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Some Physiological Aspects of the Genus Tribulus.

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PREFACE.

IN previous communications by Rimington, Quin and Roets (*Onderstepoort Jnl. Vet. Sc. & Animal Indust.* Vol. 3, No.1, pp. 137-157—1934, and Vol. 4, No. 2, pp. 463-478—1935) it was shown that in the pathogenesis of Geeldikkop as caused by species of Tribulus and other similarly acting plants, the elaboration of an icterogenic principle within such plants should be considered as the primary cause of the condition. When ingested this factor then provoked stasis on parts of the digestive system as well as a severe obstruction in the bile excretory mechanism of the liver which in turn led to the characteristic intense jaundice of the whole body.

The other characteristic feature of the disease, viz., the acute sensitivity to sunlight as shown by marked swelling and later necrosis of all unpigmented and unprotected skin, was shown to be due to the plant porphyrin phylloerythrin which originated as a normal disintegration product of ingested chlorophyll mainly in the forestomachs of ruminants. Traces of this highly fluorescent pigment are normally absorbed from the small intestine only to be re-excreted in the bile thus keeping it strictly within entero-hepatic circulation and to pass out ultimately in the faeces. When, however, obstruction to the bile flow occurs, this pigment with its strongly photodynamic action also finds its way into the systemic circulation and thus also into the cutaneous vessels where in the presence of direct sunlight it is responsible for the acute oedematous swelling usually noted on the facial skin and ears of merino sheep.

Seeing, therefore, that Tribulus plants are on the whole rich in chlorophyll, carotinoids and other related pigments and seeing also that some of these pigments and their breakdown products enter into the aetiology of the symptom complex of geeldikkop, a systematic study was undertaken on the pigment metabolism of Tribulus collected under various climatic conditions. This article is the outcome of this study, treating as it does only with this one aspect of the problem. The other aspect, viz., the elucidation of the primary icterogenic factor is being investigated along other lines and consequently will be reported on separately.

Introduction.

For the farmer in the Karroo and the karroid areas the genus Tribulus is of great interest, as it includes plants which are at times excellent fodder plants and at other times suddenly prove fatal to sheep causing the dreaded " dikkop ". It is up to now not all clear whether the causing of " dikkop " is confined only to certain species or, what is more likely, whether all the species may under particular edaphic and meteorological conditions produce dikkop. It is unfortunate that the systematics of the genus is not properly worked out, thus it is often impossible to say which particular species is actually found in the veld and whether other plants which have decidedly another habit of growth are different species or only a physiological variety. According to the literature (Theiler 1918, Quin and Rimington 1933, 1934, 1935) the view is more wide spread that it is a question of physiological state, and not of different systematic species. The author having observed the growth of Tribulus on different veld (alluvial soil, farm yards, limestone, dolerite brown soil and sandveld) was at first rather inclined to think of different systematic species, with their respective hybrids, especially *Tribulus terrestris* and *T. parvispinus*. Since Schweikerdt (1937) most recently puts these two species together again, and only accepts *terrestris*, the view had to be abandoned and in the present paper the different varieties will be called physiological strains. Cultivation experiments which are in progress on the Veld Reserve at Fauresmith ought to bring about a final conclusion.

When Dr. Quin and Dr. Rimington asked the author to collaborate in the Tribulus question not much was known about the actual poisonous principle and every possible poison source in the plant had to be considered. As the Fauresmith district is about on the northern boundary of the area of dikkop outbreaks, outbreaks did not occur regularly every year, and poisonous material could only be collected sporadically and tested for different possible constituents which might have been the cause of its toxicity. In the course of the years more definite views were expressed as to the possible poisonous qualities (Rimington and Quin 1934, 1935). It is clear that with the progress of the work of the Onderstepoort workers the questions put to the botanist changed as well. Thus the results put forward in the Tribulus question are really the product of different views held by these workers. Right from the start it was mentioned (Rimington 1933, 1934) that phylloerythrin played a rôle in dikkop. This led to the investigation of the green plant pigments. The carotinoids were as well investigated, as it was seen that material collected in the veld during the occurrence of dikkop contained an unknown yellow pigment. It was thought that it was not impossible that the breakdown products of the normal carotinoids were in some way connected with the poisonous principle of the Tribulus (Polyterpenes-diterpenes). The question of physiological variety was tackled in culture experiments. An investigation of the anatomy of the plant was also done, as well as some microchemical reactions. All these questions will be dealt with in separate paragraphs.

The Anatomy of Tribulus.

The leaves of Tribulus do not offer any definite peculiarity. In transverse section the following is seen: the epidermis is slightly cuticularized and has stomata on both sides. The stomata on the upper side are slightly elevated. The leaves are covered with trichomes inserted into a pedestal. Below the upper epidermis is a tight layer of long thin palisade cells (1 : 10) which reach to about the middle of the leaf, in the middle are found very large sheaths for the bundle and large cells with calcium oxalate. The cells of the sheaths are exceptionally large. Cutting transversely, bundles may be cut transversely or parallel to the vessels; in both cases the sheath of the bundle is prominent. Below the bundle another layer of palisade cells is found, slightly shorter than those of the top layer, only occasional cells of spongy parenchyma are seen below the lower epidermis.

There are in the middle of the leaf a good few cells of calcium oxalate, but to the author's mind not enough to correspond to the large amount of $(COO)_2$ found in the macroanalysis (see page 384). There must be still some more acid potassium or sodium oxalate in the cells.

Considering the recently advanced hypothesis of the presence of resinic acid in the poisonous Tribulus, it is worth while mentioning the entire absence of any chiliferous vessels containing resin or lysigenous or schizogenous ducts in the leaves, stems and roots If any resinous product is found it must be in the plasma of ordinary cells. On the material collected in 1937 no glandular hairs could be detected, but only trichomes. The leaves did not contain any idioplastes with oily or fatty contents, only the upper epidermis containing small amounts of anthocyan, tinging it purple. Neither the stems nor the roots of Tribulus show any ducts or idioplastes, or cells with oxalic acid. The cortex tissue of the roots has a tendency to work loose from the xylem in a similar way to that described for grasses (Henrici 1929), only no actually dissolved tissue could be found.

Systematics of Tribulus.

After the species of Tribulus found around Fauresmith having for some years been identified as four to five different species (*murex*, *terrestris*, *parvispinus*, *zeyheri* or *cystoides*) today Schweikerdt accepts only the two species *terrestris* and *zeyheri* for the Fauresmith district. Since that time *Tribulus pterophorus* has been found in the district on the banks of the Orange River at Zoutpansdrift. The species *terrestris* is characterised (Schweikerdt, p. 161) by free intrastaminal glands, which are never connate to form a shallow cup; hemispherical stigma which are never slender; petals 2-12 mm. long and sepals 2-6 mm. long. All the other species have intrastaminal glands connate to form a shallow cup at the base of the ovary; stigma slender, usually pyramidal. *Terrestris* seems to be a most plastic species: striking details seen in its growth by the author will be given under Morphology, but for the complete description Schweickerdt's paper (p. 172) should be consulted.

Morphology of Tribulus.

The morphology of Tribulus will be touched only in so far as it seems necessary to elucidate the physiological growth varieties as encountered in the veld. The most striking feature of the three species under observation is no doubt the very small amount of root material in comparison with the corresponding aerial parts. In material collected on the farm Poortjie in the Fauresmith district 30 lbs. of fresh *Tribulus parvispinus* yielded only 1¼ lbs. of roots, for *Tribulus terrestris* on the Reserve itself the ratio was still worse. The plant for morphological reasons is bound to wilt as soon as the rain stops for a few days. And yet Tribulus has two typical forms which differ so much in growth habit that one is inclined to look on them as different species. The one form, so to say, which is encountered always in the Railway enclosure near the Reserve and in rainy weather on the Reserve itself and in the veld is a spreading, many-branched prostrate form. The branches may be 2 metres long, and are usually branched again. The flowers are of medium size, the sepals generally shorter than the petals. This type generally has two different kinds of fruits, typical " dubbeltjies " breaking up into five cocci lengthwise, the cocci having each 4-6 spines, though sometimes only two, and in the middle a crest of bristles and tubercles. Very often not all the five cocci are properly developed, the fourth and fifth having no spines whatever, or the fifth being suppressed entirely, so that the ripe fruit actually consists of only three cocci with spines, and a small fourth without spines. As a matter of fact the author saw more plants with three or four cocci than with five. The whole plant is generally sparingly hairy, the hairs are coarse, sharp and have partly a pedestal. If this plant wilts the secondary branches turn up vertically, as well as the pinnate leaves, which fold up. Another very small type of fruit is at times found on these plants, consisting of 3-4 cocci, practically covered all over with sharp hairs but not with proper spines.

The other type of Tribulus found everywhere in the veld of the Fauresmith area, on limestone or on alluvial or dolerite soil, is a very small plant, generally growing erect or with branches only about six inches long. On limestone especially the plant is absolutely covered with silky hairs. The flowers are generally small, the sepals being as long as the corolla. Very occasionally this type is also found with a medium-sized flower. Leaves and the few secondary branches always stand vertical as soon as it is dry. The completely upright form has never been seen with spread leaves. When dikkop has been reported in the surroundings of Fauresmith, or when plants said to cause dikkop have been sent from the Cape to Fauresmith, this type of plant has always been found. The type, however, is not necessarily poisonous, in drought years it grows in the plots of the Veld Reserve. The cocci of the fruit have sometimes only two spines. Fruit on the whole are not found regularly on this type.

It is quite obvious that in South West Africa or in the other natural habitats of the Tribulus with large flowers these species may prove fatal. But in the south-western Free State they do not occur to such an extent as to be an economic problem, except perhaps *T. pterophorus* on the banks of the Orange River.

Seeds are collected from all possible sources and sown on the Reserve. In the first generation only plants with long stems were obtained, but nearly all had the two kinds of fruit, the large fruit and the small one. As mentioned before on the Reserve generally the two forms are found. They are never poisonous. In 1932 the upright form grew in the worst drought. An uninitiated person could not discover the plants on the soil, but three sheep lived on this plot for six weeks and gained on the average 14 lbs. liveweight in this period.

Just as geel dikkop itself is only confined to a definite area of South Africa (between Victoria West and the north of Fauresmith), although the genus is widely spread, in the area itself the poisoning is only observed on a quite definite habitat. In the Fauresmith district there are only about five farms which are notorious for their regular heavy outbreaks of dikkop. On other farms there may be occasional cases, but never heavy outbreaks. Time and again it has been pointed out that geel dikkop can occur on such different soils, under different conditions. In the Fauresmith district the habitat of poisonous Tribulus seems to be restricted to two soils, limestone formations and sandy (often alluvial) red soil with low water holding capacity, often encountered on dry river banks. The low water holding capacity seems at first sight the only common feature of the two soils. For years on the limestone formation at Poortje, only small forms with short stems and small flowers or the upright form was found. Only in the very wet February 1937 the spreading form with long procumbent branches was recorded. The form on limestone is covered with silvery hairs. The stems are very red.

In going through the masses of Tribulus collected during the last five years, and trying to have it identified, one cannot help thinking it to be a thankless job. Working in the veld and observing the growing habits, the impression is surely obtained that we are not working with well defined species, but with hybrids. The eternal changing of the size of the petals compared with the calyx and the variable fruit and the peculiar two fruit types give enough food for thought in this direction.

Content of Chlorophyll and Derivatives of Chlorophyll in Tribulus.

1. *Method.*

At the same time when Rimington and Quin (1934) found that phylloerythrin was the cause of photosensitisation in sheep it was obviously the thing to look for this pigment in Tribulus. An investigation of the leaf pigments, green and yellow, of Tribulus was undertaken. It was also thought that in case the systematics of the genus should prove inadequate, it would be possible to isolate physiological varieties. In working on the chlorophyll of Tribulus collected in different localities, using the classical method of Willstaetter and Stoll for fresh plants (1913, p. 138 ff.) it soon appeared that large differences existed between plants from different localities, but that a great similarity occurred in the amount of chlorophyll in plants which had similar growth habits and grew on similar soil.

An annual plant such as Tribulus which finishes its growth and reproduction within a few weeks to three months, can be expected to have large changes in its chlorophyll content during the season. To study these changes regular samples of two Tribulus varieties were taken, the one of a very luxuriant strain growing in the railway enclosure below the Reserve, which always had long procumbent stems and belonged to *T. terrestris*. The other strain was collected on the Reserve itself, it was also a procumbent plant, but never so juicy as the first strain. Then in and out of the Fauresmith districts where outbreaks occurred within the reach of an officer connected with the Tribulus investigations, as many samples as possible were collected and analysed, careful notes being taken of the growth habit, water content and if possible of fruit and flower of the particular strain. A few samples from Victoria West had to be analysed in the dry state, as they arrived after a long journey. The standard used for the work was obtained by the courtesy of Professor A. Stoll, Basle, being a preparation of copper chlorophyll, corresponding in intensity to 120 per cent. of the same amount of chlorophyll. 0·038 grams of copper chlorophyll were dissolved in ether, saponified with methylalcoholic potassium, washed down with water, and the final solution made up to 1,000 c.c. Then comparison was made in a Leitz colorimeter.

The sampling of the plants was done as quickly as possible into closed jars, and a sample in a closed weighing-bottle set aside already in the veld for the determination of fresh matter. The leaves were ground immediately with a little chalk and pumice stone and extracted in the usual way (Willstaetter and Stoll 1913, p. 138 ff.) When there was much chlorophyll present, as in the series of November 1934 after heavy rains, the pigment came down in 35 percent. acetone and had to be regained with ether, small amount of the 35 per cent. acetone being shaken with a fair volume of ether. In all other cases the separation went very smoothly, only the very wilted plants offering some difficulty in the final separation.

Besides taking quantitave readings of the amount of chlorophyll, the solutions were tested with a Zeiss hand grating spectroscope, the bands being compared with the existing ones in the literature. Most solutions made up of 10 grams fresh leaves were far too dark for direct observation and had to be diluted 12-30 times.

For the purpose of ascertaining the position of the bands, separation of chlorophyll a and b was necessary. At the start this was done by Willstaetter and Stoll's method (1913, p. 153 ff.) Later is proved quicker to separate with an activated sugar column (Winterstein 1932, p. 1402, and Treibs 1932, p. 1351). As chromatograms were used for the separation of the carotinoids, most of the later separation work on the pigments was done in this manner. The icing sugar was heated to 150°C to activate. It proved advisable to cover it with a thin layer of talcum. The total column was much longer (30 cm.) and wider than the columns for the yellow pigments. Chlorophyll a was retained partly in the talcum, partly by the top layer of sugar in three layers. Chlorophyll b went through. As solvent equal parts of benzene and petrolether were

used. The chromatogram was eluted with ether plus 1 drop of ethylalcohol. All decomposition products if present went through with chlorophyll b.

Content of Chlorophyll.

From the start it was obvious that the fresh green plants from the railway enclosure with a high water content—nearly unknown in South African plants—had absolutely normal chlorophyll in large amounts (Table 1). But within a few weeks the content of water and chlorophyll calculated on dry matter decreased and adjusted themselves to South African conditions, the plant became more solid, the chlorophyll content became similar to that of other areas, although being always a so called strain with higher chlorophyll content. The strain from the reserve had much less chlorophyll, it showed however the same seasonal variation, higher in the young plant, decreasing slowly, even keeping stationary under the influence of good rains, and decreasing rapidly at the end of the season. An intermittent drought always resulted in a decreased chlorophyll content, behaviour which is already known for South African plants (Henrici 1927). Contrary to the series of the railway enclosure, the plants wilted badly and in no case the water content was high.

The plants collected on the different farms in the district Fauresmith, at Onbekend (Middelburg district, Cape), or at Victoria West belonged to strains either with low chlorophyll content or with a content of 1-1·5 per cent. They all showed the seasonal decrease.

Although most of the Tribulus strains collected during outbreaks of dikkop had a small chlorophyll content of 0·08-0·5 per cent. of the dry matter, not all the strains with a low chlorophyll content actually occur on veld where dikkop is observed. Furthermore it is interesting to note that all strains with a medium and high chlorophyll content were not collected where a dikkop outbreak occurred. A priori one would think that when a cleavage product of the chlorophyll is one of the responsible causes of dikkop, individuals which contain a lot of chlorophyll should cause dikkop. The present investigation shows clearly that this is not the case. The collection and working up of Tribulus during an outbreak of dikkop was always done so quickly that no material losses of chlorophyll could occur. In the Fauresmith area Tribulus dikkop is only produced by wilted Tribulus, but not all wilted Tribulus produced dikkop. In more Southern areas dikkop may be apparently produced by plants showing only incipient drying. Wilting certainly decreases the chlorophyll content, but even in the fresh state the chlorophyll content of a strain which later proves fatal in the wilted state, is never high. Table 1 is very instructive on this point.

It must be emphasized that the wording high, medium and low chlorophyll content is relative and only applies to Tribulus. The figures often encountered were 1·5, 0·5 and 0·08 per cent. chlorophyll a + b per dry matter. Willstaetter (1924, p. 15) gives for Sambucus chlorophyll a + b 0·93 per cent. on dry matter basis, which is a fair mean between the two higher values encountered. The value 0·08

is even in an absolute sense very small, the enormous value of over 5 per cent. is certainly quite exceptional and can only be explained by the chlorophyll formation taking place much quicker that the formation of any solid substance. All other values are absolutely normal for the particular strain.

So far only the actual amount of chlorophyll has been considered. With regard to purity of the chlorophyll, it was obvious from the start that the wilted plants which caused dikkop contained some other pigments besides the chlorophyll. The acetone extraction was not bright green, but had a decided olive, even brownish tint. The chlorophyllines were not pure although the major portion was still normal for the red bands and on saponification the normal brown phase was obtained. In the green blue part of the spectrum a sharp and strong band could be detected in place of the ordinary weak band of chlorophyll. The band extended from about 5250-5400 Angström, varying somewhat according to concentration. This band was never observed in a non-poisonous Tribulus with low chlorophyll content. The non poisonous Tribulus always showed the normal chlorophyll spectrum, Willstaetter's band 6 rather weak, if visible at all. The author would be inclined to look for the pigment causing this band in the phaeophorbides or phaeophytin, for which a strong band in this position is characteristic, but the second strong band for phaeophorbides could not be traced. For the time being the only conclusion which could be drawn was that there were cleavage products present in wilted Tribulus, which causes dikkop, but only in those. An observation may be mentioned here. If after some months of storing the plant powder of Tribulus was used again to isolate chlorophyll, the pigment of the fresh or wilted non poisonous Tribulus was normal, in percentage perhaps a little less after years of storing. Never was any sign of deterioration seen, whilst when the Tribulus which had caused dikkop was treated in the same way, very little, often only traces of chlorophyll were left after a few months' storing, and a fair amount of decomposition products were present. As the plants were treated in absolutely the same manner, it can only be suggested that something in the plants collected during outbreaks of dikkop caused the difference. The question of Tribulosis is so complicated that smallest indications of that sort have to be taken notice of.

Another point was clear as well. Phylloerythrin was not present in the wilted nor in any of the Tribulus plants. To test this, the plant material (fresh, green, wilted, wilted poisonous Tribulus) was extracted with a mixture of ether and acetic acid (2 : 1), and the extract shaken with acid of different strengths. The acid extraction did not show the spectrum of phylloerythrin.

The presence of phaeophytin was considered, as this pigment is supposed to occur in dry plants, for instance in herbarium specimens. Lately it has been recorded in leaves under the influence of excessive heat or cold (Röben 1933, Röben und Stern 1935).

The colour of phaeophytin made by treating the chlorophyll with concentrated acid agrees with the colour of the pigment in the wilted poisonous plant.

It is therefore quite likely that phaeophytin is present in the poisonous wilted plants. Phaeophytin is derived from chlorophyll by the action of acid, especially easily by oxalic acid which splits off the magnesium of the chlorophyll molecule. Would such a reaction be possible in the living plant? Mineral acids are of course out of question, but oxalic acid is present in Tribulus, and what is rather interesting, the amounts present in older wilted Tribulus are about four times as high as those in fresh Tribulus. The Tribulus from the Railway enclosure contained in the first determination 0·21 $(COO)_2$, while wilted Tribulus contained so far 0·9-1·3 per cent. The oxalic acid was determined by Dakin's modifications of Salkowski-Autenrieth and Barrth's method (Hawk 1927, p. 769 ff.), the Calcium by the usual method of MacCrudden.

Cross sections through the leaves were treated with H_2SO_4. In the few crystal druses to be seen—there were remarkably few—gypsum needles were observed. Part of the oxalate is certainly present as calcium oxalate, but is does not seem all. Tribulus leaves contain a lot of calcium, between 2·5-4·5 per cent. CaO; it would be more than enough to neutralize the oxalate, but apparently most of it is not bound to $(COO)_2$ or more crystals should be encountered, and the plant juice should be less acid. It is likely that acid K or Na salts of oxalate are in solution which during wilting penetrate to the chlorophyll and destroy it. There is no doubt that during wilting the semipermeability of the protoplasm is changed to permeability, and the acid salt obtains access to the pigment in the chloroplasts, and split the magnesium off. So much for the plant. It has been maintained that such a decay of the chlorophyll would immediately be stopped in the stomach of the sheep, as the first enzyme pepsin only acts in acid medium. This may be so, but by that time the cleavage product is already irreversibly formed, and the flora of the intestinal tract need only continue their work to prepare phylloerythrin from phaeophytin (Rimington and Quin 1935).

To separate phaeophytin quantitatively from the chlorophyll, will be the next step to be taken. Phaeophytin however is not the only cleavage product occurring in Tribulus during wilting. To get an idea what the breakdown products in the plant were, the method of Willstaetter and Stoll (1913, p. 262-273) was adapted, the ether solution of the plant pigments being extracted with hydrochloric acid of different strengths. (Tables 2 and 3.)

It was fully realised that under the influence of concentrated acid phaeophytin may be formed; but it was obvious from Willstaetter's table that this would only happen with acid of 29 per cent. or over, that is to say, no interference was to be feared with lower concentrations. The point was tested with crystallized chlorophyll dissolved in ether. With no strength of hydrochloric acid did a change of colour take place in the ether. The acid up to 28 per cent. did not extract any pigments from the ether. 29 per cent. HCl was very slightly coloured green. The result is exceedingly interesting (Table 2). No Tribulus from the railway enclosure nor from the reserve (although some of the latter was wilted), tinted weak 8 per cent. acid; but half of the wilted Tribulus which was

collected during outbreaks of dikkop tinted it faint blue green. 16 per cent. HCl was tinted blue green by all but one of the poisonous wilted plants and tinted green (a different shade) by two pigments of wilted not poisonous plants. 22 per cent. HCl took a blue green pigment from all wilted plants. Some extracts from fresh plants gave a colour to 22 per cent. HCl, but the shade was a different green, the same colour as was obtained with concentrated acid from all chlorophyll and which is due to phaeophytin. With 22 per cent. HCl in several cases the ether could be completely extracted, being absolutely yellow from the carotinoids.

Summarizing it can be said that there are certainly cleavage products of the chlorophyll present in the wilted Tribulus collected during the outbreaks of dikkop and the plant possesses in this stage a constituent which breaks down the entire chlorophyll. In the isolation of chlorophyll, they cling to the pigment to the very last stage. Run through the sugar column they cling to chlorophyll b. Another means has still to be found to separate them.

Content of Carotinoids.

1. *Method.*

Although is was soon obvious that the icterus of the dikkop was due to bilirubin (Quin 1933, p. 505 ff.) and not to a plant pigment, as first suggested, it was thought of interest to know what yellow pigments were present in Tribulus, the more so as the carotinoids are also built up by the isopren radical, and constituents of isopren (resinic acid) may play an important rôle in the poison principle of Tribulus (Rimington and Quin 1935 b). At the time when it was certain that no plant pigment causes the icterus, the present investigation was already far advanced and certain results obtained. The pigments were investigated quantitatively and spectroscopically. Either fresh plant material or carefully air dried leaves were used for the isolation of the yellow pigments.

First Willstaetter and Stoll's method (1913, p. 231 ff.) was used.* The bichromate standard was used and compared with the standard of Azobenzol. A Leitz colorimeter was used, but in spite of all precautions, the colours did not quite match and the colour filters of the colorimeter were too dark to allow comparable readings in any other colour than yellow. For no apparent reason the azobenzol readings were too low. In a paper of Guilberts (1934) a standard for carotinoids was described which agreed well with the values of the bichromate standard, and which was in tint of colour exactly the tint of the carotene prepared from plant leaves. It consisted of 3·06 grams of Naphtol yellow S and 0·45 grams of orange G. dissolved in 1 litre of water as stock solution. Of the stock solution 5 c.cs. are diluted to a 1000 for the standard. The ratio tables of the bichromate standard could be used for this standard as well, for the calculation of xanthophyll. As this standard proved to be the best as colour match, in the later work is was solely used.

* As for the chlorophyll determination the method will not be described in full detail, the original being closely followed. Only when alterations proved necessary or when difficulties were experienced, these are fully described.

Different extraction methods were tried out during the investigation, especially when only carotinoids and not the chlorophylls were used. Besides Willstaetter and Stoll's method (1913) Zechmeister's (1932 and 1934) extraction and separation were used. As his tables are at times difficult to follow, the method will be more fully described. The main point with Zechmeister's method is the first extraction which is alternatively done with petrolether boiling point 60-80°C. and methylalcohol. 10 Grams of air dried plant powder were shaken in a wide mouth stoppered bottle alternatively with small quantities of the two solvents and the two solvents being finally united. The amount of alcohol must be measured carefully so that the water content can be made up to 10 per cent. The exhaustive extraction needed generally more solvent (600 c.cs.) than Willstaetter prescribes. It may be mentioned here that in different prescriptions for the extraction of plant material the amount of solvent to be used varies a few thousand per cent. Even one of the Willstaetter prescriptions for a large scale extraction speaks only of six litres solvent to 2 kg. of plant material (1913, p. 76) while in the smaller extraction 300 c.cs. of solvents are used to 10 grams fresh plant material. To have an extraction as complete as possible, the author had always to use a fairly large amount of solvent. The combined petrolether-methylalcoholic extract (extract I) was separated by adding the necessary water to make the methylalcohol 90 per cent. Most of the chlorophyll went into the fraction of petrolether (I), the alcohol fraction (I) was repeatedly shaken with petrolether (II) which sometimes removed all the chlorophyll. All petrolether-extraction as well as all methanol fractions were united respectively. At this stage the latter were often very cloudy but cleared sometimes on standing overnight. Under these conditions the xanthophylls are in the alcohol phase, and the carotenes in the benzine phase. If lypochroms or lycopin should be present, they would be in the benzine phase; it may be already stated here that none was found in the leaves of Tribulus.

The methylalcoholic fraction (I) is then saponified in case there is still some chlorophyll present which can easily be controlled in the spectroscope. In case no or very little chlorophyll is present, only 1-2 c.c. 2N NaOH is added; as plenty of flavones are present, the solution becomes cloudy and reddish yellow. When more chlorophyll was present, up to 20 c.c. NaOH were given. Experience teaches very quickly how much has to be added so that the brown phase appears immediately. The solution was left to itself for about three hours. After three hours the same amount of water as methylalcohol is added to the saponified solution and then the emulsion shaken with benzine. If chlorophyll is present, it is hypophasic and stays back in the methylalcohol-water fraction II. If no chlorophyll is present, this fraction II is yellow or yellow-red from flavones, some of them flocculating out at the contact surface between alcohol and benzine. It proved advisable to extract the large bulk of the methylalcohol layer II several times with small amounts of benzine, till the latter remained colourless after good shaking. The methanol layer II certainly contained some benzine in the emulsion, a good deal of it cleared and united with the benzine layer II. All these benzine extractions were united to extraction III, which contained all the xanthophyll (in the sense of Willstaetter and Stoll 1913).

The petrolether fraction 1 which contains chlorophyll and carotenes is mixed with an equal volume of 5 per cent. ethylalcoholic potash and kept for three hours at 40°C. An amount of water corresponding to 20 per cent. of the alcoholic potash was added to separate the layers. The benzine layer is repeatedly shaken with 90 per cent. methanol, until the latter remains colourless. It happened several times that the benzine fraction I did not mix well with the potash and consequently the saponification was incomplete. More potash was added and chlorophyll again washed out. But after a few tentative trials it was found much easier to remove the remaining unsaponified chlorophyll by shaking the benzine fraction I with some talcum plus some glowed Na_2SO_4.

The talcum immediately absorbed the chlorophyll and pure carotene solutions were obtained. Xanthophyll and carotene were compared with the naphtol yellow S standard described before.

In going through the literature, the method of Guilbert (1934) was encountered and consequently tried out. At first it seemed rather drastic to use boiling alkali in the separation, as every paper on carotenes contains a warning that these pigments are exceedingly sensitive to heat. As on the other hand the method seemed very time saving, it was worth while trying out. After some experimenting the method gave satisfactory results, differing very little from those obtained with Willstaetter and Zechmeister's method. The main point—in all three methods—is the very thorough grinding of the sample.

Willstaetter and Stoll's method (1913) is described so well, that scarcely anything has to be added. The only point which at first offered some difficulties was the final removal of the xanthophyll to the ether where it is stated that water should be added slowly. The author found it more satisfactory to add a very large quantity at once. The xanthophyll separates immediately out into the ether, although some ether may be absorbed in the water. If the water was added slowly, emulsions occurred which had to be taken up several times with ether.

The quantity of the carotinoids was determined in a Leitz colorimeter. As certain colours were observed in the freshly collected plant, it seemed advisable to check the pigments in the spectroscope. In the literature the bands of these pigments are mostly given for solutions of alcohol, benzene, chloroform and carbondisulphide. The chromatogram method (Zechmeister 1934, p. 98 ff) was used to separate the carotinoids.

As absorbent, activated MgO prepared from $Mg(OH)_2$ was used, as no suitable aluminiumoxide could be obtained (Strain H.H. 1933). The different components of the xanthophyll were adsorbed generally in four different layers and the carotene could be far enough removed from the xanthophyll or could be forced to pass the column unadsorbed, at will. The yellow pigments in the ether solution were fanned to dryness, taken up with benzene, or a mixture (1 : 1) of benzene and petrol-ether. Then the solution is run through the column. After the adsorption column had been sucked dry, it could easily be removed from the glass tube with a rubber stopper

fixed on a glass rod. This device was also useful in filling the column, but care had to be taken that the stopper is *less* in diameter than the tube so that in pulling out the stopper, no vacuum occurs which may break the column. The column was usually 10 cm. long, and ±1 cm. in diameter.

After the column has been taken out of the tube, the different layers are separated (see results) and eluted in the centrifuge. Generally 2-3 stirrings with the eluting fluid and centrifuging were enough. If the pigments are to be used for spectroscopic work, elute with ether and 1 per cent. alcohol. For colorimetric work elute with ether or petrolether. For spectroscopic work the elution is fanned down again and the pigment taken up with the solvent in which the bands have to be measured. This takes only a few minutes. At this stage, from the more concentrated solutions crystals were obtained.

The fanning down was done with an ordinary electric fan to which a long funnel made of stiff brown paper was attached, guiding the air to the surfaces of the solution. The last traces of liquid after fanning could easily be removed by putting the crystallising dish into a desiccator with $CaCl_2$.

The absorption bands were studied in the usual way in the new Zeiss spectroscope, opening of the slit $\frac{1}{2}$ (0·05 mm.), thickness of layer 10 mm., carbondisulfide, benzine and ethylalcohol being used as solvents.

2. *Results.*

I. *Spectroscopical. Table* 4.

As the concentration of the yellow pigments varied a lot in the different plants, the chromatogram did not allow of distinguishing all the bands in all cases. But in the typical MgO column of fresh or wilted not poisonous Tribulus five distinct bands (including carotene) could be seen. In the following the counting is done from the bottom to the top. Sometimes a faint yellow band preceded the actual pink band which proved to be carotene β. This faint band was too weak to be studied with certainty (carotene α?). Band 2 (pink yellow) was very strong and after eluting had to be diluted. It gave the usual absorption band in CS_2 of 521 and 485·5 (centres) and in benzine 483·5 and 452, corresponding to carotene γ β. Carotene was never observed.

The third band of chromatogram, yellow pink, was generally very dilute and showed in petrolether absorption bands with centres at 435 and 458·0 $\mu.\mu$.

The fourth band was the strongest. It was dark orange with a ring of lighter yellow in front of it. Eluted and evaporated down it formed at times good crystals, dark red brown, thickish prisms with swallow-tailed ends, sparingly soluble in CS_2. The absorption bands had the following centres 446; 475·4; 508·2. The band at 420 was not detected. This pigment seems to be Lutein.

Band 5, orange red, was not present in all the preparations. In No. 1046, however, it even formed crystals, like flat (plate-like) prisms. The following are the centres of the band in CS_2: 450·0

(not too good); 478·7; 511·6. It is doubtful whether this pigment corresponds to any known xanthophyll, perhaps it is Zeaxanthin.

The sixth band of the chromatogram, greenish brown-orange, showed very sharp bands in CS_2. The centres were 440; 467·8; and 500·6 (others 440; 470·6; 501·1). The pigment did not crystallize. The bands are those of Violaranthin.

These pigments were obtained from air dry material, a few months old. It seems that in the fresh Tribulus all normal yellow pigments and no unknown pigments are present. When wilted Tribulus collected during outbreaks of dikkop was extracted on the spot the result was different. Unfortunately at that time the technique of the chromatogram was not yet worked out, and only spectroscopic tests on ether solution could be done. For the last four years no dikkop outbreak has occurred in the Fauresmith district. The ether solution of the poisonous Tribulus was not yellow but reddish. It was made quite sure (in the spectroscope) that no traces of unsaponified chlorophyll were left which might give to the ether a reddish tint. The usual absorption bands of carotinoids were found and then a very strong band extending nearly to 550 $\mu.\mu.$, from about 520 $\mu.\mu.$ A less dark band was between 500 and 520 $\mu.\mu.$ There is no doubt that there is a pigment in the wilted poisonous Tribulus which is not present in the fresh plant. But this pigment so characteristic in its colour and absorption bands visible even to a lay man in the matter, is not stable. When the air dried plant is extracted a few weeks later, no trace can be found, but, and that is the peculiar point, a pigment is found in the chromatogram which gives the absorption spectrum of Zeaxanthin,

in CS_2 with the centre of absorption 520; 483; 450.

in C_2H_5OH with the centre of absorption 483; 451; 423.

In petrolether long needles were obtained. In methylalcohol long prisms were obtained with swallow tailed ends and sharp edges (see Zechmeister p. 292). If enough poisonous material comes to hand further investigations will have to show whether the disappearance of the unknown pigment and the appearance of Zeaxanthin is a similar case to that described by Heilbron and Phipers (1935). According to these authors fucoxanthin can only be isolated from fresh plants. Ten days after the collection of the Algae no fucoxanthin is found, but zeaxanthin appears, as a product of reduction. Tribulus is a very active plant with a high oxyreduction potential (it needs only the presence of water to reduce nitrates to nitrites in the plant powder); thus the reduction of the unknown carotinoid to zeaxanthin does not seem far fetched, but a very likely occurrence. In normal fresh or slightly wilted Tribulus zeaxanthin was never found. Zeaxanthin is certainly not a usual product in the plant leaves (Zechmeister 1934, p. 184 ff.). It cannot be accepted that the few Tribulus collected during outbreaks of dikkop were especially badly treated (on the contrary, they were cherished); so that a decomposition product occurred. Zeaxanthin is certainly a post-mortem product in this particular case, but would also appear under the best conditions of drying. Here a further observation has to be started, when material (and lots of material) is available.

In this connection Lippmaa's (1925) work on rhodoxanthin has to be mentioned, as this pigment shows a strong absorption band between 515-535 $\mu.\mu.$, while the other three absorption bands (two in the red and yellow part of the spectrum) are much weaker. According to Lippmaa rhodoxanthin occurs in the most different families under definite exterior and interior conditions, such as strong illumination, drought, plenty of sugar instead of starch, etc. All these conditions would be fulfilled in the case of wilted Tribulus, as during wilting starch is hydrolysed to sugar (Iljin 1922), but the absorption band in red and yellow was never observed. This would exclude right away Lippmaa's pigment, if Kuhn and Brockmann do not give different bands for the same pigment. Their bands in ethylalcohol and ether agree much better with the ones seen by the author in the spectroscope. Thus there is a likelihood that the unknown pigment may be rhodoxanthin.

II. *Quantitative Study of the Carotinoids. Table 5.*

In their classical investigation Willstaetter and Stoll (1913, p. 111 ff.) state that under normal conditions in green leaves the ratio of green to yellow pigments shows but small fluctuations, being about 3-6. For very young and Autumn leaves this ratio is different (Willstaetter and Stoll 1915, p. 526) when the amount of yellow pigments is relatively larger.

The question arises as to the ratio of the pigments in the investigated Tribulus. A priori it may be said, that only in a few cases can the plant investigated be regarded as in a normal state; generally they are wilted. If desiccation affect the ratio, differences have to be expected. In Table 5 the total chlorophyll content, the percentage and the ratio of the yellow pigments are given. In a special column the condition of the plant (fresh, wilted, collected during outbreaks of dikkop) is marked. Table 5 shows beyond doubt that under the given circumstances the ratio of the green to the yellow pigments is not constant, varying as a matter of fact from 8-66.

At first sight it might be thought that the extraction was incomplete, but the analyses were repeated with three different methods (see above) and the values obtained showed very small differences. The greatest care was of course taken to extract as completely as possible. Other plants were tested and gave lower figures (but also higher than Willstaetter and Stoll's), but wilted plants always gave high ratios.

A closer inspection of Table 5 certainly reveals the fact that the content of carotinoids is very low indeed. After what has been said about the destruction of chlorophyll in the wilted plant, it is not surprising that in very badly wilted plants the ratio of green to yellow pigment is relatively low, as apparently chlorophyll and carotinoids have been destroyed. Then there seems to be a group of plants which still have their normal chlorophyll content, but in

which a lot of yellow pigments have been destroyed; they show the widest ratios. There are also a few fresh plants with ratios between 10 and 20, which seem to have a fairly normal content of yellow pigments and chlorophyll.

Summarizing the Table it seems that even in the fresh plant the ratio is wider than in Europe, and that in wilting or even incipient drying which is not yet obvious to the eye, carotinoids (especially xanthophyll) are destroyed before a destruction of chlorophyll takes place, and hence the high ratio; then when the plant is badly wilted chlorophyll and further carotinoids are destroyed and smaller ratios are again obtained. From the percentage figures it seems that especially xanthophyll is destroyed. What actually happens to the pigment, one can only speculate. It is possible that isopren rests are freed and used to build up some other terpenes.

One point has to be stressed. The destruction of the pigments in Tribulus is very unlike the autumnal destruction of plant pigments in Europe, when generally first chlorophyll and its enzyme are destroyed, and then only the yellow pigments. Here just the opposite takes place. The only similarity is that in both cases a lot of water soluble yellow pigments occur (flavones according to positive reaction with caustic alcohol). The difference in both processes may be due to the lack of moisture in the South African climate.

Nitrate and Nitrite Content of Tribulus terrestris.

(Table 6.)

Tribulus terrestris is certainly a nitrate plant. Table 6 gives the accumulated data in this respect. It has to be emphasized that the amounts obtained in any material collected in Fauresmith were much smaller than those obtained in the surroundings of Pretoria by Rimington using the same method (Strowd 1920). As soon as the plants were immersed in water, nitrite appeared, these amounts also being smaller than those of Pretoria plants, the latter probably due to the fact that at Fauresmith the plants were carried into the laboratory and immediately worked up or put into water in the veld, on the whole more in their natural condition with no time lost to allow for chemical changes. The lower nitrate content is due to the poverty of Fauresmith soil in nitrogen. Most Fauresmith pasture plants, except when very young are rather poor in protein.

In spite of the nitrite content of the Tribulus, this radical is certainly not the cause of any poisonous quality of the plant, as our Reserve plants which were never poisonous to stock, always showed positive reaction to Griess' nitrous acid reagent. (Treadwell II 1924, p. 306.)

Content of Calcium and Phosphorus.

In grazing experiments on the Reserve in 1932 *Tribulus terrestris* proved to be an excellent fodder plant, in spite of the poor and wilted appearance; two sheep grazing for two months on a

half-morgen plot with nothing else but Tribulus in it increased 10 and 14 lb. Some determinations of phosphorus were done on Tribulus collected from different places. The phosphorus is very high as the following figures show: —

No.	Date.	Place Where Collected.	Percentage P_2O_5 on Dry Matter.
1221	1/12/34	Railway enclosure near Reserve.....	0·68
1232	28/12/34	Ventersvlei–Fauresmith District.....	0·91
1240	25/ 1/35	Railway enclosure near Reserve.....	0·60

The calcium content of all the samples investigated was very high.

No.	Date.	Place Where Collected.	CaO.	MgO.	
1219	19/11/34	Railway enclosure near Reserve	3·69	1·05	
1221	1/12/34	Railway enclosure near Reserve	3·78	1·08	
1240	25/ 1/35	Railway enclosure near Reserve	4·45	—	
1220	29/11/34	Reserve.....	3·06	0·99	
1237	22/ 1/35	Onbekend, Middelburg District.	2·55	—	Upright, after dikkop outbreak.
1238	22/ 1/35	Onbekend, Middelburg District.	3·26	—	Prostrate.
1239	22/ 1/35	Onbekend, Middelburg District.	3·26	—	Prostrate.
1241	28/ 1/35	Riet River.....	4·03	—	Prostrate.
1242	28/ 1/35	Dassiespoort near River.....	4·06	—	Upright.
1245	28/ 1/35	Dassiespoort near River.....	4·52	—	Prostrate.
1243	28/ 1/35	Dassiespoort Garden.....	3·57	—	Upright.
1244	28/ 1/35	Dassiespoort Garden.....	3·11	—	Prostrate.
1250	21/ 1/35	Victoria West "A".....	3·59	—	Wilted, collected during dikkop outbreak.
1251	21/ 1/35	Victoria West "B".....	3·27	—	,, ,,
1252	21/ 1/35	Victoria West "C".....	3·78	—	,, ,,

The magnesium content of the plants, done only on a few samples, was $\frac{1}{3}$–$\frac{1}{4}$ of the calcium content. No relation can be seen between habitat and calcium content. The ratio of calcium content to magnesium is very good. From the point of view of phosphorus it can be understood that *Tribulus terrestris* is excellent food.

Content of Oxalate in Tribulus.

As already pointed out in the anatomical description, *Tribulus terrestris* contains in its leaves a layer of cells with calcium oxalate.

The oxalate was determined macrochemically (Hawk 1927, p. 799), and the calcium oxalate also microchemically (Tunmann 1913, p. 141).

No.	Date.	Habitat.	Percentage $(COO)_2$	
815	31/10/32	Tietiespan	0·78	Upright, wilted. collected during dikkop outbreak.
1042	26/ 1/33	Veld Reserve	0·83	
1220	29/11/34	Veld Reserve	1·29	Dry.
1219	19/11/34	Railway enclosure Fauresmith	0·63	Spreading.
1221	1/12/34	Railway enclosure Fauresmith	0·21	Very spread.
1235	28/12/34	Railway enclosure Fauresmith	1·14	Spreading.
1240	25/ 1/35	Railway enclosure Fauresmith	1·11	Spreading.
1228	28/12/34	Riet river bank, Dassiespoort	1·25	Spreading, fresh.
1229	28/12/34	Riet river bank, Dassiespoort	0·37	Upright, fresh.
1242	28/ 1/35	Riet river bank, Dassiespoort	1·32	Spreading.
1226	28/12/34	Dassiespoort Yard	1·01	Spreading.
1227	28/12/34	Dassiespoort Yard	0·29	Upright.
1234	28/12/34	Dassiespoort Garden	0·09	Spreading, very much. Fresh as 1221.
1231	28/12/34	Koksfontein, on lime stone	0·47	Upright.
1232	28/12/34	Ventersvlei	0·92	More spreading.
1233	28/12/34	Riet River Bridge	0·83	Little spreading.
1241	28/ 1/35	Riet River Bridge	1·11	Spreading.
1237	22/ 1/35	Onbekend, Middelburg District	0·94	Upright.
1238	22/ 1/35	Onbekend, Middelburg District	1·21	Prostrate.
1239	22/ 1/35	Onbekend, Middelburg District	0·76	Fresh, spreading.

The above table shows the amount of oxalic acid. It varies a good deal. For absolutely fresh plants it is very small (1221; 1234); on the other hand it may be low for upright forms (1231, 1229, 1227). No relation between poisonous qualities of the Tribulus and its oxalate content could be detected. It must, however, not be forgotten that the oxalic acid if not bound to a mineral may at times tackle the chlorophyll when through external conditions the permeability of the cells is altered.

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APPENDIX.

PROTOCOL OF CHLOROPHYLL EX " TRIBULUS TERRESTRIS, No. 1253, POORTJIE, NOT MUCH WILTED. 25/2/1935.

Slit of spectroscope $\frac{1}{2}$; daylight, depth of layer 12 m.m. The chlorophyll was prepared of 50-gram dry plants, but then diluted about 1,000 times. For certain bands a further dilution was necessary. Chlorophyll (*a*) and (*b*) were separated by a chromatogram of talcum and activated icing sugar.

Chlorophyll (*a*) gave 3 distinct absorption bands. In ether. No. 1 the lowest, No. 3 the highest in the chromatogram. The different absorption bands gave different absorption bands which all corresponded very well with Willstaetter's bands.

Band centres.

No. 1. End absorption 4328 and 7200.
Bands 4100; 4328; 6646; 6600-6702.

No. 2. 5360; 5800; 6168; 4649; 4310; 6600;-6710. More dilute 5100 (faint) 6200.

No. 3. 5350; 5100; 5720 (good); 6130.
6571-6695 more diluted; end absorption 4301 and 6800 faint band 4550.

Chlorophyll (*b*) in benzene.

5350 (band 6, weakest band); 5345; 5643-5861 (band 5).

Further diluted 6154 (band 3); 4782 (band 4); 4665 (band 8); 6628 (band 1).

The bands below 4600 could not be seen well.

The band numbers in brackets are the numbers of Willstaetter.

The observed intensity order is Bd. 6; Bd. 5; Bd. 3; Bd. 4; Bd. 8; Bd. 1.

TABLE I.

Chlorophyll Content (a+b) of Tribulus terrestris from different localities.

No.	Date.	Locality.	Remarks.	Chlorophyll content in percentage of dry matter.	Water content in percentage of fresh matter.	Special remarks on the spectre of the chlorophyllides. Figures for normal spectres are not given. All bands taken layer 12 mm.; slit ½, 0·05 mm. daylight.
1032	10/12/32	Veld Reserve	—	0·38	42·3	—
1033	29/12/32	Veld Reserve	—	0·21	40·0	—
1034	9/ 1/33	Veld Reserve	—	0·59	60·2	—
1042	24/ 1/33	Veld Reserve	—	0·18	33·7	—
1046	6/ 2/33	Veld Reserve	—	0·25	47·6	—
1069	8/ 5/33	Veld Reserve	—	0·56	60·0	—
1220	29/11/34	Veld Reserve	—	0·87	67·8	—
1595	22/11/35	Veld Reserve	—	0·41	—	—
1219	19/11/34	Railway enclosure Fauresmith	—	1·23	80·74	—
1221	1/12/34	Railway enclosure Fauesmith	—	5·14	89·89	—
1235	28/12/34	Railway enclosure Fauresmith	—	1·48	65·4	—
1240	25/ 1/35	Railway enclosure Fauresmith	—	0·89	68·82	—
1274	5/ 3/35	Railway enclosure Fauresmith	—	0·54	35·2	—
815	31/10/32	Tietiespan near homestead	Collected during dikkop outbreak	0·31	46·9	Very strong band 5300–5375, and 5132.
817	31/10/22	Tietiespan near homestead	Collected during dikkop outbreak	0·10	48·0	—
1226A	28/12/34	Dassiespoort yard	Collected during dikkop outbreak. Spreading	1·51	68·65	Very strong band 5273–5343, 580–60000.
1227	28/12/34	Dassiespoort yard	Collected during dikkop outbreak. Upright	0·29	66·01	Very strong band 5280–5453, weaker band 5130–5265, 5647–5806, 5885.
1228	28/12/34	Dassiespoort river bank	Spreading	0·52	68·73	—
1229	28/12/34	Dassiespoort river bank	Upright	0·95	63·4	Strong 5160–5300.
1230	28/12/34	Dassiespoort river bank	Wilted	1·03	31·3	—
1234	28/12/34	Dassiespoort garden	Widely spread	1·30	67·4	—
1242	28/ 1/35	Dassiespoort river bank	—	0·56	66·13	—
1245	28/ 1/35	Dassiespoort river bank	—	0·49	68·97	—
1243	28/ 1/35	Dassiespoort garden	—	0·31	58·30	—
1244	28/ 1/35	Dassiespoort garden	—	0·62	57·16	—
1231	28/12/34	Koksfontein, limestone	Collected during dikkop outbreak	0·18	57·44	5127 and 5230.
1232	28/12/34	Kalabasdrift	—	0·69	65·9	—
1233	28/12/34	Rietriver bridge	—	0·20	62·1	—
1237	22/ 1/35	Onbekend, Middelburg District	Collected during dikkop outbreak. Upright	0·18	37·19	5135; 5831.
1238	22/ 1/35	Onbekend, Middelburg District	Spreading	0·27	48·0	—
1239	22/ 1/35	Onbekend, Middelburg District	Widely spreading	0·55	54·94	—
1250	21/ 1/35	Victoria West	Collected during dikkop outbreak	0·37	8·6*	5212; 5925; 5810.
1251	21/ 1/35	Victoria West	,, ,, ,,	0·13	9·0*	5035; 5810; 5925.
1252	21/ 1/35	Victoria West	,, ,, ,,	0·08	8·4*	5315–5420 (very strong); 5142; 5890.
1253	25/ 2/35	Poortjie	Fresh, spreading	0·85	74·49	—
1254	25/ 2/35	Poortjie	Collected during dikkop outbreak. Upright	0·34	38·80	5062 very strong; 5200–5300 very strong. 5800–5900.
1255	25/ 2/35	Poortjie	Collected during dikkop outbreak. Upright, near kopje	0·50	57·19	5175–5290 very strong.
1808	26/ 3/36	Poortjie	—	0·20	—	—
1785	3/ 2/37	Poortjie	Fresh, spreading	0·80	74·14	—

* Received here rather dry.

TABLE II.

TRIBULUS.—HCl-figure to see whether cleavage products are present.

Plant Powder extracted with 40% acetone, 96% acetone, ether extract of acetone treated with equal amount of HCl.

No.	Date.	Locality.	Look of plant.	Condition.	8 Per cent. HCl.	16 Per cent. HCl.	22 Per cent. HCl.	Conc. HCl.
815	31/10/32	Tietiespan	Upright	Wilted, collected during dikkop outbreak	Faint green colour	Blue green	Blue green	Blue green, (ether from start olive green.
1032	10/12/32	Reserve	Creeping	Wilted	No colour	No colour	Faint blue green	Blue green.
1033	29/12/?2	Reserve	Creeping	Wilted	No colour	No colour	No colour	Green.
1034	9/ 1/33	Reserve	Creeping	Fresh	No colour	No colour	Faint green	Faint green.
1042	24/ 1/33	Reserve	Creeping	Wilted	No colour	No colour	Very faint green	Darker green.
1046	6/ 2/33	Reserve	Creeping	Wilted	No colour	No colour	No colour	Faint green.
1069	8/ 5/33	Reserve	Creeping	Fresh	No material	—	—	—
1219	19/11/34	Railway enclosure	Creeping	Fresh	No colour	No colour	Green	Green.
1220	29/11/34	Reserve	Creeping	Wilted	No colour	Greenish tint	Green	Dark green.
1221	1/12/34	Railway enclosure	Creeping	Fresh	No colour	No colour	Faint green	Green.
1226A	28/12/34	Dassiespoort yard	Creeping	Fresh, collected during dikkop outbreak	No colour	Blue green	Blue green	Dark blue green.
1227	28/12/34	Dassiespoort yard	Upright	,, ,,	No colour	Tinted	Green	Total green in acid.
1228	28/12/34	Dassiespoort river	Creeping	,, ,,	No colour	Faint green	Green blue	Green.
1229	28/12/34	Dassiespoort river	Upright	,, ,,	Faint green	Blue green	Blue green	Blue green.
1231	28/12/34	Koksfontein	Upright	Rather dry, collected during Dikkop outbreak	No colour	Tinted	Faint blue	Blue green.
1232	28/12/34	Kalabasdrift, Ventersvlei	Creeping	Drier than Dassiespoort samples	No colour	No colour	Faint green blue	Faint blue green.
1233	28/12/34	Riet river bridge	Creeping	,, ,,	No colour	No colour	No colour	No colour.
1234	28/12/34	Dassiespoort garden	Creeping	Very juicy	No colour	No colour	No colour	Faint green.
1235	28/12/34	Railway enclosure	Creeping	Fresh	No colour	No colour	No colour	Faint green-blue.
1237	22/ 1/35	Onbekend, Middelburg	Upright	Wilted, collected during Dikkop outbreak	Greenish	Light green	Green	Deep green.
1238	22/ 1/35	Onbekend, Middelburg	Creeping	Less wilted than 1237	No colour	No colour	No colour	Faint green.
1239	22/ 1/35	Onbekend, Middelburg	Creeping	Fresh	No colour	No colour	Faint green	Blue green.
1240	25/ 1/35	Railway enclosure	Creeping	Fresh	Faint green	Faint green	Green	Deep green.
1241	28/ 1/35	Riet river bridge	Creeping	Wilted	No colour	No colour	Pale green	Green.
1242	28/ 1/35	Dassiespoort river	Creeping	Wilted	No colour	Light green	Light green	Blue green.
1243	28/ 1/35	Dassiespoort garden	Upright	Wilted	Faint green	Blue green	Blue green	Dark blue green.
1244	28/ 1/35	Dassiespoort garden	Creeping	Wilted	No colour	Faint green	Blue green	Deep green.
1245	28/ 1/35	Dassiespoort river	Creeping	Wilted	Light green	Blue green	Blue green	Blue green.
1250	21/ 1/35	Victoria West	Upright	Wilted, collected during Dikkop outbreak	No colour	Faint green	Green	Green.
1251	21/ 1/35	Victoria West	Upright	,, ,,	No colour	No colour	Pale green	Green, has very little chlorophyll.
1252	21/ 1/35	Victoria West	Upright	,, ,,	No colour	Faint green	Green	Green.
1253	25/ 2/35	Poortjie, Fauresmith	Creeping	Fresh	No colour	Just faint tint	Blue green	Dark blue green.
1254	25/ 2/35	Poortjie, Fauresmith	Upright	Wilted, collected during Dikkop outbreak	Faint tint	Blue green	Dark blue green	Blue green, ether extracted.
1255	25/ 2/35	Poortjie, Fauresmith	Upright	,, ,,	No colour	Lightblue green	Dark blue green	Dark blue green, complete ether extracted.
1274	5/ 3/35	Railway enclosure	Creeping	Fresh	No colour	No colour	Slightly coloured	Blue green.

TABLE III.

Cleavage Products of Chlorophyll. Absorption bands in acid of different percentages.

Slit ½ ; 0·05 mm. daylight. Depth of layer 12 mm. Extracts of 1 gram dry material.

No.	Date.	Locality.	In 8 per cent HCl. (ether extracted).	In 16 per cent. HCl. (ether extracted).	In 22 per cent. HCl. (ether extracted).
815	31/10/32	Tietiespan	4960 ; 5120	4837–5042 ; 5193 ; beyond 4560 and 6500	4808–5000 ; 5193 ; beyond 6300 and 4500 diluted 3 times beyond 6500.
1231	28/12/34	Koksfontein	Too weak	Too weak	4890–5026 ; 5200 ; beyond 6550.
1228	28/12/34	Riet river bank	Too weak	4835–4985 ; 5200, 5825 ; beyond 4500 and 6500	4850–5060 ; 5184 ; beyond 6500 and 4500.
1243	28/ 1/35	Dassiespoort garden	4900	4812–5008 ; max. 5955 ; 5200 ; beyond 6500	4470 ; 4826–5020 ; 5182–5424 ; 5195 ; 5776 ; 6416–6400.
1250	21/ 1/35	Victoria West	Too weak	Too weak	Too weak.
1254	25/ 2/35	Poortjie	Too weak	4915–5080 ; 5120 ; beyond 6500	4385 ; 4824–4997 ; 5200, beyond 6500.
1255	25/ 2/35	Poortjie	Too weak	4852–4965 ; 5144 ; 5762 ; beyond 6500 and 4300	4859–4928 ; 5162 ; beyond 4548 and 6500.
1245	28/ 1/35	Dassiespoort river	—	—	4855–5048 ; 5657–5765 ; and 4590, 5146–5397 ; 6064–6400 ; and 6500.
1245	28/ 1/35	Dassiespoort river	—	—	Conc. HCl. 4845–5035 ; 5159 ; 5278–5456 ; 5654–5731 ; 5731–5891 ; 6660 end absorption and 4560.

TABLE IV.

Absorption Bands of the Yellow Pigments of Tribulus terrestris.

(Daylight slit 0·05 mm. layer thickness 12 mm., initial material used differs in the single cases, but the end concentration corresponded to ± 2·7 mgm. Carotene in 1000 ccs. which could be diluted still further or evaporated down.)

No.	Date.	State of Plant.	Centres. Adsorption bands 1 and 2 of chromatogram gave the following absorption.	Centres. Adsorption band 3 of chromatogram gave the following absorption.	Centres. Adsorption bands 4 and 5 of chromatogram gave the following absorption.		Ether extract of freshly prepared plant gave the following absorption. bands.
			In Benzine.		*In Benzine.*		
1032	10/12/32	Badly wilted.....	4518; 4834, in CS_2 4855; 5210 probably mostly Carotene	—	4451; 4768 (Guilbert Method) 4483; 4779 (Willst) probably Lutein, but band 4200 not seen)		No absorption beyond 5000.
1034	9/ 1/33	Fresh.....	In CS_2 4857; 5189 Carotene In ether 4552; 4853	In CS_2 4500; 4750; 5078; Lutein Flat prisms, red brown, close clusters. Swallow tails on evaporation ex CS_2	In CS_2 4700; 5011 Violaxanthin	—	No absorption beyond 5000.
1042	24/ 1/33	Wilted.....	In alcohol 4525; 4887 Carotene In CS_2 5200; 4855..	In alcohol 4458; 4776; 4200 Lutein In CS_2 4750; 4450; 5077	In alcohol 4715; 4420; 4175 Violaxanthin In CS_2 4400; 4693; 5010	— —	No absorption beyond 5000. —
1046	6/ 2/33	Wilted.....	In CS_2 4870; 5180 Carotene mostly	In CS_2 4460; 4754; 5082 Thick prisms ex CS_2 Lutein	(4) In CS_2 4500; 5116; 4787; plate-like prisms ? Zeaxanthin.....	(5) In CS_2 4400; 4678; 5006; very sharp Violaxanthin.....	Absorption towards 5350. —
1069	8/ 5/33	Fresh.....	In CS_2 4850; 5210..	(3) In CS_2 4460; 4760; 5080	Bds. 3 and 4 were not very sharp Reading difficult. (4)	In CS_2 4400; 4700; 5006	No absorption beyond 5000.
1219	19/11/34	Fresh.....	In CS_2 4855; 5210 Carotene	In C_2 4450; 4741; 5081 Lutein Large long crystals in ether-alcohol	In CS_2 4400; 5000; 4690 Violaxanthin —	There were many bands in this chromatogram and a second and third chromatogram had to be made. These are the final results. For a long time Lutein and Violaxanthin adsorption bands were mixed. But there was no other pigment present, as first thought.	No absorption beyond 5000.
1226A	28/12/34	Fresh.....	In benzine 4523; 4831 In CS_2 4520; 4830..	In benzine (3) 4350; 4580 In CS_2 4750; 4450; 5080	In benzine (4) 4483; 4782 In CS_2 4400; 4690; 5000 (band very weak)	(5) 4490, 4788..... —	No absorption beyond 5000. —
1230	28/12/34	Collected during dikkop outbreak, wilted	In CS_2 4520; 4830..	In CS_2 4450; 4750; 5080 Lutein	In CS_2 5200; 4820; 4500 Zeaxanthin	4400; 4690; 5035'.. Violaxanthin	Absorption at 5350 and 5570.
1237	22/ 1/35	Collected during dikkop outbreak, wilted	—	—	—	—	Absorption at 5350 and 5570.
1252	21/ 1/35	Collected during dikkop outbreak, wilted	In benzine 4517; 4817 Carotene	In benzine 4479 and 4516; 4790 Lutein	In benzine 4504; 4830 Zeaxanthin	—	Absorption 5350 and 5570.
1253	25/ 1/35	Spreading, but wilted....	—	—	—	—	Absorption 5350; 5570; 5090.
1254	25/ 2/35	Wilted, upright.....	—	—	—	—	Absorption 5350; 5570.
1255	25/ 2/35	Wilted, upright.....	In CS_2 5210; 4855 Carotene	4450; 4750; 5080 Lutein	5200; 4830; 4500 Zeaxanthin	—	Absorption 5350; 5570.

TABLE V.

Carotinoids of Tribulus terrestris from different localities and under different conditions.

No.	Date.	Locality.	Condition of Plant.	Chlorophyll in percentage of dry matter.	Total yellow pigments in percentage of dry matter.	Ratio of green to yellow pigments.	Xanthophyll in percentage of dry matter.	Carotene in percentage of dry matter.	Remarks.
1032	10/12/32	Veld Reserve	Badly wilted	0·38	0·0194	19·5	0·0095	0·0099	—
1042	24 1 33	Veld Reserve	Wilted	0·18	0·0026	69·8	0·0010	0·0016	—
1069	8 5 33	Veld Reserve	New plants fresh, green	0·56	0·0247	16·1	0·0247	0·0·00	—
1220	29/11/34	Veld Reserve	Plant not as fresh as from other places	0·08	0·0055	14·5	0·0029	0·0026	—
1595	22 11 35	Veld Reserve	Spreading	0·41	0·041	10·0	0·0262	0·0146	—
1226a	28/12/34	Dassiespoort yard	Fresh	1·51	0·034	44·4	0·0270	0·0070	Small flower.
1229	28/12/34	Dassiespoort river bank	Fresh	0·95	0·0143	66·0	0·0090	0·0053	Medium flower.
1231	28/12/34	Koksfontein, lime stone formation	Rather dry eaten by locusts	0·18	0·0078	23·0	0·0043	0·0035	No flower.
1233	28/12/34	Riet river bridge	Rather dry	0·20	0·0277	7·20	0·0171	0·0·06	Small flower.
1237	22/ 1/35	Onbekend, Middelburg District	Collected after dikkop outbreak, very wilted	0·18	0·0158	10·4	0·0099	0·0059	Upright small flower.
1238	22/ 1/35	Onbekend, Middelburg District	Better than 1237	0·28	0·0210	13·3	0·0142	0·0068	Prostrate.
1239	22/ 1/35	Onbekend, lands	Fresh	0·55	0·0096	57·3	0·0056	0·0040	Widely spreading.
1250	21 1 35	Victoria West	Collected during dikkop outbreak, wilted	0·37	0·0237	15·6	0·0151	0·0086	Upright.
1251	21/ 1/35	Victoria West	" "	0·13	0·0044	28·8	0·0017	0·0027	Upright.
1254	25/ 2/35	Poortjie	" "	0·34	0·048	7·08	0·030	0·018	Upright.
1808	26 3 35	Poortjie	" "	0·20	0·0056	35·5	0·0033	0·0023	—
1785	3/ 2/37	Poortjie	Fresh	0·80	0·044	18·3	0·026	0·0186	Spreading.
815	31/10/32	Tieticspan	Collected during dikkop outbreak, very wilted	0·31	0·0073	42·4	0·0033	0·00·0	Upright.

TABLE VI.

TRIBULUS.

No.	Date.	Locality.	NO_3 content.			NO_3 content after treating with 0·15 gm. aspartic acid.		Difference expressed as NO_3 probably nitrite.			Remarks.
			Determined.	Calculated.	Determined.	Determined.	Determined.	Per 100 gm. fresh matter.	Per 100 gm. dry matter.	Water content.	
			Per 100 gm. fresh matter.	Per 100 gm. dry matter.	Per 100 gm. dried material.	Per 100 gm. fresh matter.	Per 100 gm. dry matter.				
815	30/10/32	Tietiespan.....	0·41	—	0·476	0·194	0·365	0·206	0·111	46·9	Collected during dikkop outbreak, wilted.
816	30/10/32	Tietiespan.....	0·30	—	0·479	0·209	0·459	0·081	0·020	54·5	Fresh.
817	30/10/32	Tietiespan.....	0·381	—	0·734	—	0·574	—	0·160	48·0	Collected during dikkop outbreak, wilted.
—	8/12/32	Dassiespoort.....	—	—	After alcohol extract. 0·167	—	0·049	—	0·118	43·2	Wilted.
1032	10/12/32	Veld Reserve.....	0·395	—	—	0·275	—	0·120	—	42·3	Wilted.
1033	29/12/32	Veld Reserve.....	—	—	0·977	—	0·738	—	0·239	... 40	Wilted. Strong reaction for nitrite.
1034	9/ 1/33	Veld Reserve.....	0·113	0·167	0·167	0·115	0·161	0·0	0·006	60·2	Fresh no reaction for NO_3.
1042	24/ 1/33	Veld Reserve....	0·145	0·219	—	—	—	—	—	33·7	Wilted, Strong reaction for nitrite.
1046	6/ 2/33	Veld Reserve.....	0·054	—	0·099	—	—	—	—	47·6	Wilted, Strong reaction for nitrite.