Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 7, Number 2, October, 1936.

Printed in the Union of South Africa by the Government Printer, Pretoria.

Chemical Studies upon the Vermeerbos, *Geigeria Aspera*, Harv. II. Isolation of the Active Principle, "Vermeeric Acid."

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In the preceding communication (Rimington and Roets, 1936) the isolation has been described of a lactonic bitter principle, $C_{15}H_{20}O_4$, named "Geigerin" from the Vermeerbos *Geigeria aspera*, Harv. (Compositae). The present paper deals with the isolation from this plant of the active principle causing vermeersiekte, or vomiting disease, and its characterisation as a dibasic acid $C_{18}H_{28}O_7$ which readily passes over into the dilactone $C_{18}H_{24}O_5$. It is proposed to call the free acid "Vermeeric acid" and its dilactone "Vermeerin". Both substances show certain marked points of similarity with Geigerin, and it is probable that their structures are closely similar. Empirically, Vermeerin differs from Geigerin by the elements C_3H_4O but it still gives the colour test with hydrochloric acid (reddish solution with absorption bands in the visible spectrum) although with much less intensity than does the latter and it also affords on alkaline permanganate oxidation, a substance yielding acetaldehyde on treatment with cold dilute acids.

During the course of the investigation there were also isolated two substances which appear to be flavones and which were only separated from Vermeeric acid with difficulty. They are not considered to be in any way related to the toxic principle.

Pharmacological trials have shown that rabbits are entirely unsuited for the testing of vermeersiekte activity. Frogs, on the other hand, would appear to offer considerable possibilities, especially since the doses required to cause paralysis and death are of the order of a few milligrams only. Quantities of 10 to 15 gm. of vermeeric acid given orally to sheep cause death from acute vermeersiekte within 5 to 24 hours. The pharmacological examination of this substance is reserved for a future publication.

Preliminary Tests.

Quite early in these experiments, when attention was being directed towards the isolation of the lactone Geigerin, it was noticed that the final fractions contained a considerable quantity of an

acidic oil with a pleasantly aromatic odour. Dosing of this material or the ether extract of 80 gm. of dried plant to rabbits was without effect although frogs which received about 50 miligrams injected into the dorsal lymph sac suffered from paralysis and respiratory distress, death usually occurring fom asphyxia within an hour. The neutral fractions of the extracts were almost devoid of activity.

Experiments upon sheep showed that the active principle could be extracted by boiling ether and transferred, by shaking, to dilute aqueous sodium carbonate solution, thus: 1·5 kilos of dried plant (Vereeniging Estates, 1st Lot; effective dose for sheep ±750 gm.) were extracted by ether and the extract shaken with 1 per cent. sodium carbonate solution. The ether soluble residue was dosed with negative result. The clear, brown aqueous liquid was then acidified and shaken with chloroform which removed a pale yellow acidic oil. This was given to a sheep per os with the following result. Dosed 3 p.m.:—

Next morning,	9 a.m.	Listless, not eating; temperature 101°.
	11.30 a.m.	Walking with a stiff gait; temperature 103°.
	2 p.m.	Prostrate; forced respiration. Pulse 160, hard and jerky. Some signs of nervous hypersensibility.
	2.15 p.m.	Died.

Post mortem.—Gastro-intestinal inflammation, ulcers in the omasum associated with particles of a brown material. Heart flabby with haemorragic patches covering the left auricle. Liver friable, and somewhat fatty with central stasis. Kidney medulla slightly engorged. Some fresh ingesta found in the upper portion of the trachea.

In a repetition of this experiment, the sodium carbonate solution was shaken repeatedly with fresh chloroform in order to remove completely all non-acidic material since Geigerin is appreciably soluble in aqueous solutions. There was recovered in this way 6·2 gm. of Geigerin crystals, but the residual acid fraction weighing 15·5 gm. was still very toxic, the test sheep dying within 5·5 hours after dosing. A small chloroform-insoluble, dark, amorphous material which separated when the carbonate solution was acidified was dosed separately but proved to be inactive.

Various methods of purification of the active fraction were resorted to, such as high vacuum distillation, but a certain degree of lability of the principle, especially at high temperatures, led to rapid loss of toxicity.

At this stage, it was noticed that as the solvent evaporated, chloroform solutions of the acidic oil deposited a small quantity of a yellow material which was practically insoluble in chloroform when

the mixture was stirred again with fresh solvent. In this way a further purification was achieved and on an accumulated sample of the active chloroform-soluble material certain tests were carried out as follows:—

Toxicity.—10 gm. dosed to a sheep caused death from typical acute vermeersiekte within 24 hours, the onset of symptoms being noticed about 8 hours after dosing.

7 gm. caused symptoms of stiffness and vomiting but the animal made a gradual recovery.

HCl Colour Reaction.—With concentrated hydrochloric acid a brownish-red colour developed slowly but more rapidly on warming and the solution exhibited absorption bands at approximately 542, 502 and 465 mμ. The bands were less distinct and the colour more brownish than is the case with Geigerin.

Optical Rotation.—A 0·52 per cent. solution in alcohol was optically inactive (2 dm. tube).

Equivalent by Titration.—0·3564 gm. of the oil dissolved in alcohol and titrated to phenol phthalein required 12·23 c.c. of 0·1 N sodium hydroxide. The sodium salt dissolved in alcohol and precipitated by ether was analysed with the following result:—

Micro-analysis.—

	Na
Crude Na salt of toxic acid.	Found 10·59

It can be mentioned that the calculated value required by the formula $C_{18}H_{26}O_7Na_2$ (based on results to be described later) is 11·50.

Separation of Two Flavones.

A modification introduced into the procedure for the preparation of the toxic fraction resulted in a very much more complete and easy separation of the yellow material referred to above.

An ether extract of 1·5 kilos of plant powder was treated in the usual way, but in order to prevent emulsification and accelerate the separation of the phases when the sodium carbonate solution was shaken with chloroform, an addition of about 50 gm. of sodium chloride was made. This allowed of much quicker working and of more perfect elimination of colouring matter, etc. The final chloroform solutions deposited about 80 mgm. of yellow crystalline material from which the toxic oil was easily separated by washing with chloroform. The yellow residue was dissolved in hot absolute alcohol and the solution, to which a drop of hydrochloric acid was added, was placed in the ice-chest. A crop of pale yellow crystals separated and on dilution of the mother liquors further material was obtained. By repeated recrystallisation, two pure fractions were eventually obtained, in greatest quantity a substance "A" crystallising in diamond-shaped pale yellow plates with M.P. 269-271° (Fig. 1) and a small amount of a substance "B", somewhat paler in colour and crystallising in square plates with slightly rounded corners,

M.P. 243-4° (Fig. 2). The material A exhibited in a very striking manner the modification of crystalline form caused by traces of impurities. Thus, during the progress of purification, all stages were observed between somatoids, flat boat-shaped crystals, aggregations of plates tapering to a point at the end (see Fig. 3) and the perfectly-formed diamond-shaped crystals of the pure substance.

On account of the properties of these two substances, listed below, it is suspected that they may be flavones.

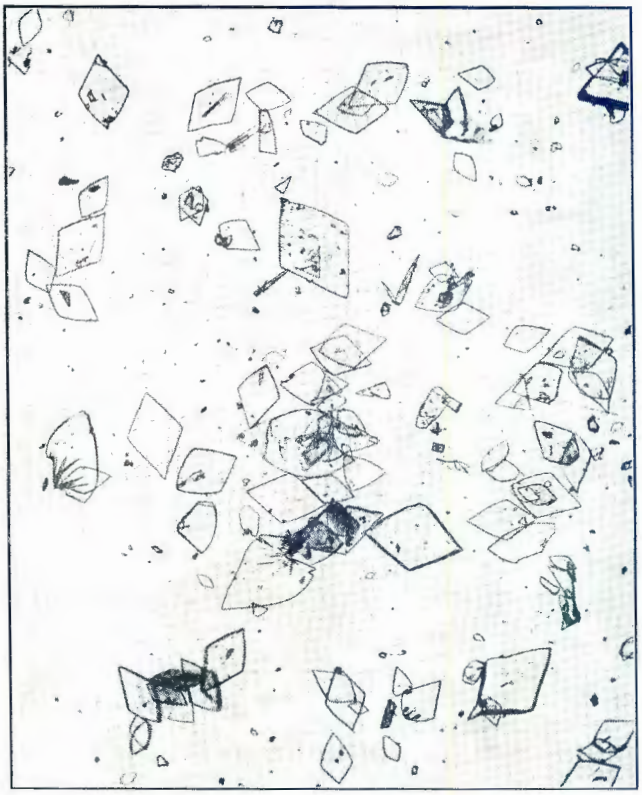

Fig. 1. Substance " A " from *Geigeria aspera*, M.P. 269-71°, ×130.

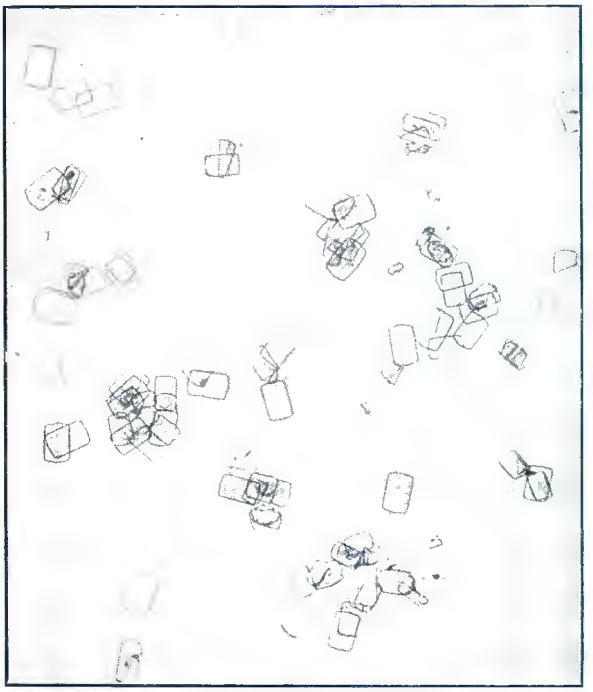

Fig. 2. Substance "B" from *Geigeria aspera*, M.P. 243-4°, ×175.

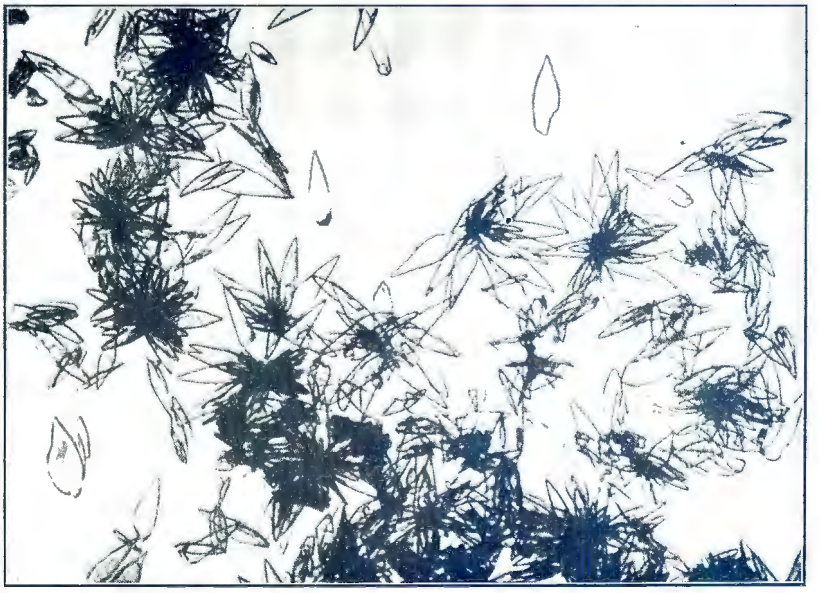

Fig. 3. Somatoids of substance "A" from *Geigeria aspera*, ×85.

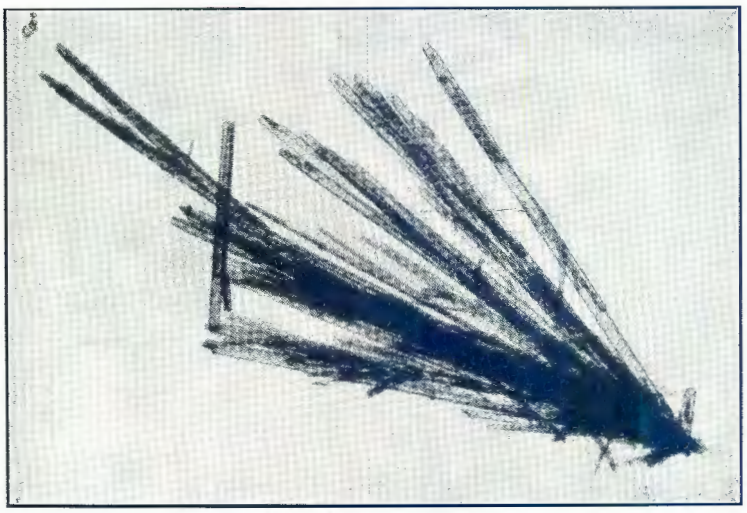

Fig. 4. Crystals from solution of substance " A " in concentrated hydrochloric acid, ×80.

Properties of Substances A and B.

A.	B.
Insoluble in water.	Insoluble in water.
Insoluble in chloroform (when pure).	Insoluble in chloroform (when pure).
Sparingly soluble in hot alcohol.	Sparingly soluble in hot alcohol.
Easily soluble in concentrated HCl, solution deposits long, bright-yellow needles (Fig. 4).	Easily soluble in concentrated HCl
Very soluble in dilute Na_2CO_3 solution with bright yellow colour.	Very soluble in dilute Na_2CO_3 solution with bright yellow colour.
Ferric chloride coloured olive green.	Ferric chloride coloured dark olive green.
Reactions for ketonic groups negative.	Reactions for ketonic groups negative.
Fehling's solution turned dirty green on boiling.	Fehling's solution turned dirty green on boiling.
Pale yellow diamond-shaped plates M.P. 269–71°.	Pale, square plates M.P. 243–4°.

Analyses indicated the probable formulae $C_{19}H_{18}O_8$ and $C_{19}H_{18}O_7$ for A and B respectively. The materials were insoluble in camphor.

*Micro-analysis.**

	C.	H.	CH_3O.
Substance A. Found.....	61·02	4·51	15·36
$C_{19}H_{18}O_8$ requires.....	60·92	4·86	15·42 (for $2CH_3O$).
Substance B. Found.....	63·58	5·16	—
$C_{19}H_{18}O_7$ requires.....	63·68	5·03	—

* Micro-analyses by Dr. O. Backeberg, University of the Witwatersrand, to whom we wish to express our thanks.

The quantity of B was insufficient to permit of methoxyl determination.

These two materials could also be obtained by dissolving the crude oil in 50 per cent. by volume hydrochloric acid, and setting the solution aside when the yellow compounds slowly crystallised out.

It may be recalled that W. Karrer (1934) recently reported the isolation of a flavone Thapsin from the drug *Digitalis Thapsi*. It had M.P. 224° (uncorr.) and was assigned the formula $C_{15}H_6O_4(OCH_3)_4$ and shown to possess 4 methoxyl and 2 hydroxyl groups. As soon as sufficient material has been accumulated, a further study of these substances present in *Geigeria aspera* will be undertaken.

Final Purification of the Toxic Fraction.

A phenomenon which proved to be of importance in the final purification of the active material was observed at this stage. Various analyses of final toxic fractions had shown some diversity and it was also observed that preparations which were at first quite fluid gradually lost their mobility on standing in open dishes and that such " old " samples were no longer completely soluble in sodium carbonate solution. Traces of the " A " and " B " materials were also difficult to remove and imparted a dark yellow-brown colour to the preparations.

By dissolving such imperfectly purified material in chloroform and adding, with vigorous stirring, three volumes of petroleum ether, the bulk of the impurities could be removed in the dark resinous precipitate which formed. By a sufficient repetition of the process, preparations of the toxic material could be obtained which no longer gave any colour with ferric chloride solution and were therefore free from the A and B constituents. The increase in viscosity on standing for any considerable period of time still occurred, however, and on occasion the formation was observed of a colourless crystalline substance separating slowly in the bulk of the material (see Fig. 5). The crystals showed marked twinning.

Fig. 5. Crystals of Vermeerin forming in toxic oil (Vermeeric acid) on standing, ×40.

By titurating such preparations with anhydrous ether, added in successive quantities, it was found possible to separate the active acidic oil from the crystals. Where crystallisation had not commenced spontaneously, it could be induced by stirring the oil with dry ether. The crystalline constituent proved to be a neutral principle insoluble in sodium carbonate solution. It could be easily recrystallised from chloroform-petroleum ether mixture and was so obtained in colourless needle-like prisms with M.P. 143° (Fig. 6).

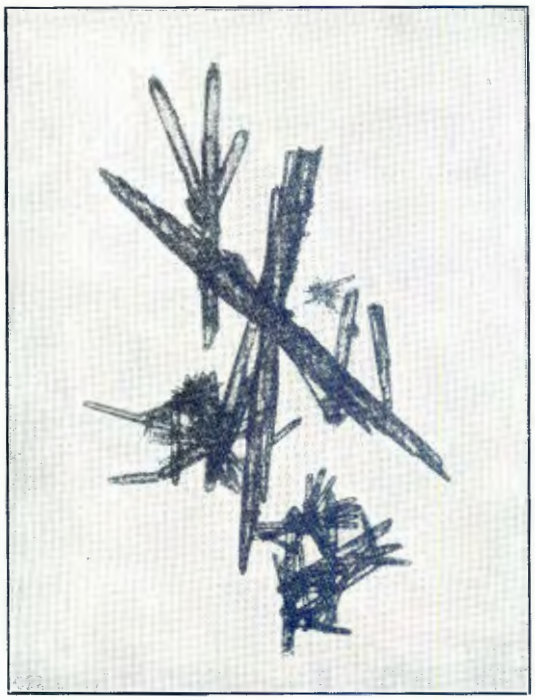

Fig. 6. Vermeerin, the di-lactone of Vermeeric acid, M.P. 143°, ×85.

It was proved that samples of the purified active oil, which originally had been freely and completely soluble in aqueous sodium carbonate solution, slowly but progressively gave rise to this material on standing. Analyses demonstrated that it was formed by spontaneous lactonisation (see below). For analytical purposes, portions of the active oil were dissolved in chloroform and their solutions shaken with aqueous sodium carbonate. The alkaline phase was then repeatedly shaken with fresh portions of chloroform to remove any non-acidic material and the acid transferred to a small volume of chloroform after acidification of the carbonate solution. From this solvent it was precipitated by addition of much anhydrous petroleum ether, centrifuged down, washed with petrol ether and subjected to

a short drying (1 hour) in a vacuum desiccator over sulphuric acid. Analyses were made with as little delay as possible and afforded consistent results agreeing with the formula $C_{18}H_{28}O_7$.

Micro-analysis:

	C.	H.
Preparation 1. Found.....	60·96	7·96
Preparation 2. Found.....	60·90	7·19
$C_{18}H_{28}O_7$ requires.....	60·68	7·87

Preparations 1 and 2 were made from different batches of *Geigeria aspera* collected at an interval of about six months. Confirmation of this empirical formula was afforded by analyses of the dilactone as recorded below.

In view of the pharmacological action of this acidic material in causing vermeersiekte or vomiting disease it is proposed to designate it " Vermeeric acid ".

The yield from the batch of material investigated (effective dose for a sheep approximately 2·5 kilos) was 0·5 per cent. The yield of flavones (A and B) was 0·01 per cent.

Properties of Vermeeric Acid.

The material obtained by the process outlined above was a slightly viscous, very pale yellow oil with a pleasantly aromatic smell and devoid of optical activity. It gave the colour reaction with hydrochloric acid which has been previously described and reduced alkaline potassium-permanganate solution in the cold. Vermeeric acid is a dibasic acid as the analysis of its sodium salt and titration experiments demonstrated. It forms a 2:4 dinitrophenylhydrazone and must therefore contain the function $>CO$ or a grouping capable of giving rise to a ketonic group such as an oxygen atom showing keto-enol tautomerism. The reasons for suspecting the presence of such a grouping in Geigerin were fully discussed in the preceding paper.

An amorphous methyl ester was prepared by means of dimethyl-sulphate. The preparation contained 0·72 per cent. ash.

Micro-analysis:

	C.	H.
Found.....	64·12	7·51
$C_{18}H_{24}O_3(CH_3O)_4$ requires.....	64·03	8·73

Vermeerin, the Di-lactone of Vermeeric Acid.

The crystalline material, M.P. 143°, formed from Vermeeric acid on standing (see Figs. 5 and 6) possessed the formula $C_{18}H_{24}O_5$ and was found to neutralise two equivalents of alkali after opening of the lactone rings. It is laevorotatory but the free acid, as was to be expected, proved to be completely inactive. Tests with Brady's reagent revealed the fact that only a very slight precipitate is formed on adding the reagent and this not immediately. From such behaviour

it would appear highly probable that the closure of one or other lactone ring involves the structure in Vermeeric acid which, as postulated, is capable of giving rise to a ketonic function in the presence of hydrochloric acid and a ketone reagent. Optical activity must be associated in some way with the closure of the lactone rings.

Micro-analysis:

	C.	H.
Found.....	67·77	7·45
$C_{18}H_{24}O_5$ requires.....	67·47	7·56

50·5 mgm. of the substance dissolved in 15 c.c. of absolute alcohol had a rotation of −0·34°.

$$\therefore \quad [\alpha]_D^{28} = \frac{-0{\cdot}34 \times 15 \times 100}{2 \times 5{\cdot}05}$$
$$= -50{\cdot}51°.$$

To this solution was added 5 c.c. of alcoholic potassium hydroxide (approximately 0·08 N solution) and the mixture left at 37° overnight. It was then back titrated after the addition of phenolphthalein.

5 c.c. KOH = 3·42 c.c. of 0·1086 N HCl
Acid back = 0·35 c.c.
∴ Neutralised 3·07 c.c.
= 3·33 c.c. of 0·1 N
∴ Equivalent weight = 151·7

The empirical formula $C_{18}H_{24}O_5$ for 2 lactone groups requires 160.

To the residual solution, the full equivalent of acid was added and the mixture examined in the polarimeter. It proved to be completely inactive.

Like Geigerin and Vermeeric acid, Vermeerin gives the colour test with hydrochloric acid, in which it is fairly readily soluble. The colour was much less intense and somewhat browner than that given by Geigerin but a distinct absorption band was visible with its centre at 546·5 mμ. Two other bands were faintly discernible as in the case of Geigerin and Vermeeric acid. On dilution of the acid, the bulk of the dissolved substance separated in pure crystalline form M.P. 143°. This might indicate that the action of hydrochloric acid, presumably causing some isomeric rearrangement within the molecule is much less marked upon Vermeerin than it is with the other two compounds, a difference possibly to be correlated with the absence of a ketonic functioning group (compare the formulae on p. 519).

During the course of the separation of crystalline Vermeerin from active fractions which had stood some length of time, a certain quantity of another substance was encountered. This was an

amorphous solid, devoid of acidic properties, but neutralising alcoholic potash on standing at 37° for 24 hours. 48·8 mgm. left in 5 c.c. alc. KOH (=3·90 c.c. HCl) required for back titration 1·85 c.c. 0·09709 N HCl.

Neutralised 1·80 c.c. N/10.

∴ Equivalent = 271·6.

The resultant solution was optically inactive.

Micro-analysis:

	C.	H.	M.Wt.	
Found....	61·56	7·89	1028	(by Rast's method).
$C_{54}H_{82}O_{20}$ requires...	61·67	7·87	1050·7	

This substance is characterised by its very sparing solubility in all organic solvents. It has not yet been closely studied but it may be here pointed out that the molecular weight is 4 times the equivalent weight, indicating that there are in all probability 4 lactone groupings in the molecule. The molecular formula $C_{54}H_{82}O_{20}$ corresponds to 3 times the molecular formula of Vermeeric acid less 1 molecule of water thus, $3 \times C_{18}H_{28}O_7 - H_2O = C_{54}H_{82}O_{20}$. Vermeeric acid contains two carboxyl groups per molecule and it may be suggested that possibly some sort of polymerisation involving anhydride formation may have taken place.

Vermeeric Acid 2:4 Dinitrophenylhydrazone.

This derivative, which established the presence in the molecule of Vermeeric acid of a ketonic group (or formation of such a group in the presence of acid) was prepared in the following manner:—

About 50 mgm. of Vermeeric acid was dissolved in aqueous alcohol of strength just sufficient to afford a clear solution and after addition of sufficient hydrochloric acid to bring to 2 N, the requisite quantity of hot Brady's reagent was added. The separation of an orange-red dinitrophenylhydrazone commenced rapidly but the precipitate was apt to be sticky. It was washed well in the usual way and dissolved in 1 per cent. aqueous sodium carbonate solution. To the filtered, dark reddish-brown solution was added an excess of hydrochloric acid and the precipitated derivative was centrifuged down and washed. It crystallised from hot dilute alcohol in reddish-orange prisms with M.P. 108–110°.

Micro-analysis:

		C.	H.	N.	
Found.	Preparation 1...	54·80	5·94	11·42	(trace of ash).
,,	,, 2...	54·96	5·71	12·52	
$C_{24}H_{34}O_{11}N_4$	requires.....	54·75	5·73	12·17	

The analysis of this derivative occasioned some surprise. The formula expected from the interaction of 1 molecule of Vermeeric acid with 1 molecule of dinitrophenylhydrazine with the elimination of 1 molecule of water would be $C_{24}H_{36}O_{12}N_4$ whereas the substance actually formed appears to contain H_2O less. Considering the ease with which

Vermeeric acid lactonises on standing, it seemed highly probable that the extra molecule of water eliminated owed its origin to closure of one of the lactone rings. That this was indeed the case was proved by microtitration of a specimen of the 2:4 dinitrophenylhydrazone following the method of Clift and Cook (1932).

7·8 mgm. dissolved in 5 c.c. of N/100 sodium hydroxide required for back titration 3·60 c.c. of N/100 hydrochloric acid,

$\therefore$ alkali neutralised = 1·40 c.c.

Assuming the presence of 1 carboxyl group, $C_{24}H_{34}O_{11}N_4$ requires ... ... 1·39 c.c.

As already pointed out, there is considerable experimental support for considering the ketonic function in Vermeeric acid to arise through keto-enol tautomerism involving an oxygen atom which in its enol form is capable of lactonising with a carboxyl group. It is thus understandable that during the preparation of the 2:4 dinitrophenylhydrazone of Vermeeric acid, only one lactone ring closes whilst one oxygen atom reacts to form the desired derivative. The 2:4 dinitrophenylhydrazone is readily soluble in sodium carbonate solution, which demonstrates the presence of an acidic function in the molecule.

Oxidative Degradation of Vermeeric Acid.

0·5 gm. of the toxic acidic oil was dissolved in 5 per cent. sodium carbonate solution, and to this solution, whilst boiling, was added gradually 40 c.c. of 0·25 N potassium permanganate. On filtration and distillation of the alkaline reaction mixture, a distillate was obtained which gave only the faintest trace of turbidity with Brady's reagent.

The residual alkaline solution was then extracted thoroughly with ether which removed a substance remaining behind as a colourless oil when the solvent evaporated. This material afforded an immediate precipitate of a 2:4 dinitrophenylhydrazone with Brady's reagent. The derivative was washed well and crystallised from hot dilute alcohol in yellow needles, M.P. 160°. It proved to be identical with acetaldehyde 2:4 dinitrophenylhydrazone.

Micro-analysis:

	C.	H.	N.
Found.....	42·99	3·73	24·40
$C_8H_8O_4N_4$ requires.....	42·86	3·60	25·00

Mixed with authentic acetaldehyde 2:4 dinitrophenylhydrazone of M.P. 159–161° it melted without depression at 159–161°. The yield was 0·23 gm.

After making acid by ether, the residual main solution was again extracted and yielded to ether a small quantity of an acid which was eventually obtained from acetone in aggregates of needles, M.P. 229–30° but the quantity was insufficient for analysis.

These results are purely preliminary but serve to demonstrate the close similarity between Vermeeric acid and Geigerin. Further work upon the constitution of these substances will be undertaken at a later date.

Summarising the chemical information so far obtained, one may represent the three substances Geigerin, Vermeeric acid and Vermeerin as follows, an indication being given of the possible involvement of one atom of oxygen in a keto-enol tautomerism.

Geigerin, $C_{15}H_{20}O_4$.

$$C_{14}H_{20}O \begin{cases} -CO \\ | \\ -O \\ \\ =O \end{cases} \quad \text{or} \quad C_{14}H_{19}O \begin{cases} -CO \\ | \\ -O \\ \\ -OH \rightleftarrows = O + H \text{ to ring.} \end{cases}$$

Vermeeric acid, $C_{18}H_{28}O_7$.

$$C_{16}H_{24} \begin{cases} -COOH \\ -COOH \\ -OH \\ -OH \\ =O \end{cases} \quad \text{or} \quad C_{16}H_{24}O \begin{cases} -COOH \\ -COOH \\ -OH \\ -OH \rightleftarrows = O \times H \text{ to ring.} \end{cases}$$

Vermeerin, $C_{18}H_{24}O_5$.

$$C_{16}H_{24}O \begin{cases} --CO \\ \quad | \\ --O \\ \\ --CO \\ \quad | \\ --O \end{cases}$$

Summary.

1. The toxic principle of the Vermeerbos, *Geigeria aspera*, has been isolated. It is a dibasic acid $C_{18}H_{28}O_7$ and has been named "Vermeeric acid". On standing in the air, Vermeeric acid gradually loses two molecules of water forming the crystalline dilactone "Vermeerin" $C_{18}H_{24}O_5$.

2. Vermeerin has M.P. 143° and $\left[\alpha\right]_D^{28} = -50{\cdot}51°$ but Vermeeric acid is optically inactive.

3. Both substances, like Geigerin, give a colour reaction with hydrochloric acid but the colour is browner, and in the case of Vermeerin it is of very slight intensity. An absorption band at 546·5 mμ could be distinguished and two other bands were faintly discernible.

4. Vermeeric acid forms a 2:4 dinitrophenylhydrazone soluble in sodium carbonate and reprecipitated by acids. This derivative appears to contain H_2O less than that expected upon the assumption of a simple reaction and it is thought probable that closure of one lactone ring simultaneously occurs. Vermeerin when treated with hot Brady's reagent reacts to such a very slight extent that the absence of any ketonic or keto-enolic function is infered in the undecomposed substance.

5. Vermeeric acid decolorises potassium permanganate in the cold. By oxidation with this reagent in alkaline solution at the boiling temperature there was obtained an acid crystallising in small prismatic needles M.P. 229–30° and a substance which when treated with cold dilute acid immediately liberated acetaldehyde, the 2:4 dinitrophenylhydrazone of which was prepared for identification.

6. Accompanying Vermeeric acid in the plant were found two flavone-like substances, the one with M.P. 269–71° being, in all probability $C_{17}H_{12}O_6(CH_3O)_2$, the other, M.P. 243–4°, having the formula $C_{19}H_{18}O_7$ (methoxyl not determined).

7. Vermeeric acid drenched to sheep in doses of 10 to 15 gm. causes death from acute vermeersiekte within 6 to 48 hours.

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