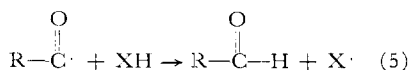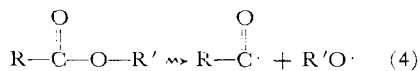(Table IV). These results confirm earlier reports (7, 17) that mono-carbonyls are formed upon irradiation of fats under vacuum. Day and Papaioannou (7) obtained evidence for alk-2-enals and alk-2,4-dienals in irradiated milk fat. In the present study, traces of the C_5, C_6, C_9, and C_{12} alk-2-enals but no alk-2,4-dienals were identified. Qualitatively, the methyl ketones did not show a significant change upon irradiation (Table IV); however they are present in much larger quantities in irradiated milk fat. The origin of methyl ketones and long chain aldehydes has been discussed by Day and Papaioannou (7). They suggested that hydrolytic cleavage of ester and enol-ether linkages were responsible for these compounds. Work in our laboratory (14) has demonstrated that hydrolysis as well as cleavage of ester linkages occurs with irradiation. Langler and Day (18) have suggested that methyl ketones are formed in heated milk fat as a result of hydrolysis. Also, long chain aldehydes occur in milk fat as bound aldehydes (9, 24). Thus, irradiation-induced hydrolysis of ester and enol-ether linkages is probably a likely mechanism accounting for the production of ketones and long chain aldehydes in irradiated milk fat. The formation of short chain aldehydes may also be explained by a similar mechanism.

$$R-\overset{O}{\overset{\|}{C}}-O-R' \rightsquigarrow R-\overset{O}{\overset{\|}{C}}\cdot + R'O\cdot \quad (4)$$

$$R-\overset{O}{\overset{\|}{C}}\cdot + XH \rightarrow R-\overset{O}{\overset{\|}{C}}-H + X\cdot \quad (5)$$

The finding of C_6 and C_8 methyl ketones is difficult to explain. The presence of these and many other even- and odd-numbered saturated and unsaturated ketones, and the probable mechanism of their formation in irradiated fats have been reported by Kohn (16).

Compounds such as ethyl acetate, benzene, chloroform, and dichlorobenzene were also detected in the control sample (Table III). Chloroform has been reported in milk fat by Wong and Patton (32). It was suggested that this compound arises from pesticide residues in milk. Dichlorobenzene, which has not been reported previously in milk, could also have its origin in pesticides.

Four components listed in Table I (peak numbers 31, 35, 37, and 39) had a characteristic candle-like odor. These compounds were present in relatively small quantities (Figure 2). The exact nature of these components could not be determined. Work is in progress in our laboratory to characterize and identify these components further.

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ANALYTICAL METHOD

Determination of the Pungent Constituents of *Piper nigrum*

BART LABRUYÈRE

N. V. Chemische Fabriek "Flebo," Hoogezand, Holland

SINCE spice oleoresins have come into the market in several forms, the need for good analytical methods for determination of the active constituents has grown. This holds especially true for pepper. For piperine (1-piperoylpiperidine), the following methods have been described in the literature:

A. Kjeldahl determination of nitrogen (1, 6, 7).
B. Polarographic determination (6, 11).
C. Iodometric procedure (5).
D. Spectrophotometric method (ultraviolet-absorption) (6, 7).
E. Colorimetric method using chromotropic acid (2, 7, 13).
F. Colorimetric method using nitric acid (10).
G. Colorimetric method using sulfuric acid and an aromatic aldehyde (8).
H. Colorimetric method using phosphoric acid (9).
I. Method presented in this paper.

Figure 1 shows the part of the piperine

Existing methods for the determination of piperine are briefly discussed. A new procedure is proposed, based upon alkaline hydrolysis at about 138° C., followed by titration of the liberated piperidine after distillation with water. The result of this method is a measure of the total pungency. A suggestion is made on the manner in which vinyl homologs of piperine are formed.
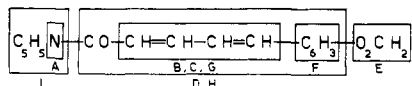

Figure 1

Method A is not specific, as indicated by the literature (6, 7, 22), because proteins and related compounds interfere. Unsaturated fats and some components of essential oils may influence the result of methods B and C unfavorably. Method D has a high specificity for piperine owing to the typical system of conjugated double bonds and is the best existing method for the determination of piperine —piperettine can also be determined. Method E uses a reaction given by all molecules which release formaldehyde under the conditions of the analysis, not merely compounds with a methylenedioxy grouping such as piperic acid and piperonal, but also products such as dextrose (18). Still other carbohydrates may interfere.

Just prior to completion of this work, Graham's methods (methods F, G, and H) came to the author's attention. The author is not yet able to judge their merits from his own experience. All compounds with a benzene nucleus which can be nitrated may interfere with method F. Method G seems inferior to methods B and C as unsaturated compounds, formed by concentrated sulfuric acid, may interfere. Method H, however, is very promising, is rather specific for piperine, is fast, allows the direct determination of piperine in pepper, and needs no other special apparatus than a visible range spectrophotometer. However, some questions must be elucidated.
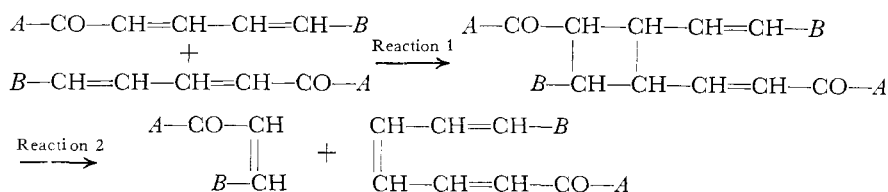
If an ultraviolet spectrophotometer is not available, only the colorimetric methods E, F, and H are acceptable alternatives for the determination of piperine. In the author's experience, method E usually gives reproducible results. According to Genest, Smith, and Chapman (7) results are high in comparison with spectrophotometric values. In applying methods D, E, F, G, and H to three samples of pepper, Graham found that the four colorimetric methods all gave the same high results in comparison with the ultraviolet method (8-70). Considering the high specificity of method H, and the low specificity of method G, this result is surprising. Graham seems to hold the

opinion that the discrepancy between the ultraviolet method and the colorimetric methods is largely due to the presence of piperettine (8, 9). The work of Genest and coworkers (7), however, shows that the "apparent percentage piperine," calculated from the absorbance at 343 mμ and without considering the absorbance at 364 mμ, includes the greater part of the piperettine. Therefore, the high results of the colorimetric methods are only to a very small extent due to the presence of piperettine. The real cause must yet be found.

A method that gives high results is not necessarily rejectable if one aims to assess the value of the product as a spice. In pepper and its derived products, compounds other than piperine may contribute to pungency. Besides the vinyl homolog piperettine (7, 19), the stereoisomer chavicine (14, 15) has been detected in pepper. Part of the results of Genest and coworkers points to the presence of methylene caffeic acid piperidide, though other results are conflicting (7).

The literature reveals many cases of dimerization of ethylenic compounds to cyclobutane derivatives and reversed depolymerization of cyclobutane compounds to ethylenic derivatives under the influence of light and heat (3, 16, 17). If piperine dimerizes under certain conditions according to Reaction 1 and then depolymerizes according to Reaction 2, the presence of methylene caffeic acid piperidide together with piperine and piperettine might be considered probable.

Thus, in principle, the presence in

methods D and H, owing to the higher specificity for piperine of the latter methods.

The purpose of this paper is to introduce a new method, the "hydrolysis method," for the determination of piperine and other bite principles of pepper and pepper oleoresins (method I). This method attacks the amidic bond of the piperidine ring, which is essential for the pungency in contrast with the methylenedioxy grouping and the aliphatic double bonds (12, 20, 21). The point of attack differs from that of all other methods. Possible interferences need not overlap those of existing procedures. For instance, piperonal, that may cause high results with both methods E and F, does not interfere. Piperic acid, which interferes with all colorimetric methods, does not influence the hydrolysis method. Thus methods D, E, F, H, and I can furnish complementary data, also in case of adulterations.

The investigation was started when the author encountered difficulties in applying method E. Some examples of these difficulties are given in Tables I and II. On heating piperine or piperine-containing products at 90° C., the author found that the piperine content, determined by method E, dropped with time (Table I). If, according to the directions of the American Spice Trade Association (2), ground pepper was extracted with alcohol on the steambath for 3 hours, a lower piperine content was found than after an extraction of 24 hours. The fineness of grinding, however, did not affect the result of the analysis (Table II).

$$A—CO—CH=CH—CH=CH—B$$
$$+ \quad \xrightarrow{\text{Reaction 1}}$$
$$B—CH=CH—CH=CH—CO—A$$

$$\begin{array}{cc} A—CO—CH \\ \xrightarrow{\text{Reaction 2}} & \| \\ B—CH \end{array} + \begin{array}{c} CH—CH=CH—B \\ \| \\ CH—CH=CH—CO—A \end{array}$$

$$A—CO—CH—CH—CH=CH—B$$
$$| \quad |$$
$$B—CH—CH—CH=CH—CO—A$$

A = piperidine nucleus
B = methylenedioxybenzene nucleus

pepper of a whole series of vinyl homologs of piperine and their stereoisomers would be imaginable. The investigations of Staudinger (20, 21) justify the supposition that these compounds, if present, contribute to the pungency of pepper. Possibly, some of these substances are determined by methods E, F, and G, but not, or not completely, by

Table I. Piperine at 90° C.

	% Piperine by Chromotropic Acid Method	
No. of Hours	1	2
0	101.3	100.8
2	98.5	99.3
4	96.8	97.4
6	95.4	96.5
24	93.2	94.0

Table II. Determination of Piperine in Ground Pepper

	% Piperine by Chromotropic Acid Method					
	3-Hour Extraction				24-Hour Extraction	
Preparation of Sample	1	2	3	4	1	2
Coarsely ground in handmill	7.4	7.3	7.5	7.3	8.1	8.1
Finely ground in electric mill	7.6	7.5	7.4	7.4	8.2	8.1
Extra finely ground in electric mill	7.5	7.6	7.4	7.2	8.0	8.0

Experimental

Apparatus. Special apparatus is not required. Flasks, condensers, and Soxhlet extraction apparatus should be provided with ground glass joints.

Materials. 2N KOH in diethylene glycol is prepared by dissolving 112 grams of KOH in 80 grams of water and diluting to 1 liter with diethylene glycol.

Boric acid, reagent grade.

Mixed indicator: 0.1 gram of methyl red, 0.5 gram of bromocresol green, and 100 ml. of ethyl alcohol (7).

Piperine, isolated from black Lampong pepper: piperine I, recrystallized five times from acetone, melting point 129.9–30.7° C.; piperine II, recrystallized four times from acetone and once from ethyl alcohol, melting point 131.0–31.6° C.; piperine III, recrystallized five times from acetone, melting point 131.2–32.7° C.; piperine IV, recrystallized four times from ethyl alcohol, melting point 130.6–32.4° C.

Oleoresin pepper I, an imitation of oleoresin pepper II, was prepared from a natural oleoresin by adding lactic acid. Oleoresin pepper II, a commercial sample, contains lactic acid. Oleoresin pepper III is a natural oleoresin from black Lampong pepper.

Procedures. Chromotropic Acid Method. The directions of the A.S.T.A. were followed (2). As a suitable piperine standard could not be found (cf. the discussion of Table IV) the author cannot guarantee the correctness of the calibration factor used for the calculation, which is 6.9% lower than that given by the A.S.T.A. Genest and coworkers also report trouble with the determination of this constant (7). Consequently more attention must be paid to the ratio of the figures than to their absolute values.

Hydrolysis Method. Determination of Bite Constituents in an Oleoresin of Pepper. Weigh in a 250-ml. round-bottomed flask well homogenized oleoresin containing about 1 gram of piperine. Add 100 ml. of 2N KOH in diethylene glycol and reflux 2 hours on a sand bath. Swirl the flask carefully from time to time. After cooling the mixture to room temperature, add 125 ml. of water from the top of the condenser and mix the contents of the flask cautiously. An adapter, which reaches below the surface of 25 ml. of saturated boric acid solution with 3 drops of the mixed indicator, is attached to the end of a descending Liebig's condenser with splash head. Distill water from the flask by way of the Liebig's condenser until the distillate is neutral to indicator paper (pH 7). If necessary, add more water to the distilling flask.

When the distillation is finished, titrate the liquid in the receiver with 0.1N HCl to the original red color.

Determination of Bite Constituents in Pepper. Extract 15 grams of ground pepper with acetone in a Soxhlet apparatus for about 16 hours, using a 500-ml. round-bottomed extraction flask. Distill the solvent over in a water bath through a descending Liebig's condenser with splash head—first at normal pressure, then at the vacuum of a Geissler pump. Do not allow the temperature to rise above 85° C. It does not matter if part of the essential oil distills over. After removal of the solvent, hydrolyze the extract which should then be treated further in the 500-ml. flask the same way as oleoresin of pepper.

Calculation. Percentage bite constituents, calculated as piperine:

$$p = \frac{2.853 \times a \times t}{g}$$

where a = ml. of 0.1N HCl
t = titer of HCl
g = mg. of oleoresin or pepper

Results and Discussion

The use of KOH in diethylene glycol was borrowed from Critchfield (4). Taking the circumstances under which the ester content of an essential oil is normally determined (2-gram sample, 1-hour reflux with 25 ml. of 0.5N alcoholic KOH), very little piperine is hydrolyzed. Even more vigorous conditions than those of Critchfield for the saponification of simple acid amides—35 meq. amide, 0.5 to 1.5 hours of reflux with 50 ml. of 1N KOH in diethylene glycol—are insufficient for total hydrolysis. The author obtained a conversion of about 80% by refluxing 8.8 meq.

of pure piperine with 25 ml. of 1N KOH in diethylene glycol for 4 hours.

Table III gives the result of a series of tests intended to establish the optimal conditions for complete hydrolysis of piperine. The tests were generally carried out according to the directions given above. Deviating conditions are mentioned in Table III. In test 11, methyl red was used as an indicator; instead of saturated boric acid solution, water was placed in the receiver in test 12.

The directions for analysis were set down on the basis of the data of Table III. The conditions are those of test 7. The mixed indicator gives somewhat better results than methyl red (test 11). The use of saturated boric acid solution is a measure of precaution to prevent loss of piperidine in the initial stage of the distillation (test 12). Table III shows that a correct relation among reaction temperature, reaction time, and excess of reagent is important.

For various products the piperine contents by both the hydrolysis and chromotropic acid methods are listed in Table IV. In the hydrolysis method, the standard directions were generally followed. In experiments 5, 8, and 11, less piperine was weighed than the directions call for; in tests 7 and 10, 120 ml. of KOH solution were used instead of 100 ml. Oleoresins I and II have a higher acid content than sample III and therefore were treated, as far as the hydrolysis method is concerned, with various proportions of oleoresin-KOH solution. The pepper of test 14 is a mixture of black Serawak and black Lampong pepper.

At present, no means are at the author's disposal to ascertain the reliability of the hydrolysis method for determining the bite constituents of pepper and oleoresins of pepper. Acid amides, ammonium compounds, and to a lesser degree proteins may cause interference. However, the author was not able to detect ammonia in the distillate after analysis of an oleoresin. Large amounts of alkali-consuming constituents interfere, and the quantity or

Table III. Piperine Content by Hydrolysis Method

Test No.	KOH Solution			Reflux Temp., ° C.	Reflux Time, Hours	% Piperine	
	Solvent	Normality	Ml.			1	2
1	DEG	1	100	158	4	96.8	96.5
2	DEG	2	100	138	4	99.4	99.2
3	DEG	3	100	132	4	94.9	94.4
4	DEG	4	100	130	4	93.0	92.6
5	DEG	2	50		4	97.6	97.8
6	DEG	2	25		4	95.9	96.6
7	DEG	2	100		2	101.2	100.4
8	DEG	4	100		2	100.3	100.4
9	EA	2	100	82	2	48.9	47.5
10	EA	2	100		4	43.6	42.8
11	DEG	2	100		2	98.8	
12	DEG	2	100		2	97.1	

DEG = diethylene glycol.
EA = ethyl alcohol.

Table IV. Piperine Content of Various Products

Test No.	Product	Hydrolysis Method Piperine, g.	KOH solution, ml.	% Piperine Hydrolysis Method 1	2	Chromotropic Acid Method 1	2
1	Piperine I	1		101.2	100.4	106.7	106.6
2	Piperine II	1		100.0	100.8	99.7	100.0
3	Piperine III	1		100.2	99.8	103.2	102.7
4	Piperine IV	1		100.2	100.1	106.1	106.1
5	Oleoresin pepper I	1	100	22.7	22.8	28.2	28.0
6	Oleoresin pepper I	4	100	21.2			
7	Oleoresin pepper I	4	120	22.7			
8	Oleoresin pepper II	1	100	21.1	20.3	29.2	28.7
9	Oleoresin pepper II	4	100	20.6			
10	Oleoresin pepper II	4	120	22.5			
11	Oleoresin pepper III	1		51.6	50.5	54.6	53.8
12	Black Lampong pepper	15		6.77	6.67	7.02	7.36
13	Black Malabar pepper	15		6.42	6.44	7.24	7.62
14	Serawak/Lampong pepper	15		6.20	6.30	7.53	7.16

Table V. Oleoresins on Dextrose Base

Test No.	Product	Hydrolysis Method Product, g.	Reflux time, hours	KOH Solution Normality	Ml.	% Piperine Hydrolysis method	Chromotropic acid method
1	Oleoresin pepper on dextrose I						18.2
2	Dextrose						18.6
3	Oleoresin pepper on salt	15	4	1	30	3.25	
4	Oleoresin pepper on dextrose II	15	4	1	30	0	
5	Oleoresin pepper on dextrose II	15	4	4	60	3.32	
6	Oleoresin pepper on dextrose II	15	4	4	100	3.42	

normality of the KOH solution may have to be varied from standard directions (Table IV, tests 5 to 10).

The four samples of piperine in Table IV have very different contents by the chromotropic acid method, whereas the hydrolysis method shows about 100% in all cases. In the author's opinion, this fact favors the hydrolysis method.

In Table IV, the colorimetric method gives much higher results than the hydrolysis method. In view of the uncertainty regarding the calibration factor, this is of minor importance. More significantly, the ratio between the results of the two methods is not constant.

Whether piperine might be directly determined by hydrolysis in oleoresins, distributed on dextrose, was examined in the experiments listed in Table V. These tests were performed before the hydrolysis method was subjected to an extensive examination, so that there are deviations from the standard directions. The oleoresins of pepper on dextrose II and on salt contain equal percentages of the same oleoresin. Table V, test 2, indicates that dextrose severely interferes with the chromotropic acid procedure. The hydrolysis method can be used for oleoresins on a dextrose base. Experiments 4, 5, and 6 demonstrate that the presence of dextrose requires a large excess of alkali.

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