析に対するオルトリン酸法に関する研究

馬場直道，切畑光統，大野 稔（京都大学化学研究所），高野武之助，河野恵子（日本除虫菊研究所宇治） 47. 8. 20 受理

ピレトリンの定量法として提案されたオルトリン酸法は，その発色反応の選択性に関して不明な点が残されていた．本研究ではピレトリンⅠ，Ⅱ及びジャスモリンⅠ，Ⅱを合成し，それらのうち前者のみが発色することを確認した．本方法により除虫菊「白雪」及びケニヤ産乾花を分析した．その結果，測定試料と標準試料の間で6有効成分の相対含量比またはピレトリンⅠ，Ⅱの全体に対する含量比が等しい場合にはオルトリン酸法による分析値は酸法による分析値と非常によく一致した．また一致しない数値に対しては上に述べた相対比を考慮することにより補正ができることがわかった．

The orthophosphoric acid method as a colorimetry for pyrethrum assay was investigated on the substrate-structure selectivity of the color reaction using synthetic pyrethroids and it was revealed that pyrethrins I and II alone reacted with the reagent (H_3PO_4-$CH_3COOC_2H_5$) to give a rose red color at 550 mμ. The whole procedure of pyrethrum assay was devised specifically and was applied to pyrethrum flowers from Kenya and Shirayuki strain, and the analytical results compared well with those by the conventional acidmetry.

Introduction

In these several years, the importance of pyrethroid as insecticide in particular for domestic use has been growing more and more with the recent recognition of a gloval pollution of the environment by a variety of pesticides. The objective of the present work is to establish a timesaving, inexpensive and highly sensitive method for determination of total pyrethrins in a number of samples of pyrethrum flower of the same strain. A number of methods have been devised for determination of pyrethroid. As a typical method, acidmetry[1], including Japanese official method, AOAC method and PBK method, has been officially adopted. But this method is time-consuming and requires large amount of samples.

In 1952, Jones *et al.*[2] used the mixture of orthophosphoric acid, glacial acetic acid and tannic acid as a reagent for determination of piperonyl butoxide. Later, Williams *et al.*[3] found that orthophosphoric acid alone reacted with natural pyrethrum extract to give a transparent rose red color and was applicable to the pyrethrum assay. In the present work, it was found that pyrethrolone and pyrethrum extract reacted with H_2SO_4-CH_3COOH and HCl-CH_3COOH also to give various red color and the origin of the coloring was discussed in another paper[4]. The reaction with orthophosphoric acid reagent, however, gave the highest molar absorbance among other reagents so far as pyrethrolone is concerned, so that this reagent was used throughout the present work.

On the other hand, pyrethrum extract contains six kinds of active rethrins whose relative insecticidal activity differs to each other. Williams purified the naturally occurring pyrethrins and cinerins by means of column chromatography and subjected these specimen to the color reaction with the reagent. As a result, he stated that pyrethrins I and II alone gave the color with the reagent but this was not the case with cinerins I and II. At that times, however, the presence of jasmolins I and II were not known as yet as minor constituents in pyrethrum extract. Accordingly, it was obscure that whether or not jasmolins I and II were responsible for the color dvelopment. In addition, the possibility that some inactive impurities present in pyrethrum extract may give the same color can not be excluded.

These situations has to be checked for the precise determination of total amounts of insecticidal constituents. For this reason, the orthophosphoric acid method has been scarcely used for determination of total pyrethrins. Accordingly, in the present work, the substrate-structure selectivity of the color reaction and conditions for color development were elucidated. A modified procedure for determination of dried pyrethrum flower by this method was described and applied to the pyrethrum flowers from Kenya and Shirayuki strain. The data were discussed from the point of the percentage of pyrethrins I and II in total active constituents.

Material and Method

Dried pyrethrum flowers from Kenya and World Standard Pyrethrum Extract (WSPE, 1972) were kindly supplied by Dr. S. W. Head, pyrethrum flowers, Shirayuki strain (1972), by the Food Office in Hiroshima Prefecture, prothrin, by Dainippon Jotyūgiku Co., Ltd. and phthalthrin and resmethrin by Sumitomo Chemical Co., Ltd. to whom the authors' thanks are due. These samples were stored at 4°C. A mixture of 85%-orthophosphoric acid and ethyl acetate (4:1 by volume) was used as color reagent. UV spectra were taken on a Hitachi EPS-3 spectrophotometer and gaschromatography were on a Hitachi K-53 with FID detector. Jasmololone, jasmolins I and II, pyrethrolone, pyrethrins I and II and trans-pyrethrolone were synthesized by the Crombie's method[5]. The structure and integrity of these alcohols and esters were identified authentically by the coincidence of IR and NMR spectra with those in the literature[6].

I. Substrat-structure Selectivity of the Color Development

In order to know which of the six constituents does contribute to the coloring with the reagent, 5 m*l* portion of the color reagent was added to about 0.5 mg of each of these alcohols and esters synthesized and the mixture was heated at 100°C for 2.5 min in water bath. As the results, in the case of pyrethrolone, pyrethrins I and II, a rose red color developed, showing the maximum absorption at 550 mμ. The color tones were identical with that of the natural pyrethrum extract. In Figure 1, absorption spectra of the colored mixtures were shown. In contrast, in the case of jasmololone, jasmolins I and II, any perceivable or measurable color did not appear. As pointed out previously by Sweeney[7], the facts mentioned above suggested that (1) since the alcholic moiety i.e., pyrethrolone *per se* was able to give this color, the acid moiety did not take part in this color reaction and (2) at least two double bonds must be necessary for a side chain of rethrolone moiety to give this color. In addition, the possibility that some impurities contaminated in pyrethrum extract may take part in the color reaction was excluded by the thin layer chromatography with Stahl's method[8]. All the results of coloring of natural and some synthetic species were summarized in Table 1.

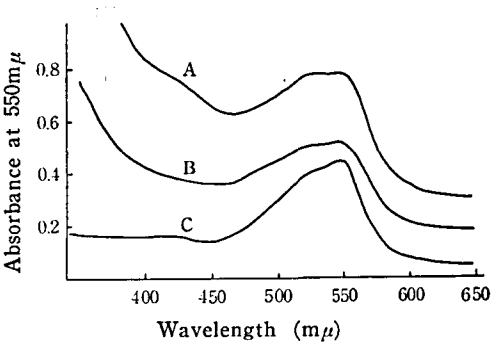

Fig. 1. Absorption spectra of colored mixtures. Curve A : synthesized pyrethrin I+ phosphoric acid reagent, B : synthesized pyrethrin II+phosphoric acid reagent, C:natural pyrethrum extract + phosphoric acid reagent. The concentrations of these three samples were not equal.

Table 1. Data from coloring test of some pyrethroids with phosphoric acid reagent.

Compound	Color	Compound	Color
pyrethrolone	red	jasmololone	colorless
pyrethrin I	red	jasmolin I	colorless
pyrethrin II	red	jasmolin II	colorless
cinerolone	colorless	phthalthrin	colorless
cinerin I	colorless	resmethrin	yellow
cinerin II	colorless	prothrin	brown

II. Condition for Color Development

For the reproducibility of accurate analytical

results, the relations between absorbance at 550 mμ and the time of shaking, time of heating, time interval from heating and cooling to photometric measurement of the colored mixture should be made clear and the optimum conditions for color development and photometric measurement should be stricly specified. Using a sample solution of pyrethrins from dried pyrethrum flowers, the conditions for coloring was examined. Figures 2, 3 and 4 show the results of the experiments. The more prolonged the time of shaking colored mixture was, the lower the absorbance at 550 mμ, so that the time of shaking was fixed as for one minute accurately. The intensity of rose red color of the sample solutions reached the maximum by 1.5 min' heating at 100°C after shaking and gradually decreased by further prolonged heating (Figure 3). To ensure the constant and reproducible coloring, therefore, it seemed best to set the time of heating to 2.5 min, taking in consideration the flat part of the absorbance curve found for coloring. After heating for developing color, the test solution was cooled within 2 min with water to room temperature for photometric measurement. The absorbance at 550 mμ decreased slowly with time of standing at room temperature and finally reached ca. 90 % of the maximum intensity after 1.5 hr (Figure 4). Consequently, the photometric measurement of the sample solutions should be carried out within 5-10 min after cooling the mixture to room temperature.

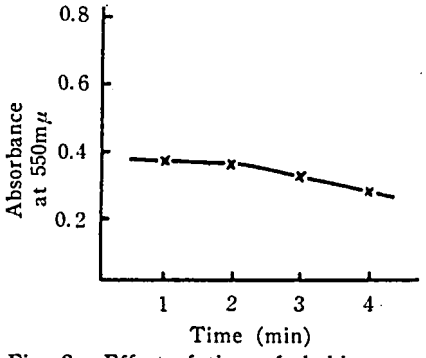

Fig. 2. Effect of time of shaking on absorbance at 550mμ.

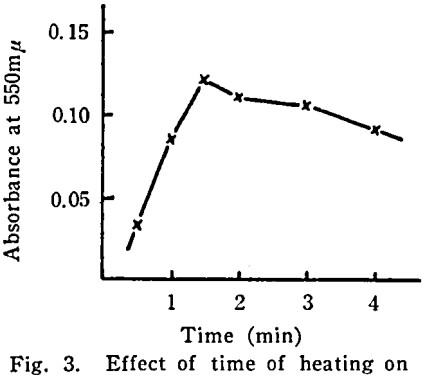

Fig. 3. Effect of time of heating on absorbance at 550mμ.

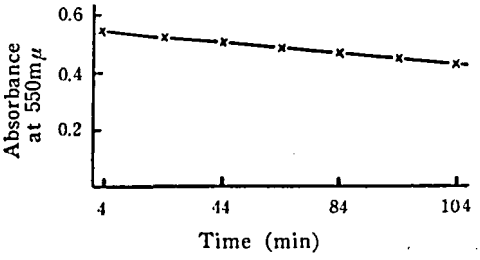

Fig. 4. Change of absorbance at 550mμ with time interval from cooling to measuring.

III. Preparation of Sample Solution from Dried Pyrethrum Flowers

Usual extraction method was modified as follows; About three grams of dried pyrethrum flowers, Shirayuki strain, after grinding, were extracted with 160 m*l* of petroleum ether in Soxhlet extractor for 2 hrs. This solution was concentrated to adout 4 m*l* under reduced pressure and purified by extraction with nitromethane (4 m*l*×3) followed by dilution with acetone to a concentration of ca. 30-90 micrograms of total pyrethrins per one m*l* of this solution. One m*l* of this solution was submitted to the coloring procedure mentioned above.

IV. Standard Pyrethrins Solution and Calculation of Pyrethrum Content in Unknown Samples

As was previously mentioned, this color reaction is effected only pyrethrins I and II and, at present, a stoichiometric relation between absolute quantity of pyrethrins I, II and absorbance at 550 mμ is not known, so that for the determination of total pyrethrins by the present methods, some standardized pyrethrins solution is required and, furthermore, the relative ratio of cinerin, jasmolin and pyrethrin in classes I and II must be identical with respect to both of

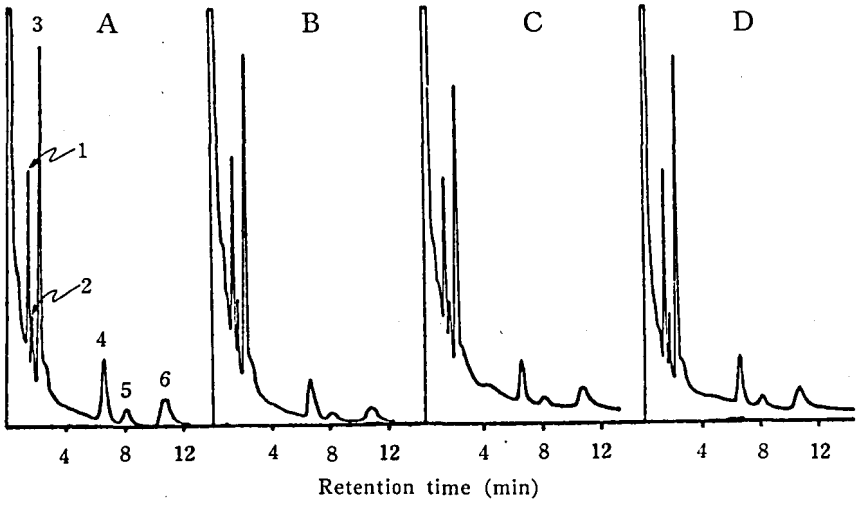

Fig. 5. Gaschromatograms of the four samples from the pyrethrum flower, Shirayuki strain. Chart A: first grade, B: second grade, C:third grade, D: third sub-grade. Peak 1: cinerin I, 2: jasmolin I, 3: pyrethrin I, 4: cinerin II, 5: jasmolin II, 6: pyrethrin II. Gaschromatographic conditions. Column: 5%-PEGS on acid washed Chromosorb W (60-80mesh), 1m×2mm, 210°C. Detector: FID, Carrier gas: N_2, flow rate: 4ml/min. Attenuation: 20×1.

the standard solution and sample solution. To know the relative ratio of the six constituents, gaschromatography was applied to the extract of the flower, Shirayuki, (first-, second-, third-grade and third-subgrade) harvested in June, 1971, as well as to WSPE (ca. 20% content of total pyrethrins) and other samples. Gaschromatograms of the samples from Shirayuki were given in Figure 5. In this case, four samples gave an almost identical peak pattern, suggesting that the relative ratio of constituents had no variation so far as these samples of the domestic Shirayuki flowers were concerned. Thus, if "pyrethrins" content of one sample arbitrarily chosen from many lots were known by some method (for example, acidmetry), a standard calibration line may be drawn for phosphoric acid method. In the present research, an extract from pyrethrum flowers of second grade (whose pyrethrins content was 1.40% by Japanese official method) and WSPE were used as the standard solution for calibration. In Figure 6 the two calibration curves were shown. Then, the total pyrethrins content (%) in unknown samples can be calculated by the following expression;

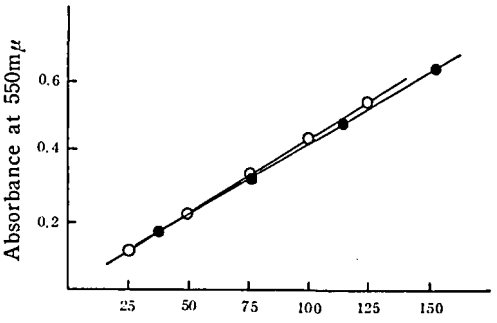

Fig. 6. Standard calibration curves.
-○—○-: Shirayuki strain of second grade (1.40%)
-●—●-: WSPE (20.0%)

$$\text{Total pyrethrins content} = \frac{Y}{40 \times X} \quad (\%)$$

where X is the amount (g) of dried pyrethrum flowers taken (ca. 3g) and Y is the pyrethrins content (microgram) of unknown sample.

Result and Discussion

By the strict adhesion to the procedures specified above, total pyrethrins content of several samples from Shirayuki strain were determined

and compared with the values obtained by acidmetry. The results were summarized in Table 1. The values given in the column designated as method A were those by the Japanese official method, while under method B were summarized the analytical values by the present method with the use of an arbitrary sample solution of Shirayuki extract whose total pyrethrins contents were estimated by the acidmetry, as the standard. Samples I, II and III were arbitrarily picked up from Shirayuki crops harvested in June, 1971. Samples IV and V are those of the same strain stored at 4°C for one and two years respectively.

Table 2. Pyrethrins content (%) in dried pyrethrum flowers by the Japanese official method (A) and phosphoric acid method (B).

	I	II	III	IV	V
A	1.40	1.31	1.20	0.90	1.00
B	1.40	1.29	1.12	0.28	0.63

As can be seen from the Table, samples I, II and III gave approximately the same analytical values in total pyrethrins content by both method A and B. In cases of the samples IV and V, however, the present method B gave much lower values as compared with those by acidmetry.

Then, the extracts of samples IV and V were subjected to gaschromatography under the same conditions as described in Figure 5. In these cases, the peak heights of pyrethrins I and II were found considerably lower than those of cinerins I and II, in contrast to the cases of the crop harvested in 1971 (Figure 7). These observations might be viewed simultaneously as a consequence of preferential degradation characteristic of pyrethrins I and II alone, and this kind of degradation might be of such a nature that can not be estimated by acidmetry. Accordingly, it may concern probably with some chemical changes on the pentadienyl side chain in pyrethrolone moiety, i. e., dimerization[9], sigmatropic rearrangement of the side chain[10,11,12]. The problem that whether or not such degraded species (false pyrethrins) give the same color is under investigation.

In Table 3 are listed total pyrethrins contents

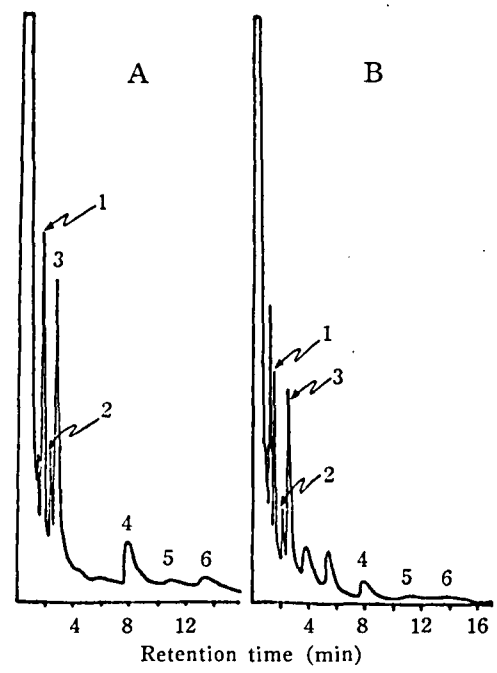

Fig. 7. Gaschromatograms of the pyrethrum flowers stored for one year (chart A) and two years (chart B). Analytical conditions were the same as described in Fig. 5. peak 1: cinerin I, 2: jasmolin I, 3: pyrethrin I, 4: cinerin II, 5: jasmolin II and 6: pyrethrin II.

of several samples from Kenya (samples No's 1-8 harvested in 1971) and domestic Shirayuki strain (samples No's 9-11 harvested in 1972) assayed by the phosphoric acid method and two types of acidmetry. The values given in the column designated as A, B and C were those by the AOAC, Japanese official and the phosphoric acid method respectively. In the column D were given the percentages of pyrethrins I and II in the six kinds of rethrins obtained by gaschromatographic analysis. The values corrected from the data in column B and D were given in column E. As can be seen in this Table, the values obtained by the phosphoric acid method is, as a whole, in good agreement with those by AOAC and Japanese official method except samples No's 3, 5 and 7. The important feature is that samples 3, 5 and 7 showed higher percentages of pyrethrins I and II than that of WSPE also gave higher values of total pyrethrins by the phosphoric acid method than those by

Table 3. Total pyrethrins content and the percentage of pyrethrins I and II in total rethrins. Column A: AOAC method, B: Japanese official method, C: phosphoric acid method, D: percentages of "pyrethrins I and II" in total rethrins and E: values corrected by the following calculation: (values in column C) × (70.3/values in column D).

	A%	B%	C%	D%	E%
1	1.19	1.45	1.14	65.0	1.23
2	1.38	1.37	1.39	65.9	1.48
3	1.63	1.71	1.92	75.6	1.79
4	1.97	2.07	1.90	66.6	2.00
5	1.13	1.45	1.26	75.3	1.15
6	1.02	1.44	1.04	64.9	1.12
7	1.37	1.58	1.59	77.4	1.44
8	1.27	1.38	1.27	70.8	1.27
WSPE	19.9	20.6	—	70.3	—
9	—	2.04	1.96	68.0	2.05
10	—	1.68	1.69	70.6	1.68
11	—	1.71	1.82	72.1	1.79

acidmetries. Such a deviation, however, can be anticipated on the basis that pyrethrins I and II alone are estimated by the phosphoric acid method and that the relative ratios of the six rethrins are not always equal in the standard solution and test samples. Then, the correction was made on the results by multiplying these values (column C) by the factor (the percentage of pyrethrins I and II in WSPE (70.3%)/those of each samples). The results of the correction are presented in column E. What is evident from the Table is that, after the correction, the differences between the assayed values by the phosphoric acid method and by the acidmetries decreased further than before the corrections. As a whole, the corrected values were found within the range from zero to at most +10% of those given by the acidmetries.

References

1) Hortz, W.: "Official Methods of Analysis of AOAC", 10th Ed., AOAC, Washington, (1956), pp. 50. H. A. Seil, Soap., 10, (No5). S. Takei and K. Wakazono, Nippon Nogeikagaku Kaishi, 16, 399 (1940).

2) Jones, H. A., H. J. Ackermann and M. E. Webster: *J. Assoc. Offic. Agr. Chem.*, 35, 771 (1952).

3) Williams, H. L., W. E. Dale and J. P. Sweeney: *J. Assoc. Offic. Agr. Chem.*, 39, 872 (1956).

4) Baba, N., M. Kirihata, T. Takano and M. Ohno: *Bull. Inst. Chem. Res.*, Kyoto Univ., 50, 150 (1972).

5) Crombie, L., P. Hemesley and G. Pattenden: *J. Chem. Soc.*, 1016, 1969. Crombie, L., P. Hemesley and G. Pattenden, Tetrahedron Letters., No. 26, 3021 (1969). Crombie, L., S. H. Harper and D. Thompson, *J. Chem. Soc.*, 2906, 1951. Doebner, Ber., 35., 1137 (1902). Gluskov and Magidson, C. A., 58, 442 (1963).

6) Bramwell, A. F., L. Crombie, P. Hemesley and G. Pattenden: Tetrahedron., 25, 1727 (1969).

7) Sweeney, J. P. and H. L. Williams: *Agr. and Food Chem.*, 5, 670 (1957).

8) Stahl, E. and J. Pfeifle: Pyrethrum Post., 8, No. 4, 8 (1966).

9) Freeman, S. K.: Soap and Chemical Specialities., 32, 150 (1956).

10) Elliott, M.: *J. Chem. Soc.*, 888, 1964.

11) Brown, N. C., D. T. Hollinshead, R. F. Phipers and M. C. Wood: Pyrethrum Post., 4, No. 2, 13 (1957).

12) Goldberg, A. A., S. Head and P. Johnston: *J. Sci. Food Agric.*, 16, 43 (1965).

Summary

1) It was reaffirmed that pyrethrins I and II alone gave the color with the phosphoric acid reagent.

2) The optimum conditions for color development and photometric measurement were strictly specified.

3) Additional nitromethane extraction process was involved in the conventional procedure of preparation of sample solution for the purpose of purification.

4) Several pyrethrum flowers from Kenya and Shirayuki strain were assayed by the phosphoric acid method using WSPE and extract of flowers of second grade from Shirayuki strain as the standard. It was found that dried flowers which were stored for a long time gave extremly lower assay values compared with the flowers which were stored for less than a few months from

the harvest time. In addition, on the gaschromatograms of these samples, the peak intensity of pyrethrins I and II was much lower comparing with those of standard extract. It was also found that samples which showed higher percentages of pyrethrins I and II than those of the WSPE also gave higher content of total pyrethrins than those by the acidmetries.

5) The assay values were corrected by multiplying these values by the ratios (the percentage of prethrins I and II in WSPE/those of each samples) which were obtained gaschromatographically. By such a correction, fairly good agreement of the assay values between the phosphoric acid method and acidmetries was obtained.

Acknowlegment The authors should like to thank Prof. Y. Inouye for his helpful discussion and advice. We are also pleased to acknowledge support of the Ministry of Agriculture and Forestry.

Teratologic Evaluation of Tsumacide (*m*-Tolyl-*N*-Methylcarbamate) in the Rat. Mineo Yasuda (Department of Anatomy, Kyoto Prefectural University of Medicine, Kyoto) Received September 21, 1972. *Botyu-Kagaku*, 37, 161, 1972.

24. ラットにおけるツマサイドの催奇形性の評価

安田峯生（京都府立医科大学解剖学教室，京都市） 47. 9. 21 受理

ツマサイド（*m*-tolyl-*N*-methylcarbamate）の催奇形作用をラットを用いて検索した．MTMCを80および4000ppmの割合で飼料にまぜ，妊娠8日より15日までSD-JCLラットに連続経口投与した．妊娠末期胎仔および生後6週の育成仔を観察したところ，ツマサイドの催奇形作用は検出されなかった．

Tsumacide® (*m*-tolyl-*N*-methylcarbamate) is an insecticide for control of planthoppers and leafhoppers in paddy rice fields, and has been widely used in Japan. Reported here are effects of this product having been given to pregnant rats upon the fetal and postnatal development of the offspring.

Materials and Methods

SD-JCL rats, obtained from Japan CLEA Co., Ltd., were used. They were housed in wire-bottom cages. The diet, ground CE-2 laboratory chow made by Japan CLEA Co., Ltd., was supplied in unrestricted amounts, and bottled water for drinking was freely available. The room temperature was maintained at 22±1°C and relative humidity at 55±5%. The room was kept dark from 10 AM to 9 PM.

Virgin females of 12 to 16 weeks of age, approaching estrus, were caged with males from 9 AM to 3 PM. Pregnancy was considered to have started (day 0) at 12 AM when sperm were found in the vaginal smears at 3 PM. On day 8, pregnant rats were randomly allotted to test groups.

Tsumacide, assay 97.6%, was supplied by Mitsubishi Chemical Industries Ltd. From day 8 to day 15 of pregnancy, Tsumacide was mixed with the diet at levels of 80 and 4000 ppm; the high level of 4000 ppm was estimated to be near the maximum nontoxic dose for adult female SD rats after a preliminary subchronic toxicity study of 2 weeks. In this preliminary study, body weight, general behavior and gross autopsy findings were used as indices of toxicity.

Rats were weighed at the time of sperm detection, and everyday from day 8 to day 21 of gestation. Daily consumption of food and water was also measured from day 8 to day 21.

About three-quarters of the dams in each group were killed on day 21 of gestation by cervical dislocation. Immediately after death, the uterine contents were examined, and the number and position of viable fetuses, dead fetuses and resorption sites were recorded. Viable fetuses were removed from the uterus, sexed, examined for externally visible malformations and cleft palate, and weighed. Then, about half of the fetuses in each litter were fixed in Bouin's fluid and the remaining half were placed in 95%

® Trade name registered by Nihon Nohyaku Co., Ltd.