Studies on *Leptadenia reticulata;* Isolation of Some Non-saponifiable Principles by a Short-cut-method*

J. V. Anjaria, B. N. Mankad†, M. K. Bamrolia† and O. D. Gulati§

ABSTRACT

A short-cut-method was set up for the isolation of non-saponifiable principles directly from the crude plant. The method involves direct saponification of crude plant material and subsequent isolation of sterols and tocopherols. Stigmasterol and tocopherols were isolated from *Leptadenia reticulata* by a short-cut-method and were typed and characterised.

INTRODUCTION

STIGMASTEROL was isolated from *Leptadenia reticulata* (*Lr*) (Murthy and Sheshadhari, 1944). The presence of sterols in the same plant (Anjaria and Gupta, 1970) and the lactogenic property of (Anjaria and Gupta, 1967) the plant has also been reported. The present work was therefore attempted to isolate stigmasterol in pure form for its further studies on lactogenesis; and to isolate other nonsaponifiable principles like tocopherols and to have a comparative study of various isolation methods.

METHODS

Stigmasterol from *Lr* has been isolated by fractional crystalization (Murthy, *et al*, 1944 and Mukherjee, and Srivastava, 1955). Column chromatographic isolation, using alluminium oxide adsorbent, are the conventional methods routinely followed for the laboratory purposes. Digitonine precipitation method for isolation of sterols is suggested (Paech and Tracy, 1955). Isolation of stigmasterols from soyabean oil by saponifying the oil and consequent precipitation of stigmasterol by steam saturation has been done previously (Ikan, 1969). Sulphuric acid treatment (Parker, 1940) method for separation of tocopherols has been reported and the use of cotton column for separation of a tocopherols has also been reported. The yield of sterols by the conventional method was found to be low. The work of a large magnitude on hand necessitated the availability of larger amount of sterols with economical consideration which prompted the development of a short cut method.

* Contribution of Department of Pharmacology, Gujarat College of Veterinary Science & Animal Husbandry, Anand 388001.

† Present Address : Department of Chemistry, S. P. University, Vallabh Vidyanagar.

§ Department of Pharmacology, Medical College, Baroda.

Short-cut-method for Isolation of Stigmasterol and Tocopherols from Lr.

Plant Collection: Stems and leaves of authenticated *Lr* were harvested in October (80 kg), dried in shade and pulverised manually by a pestle in iron mortar. Homoginised mixture (8 kg) was made and stored.

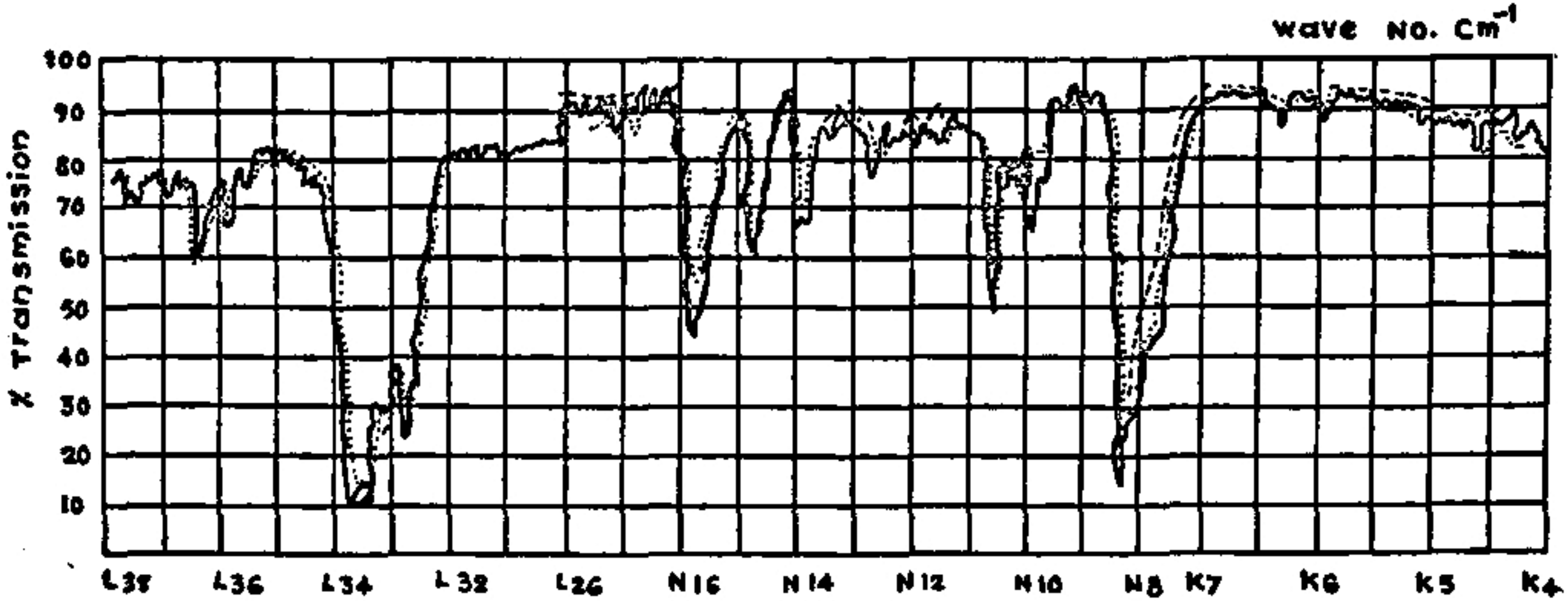
Stigmasterol: Extraction of plant material by petrolium ether and alcohol was done after the method of Murthy and Sheshadhari (1944) and consequent column chromatographic separation of stigmasterols and tocopherols was done for the comparative study. The short-cut method was attempted in the following manner.

The stage of extraction of the plant material with various solvents was omitted. 500 g of *Lr* (dried) powder was directly refluxed for 6 hours in 1000 ml of 1N alcoholic potassium hydroxide. The supernatant was expressed out after saponification, and the residue was further given two washings of ethanol. 75 per cent of ethanol was recovered from the separated material and the remaining material was extracted with about 1000 ml of petrolium ether (PE) (40—60°C) in five lots of 200 ml each. The extracted material was separated slowly on the water bath and PE was recovered till about one fourth of the volume remained. The remaining extract was washed with distilled water till alkali free, which was then slowly evaporated nearing dryness. This remaining material was picked up in 200 ml methanol and allowed to stand overnight, preferably under refrigeration (refluxing of this methanol fraction for 6 hours before refregeration is also advantageous). A gelatinous fluffy mass separated out which was removed after 24 hours. This mass was not positive to Liberman and Burchard test for sterols (LB test). The supernatant was concentrated and on reduction to half the volume another crop of fluffy mass was separated which was also negative to LB test. The remaining liquid was further concentrated to half the volume and transferred to a series of test tubes in about 8 ml volume, wherein crystals of sterols are recovered. The supernatant was decanted (siphoned off) every time, pooled and again allowed to crystalise. Crystals are washed often with methanol, till white crystals are obtained. The pooled crystals were finally picked up and purified with ethanol and recrystalised in a water glass, and dried in a dessicator preferably under vacuum and needle like crystals of sterols were recovered.

The green plant material was also processed in the similar manner and stigmasterol was isolated.

Tocopherols: The oil separated out in supernatant methanol, was evaporated to dryness and picked up in small amount of benzene. The cotton column method after Vil'yams and Semenova (1968) was followed and 18 x 1.6 cm column packed up by absorbent surgical cotton wool, in a burrette was set up. The recovered oil dissolved in benzene was charged in the cotton column and the column was eluted with benzene at a steady rate of about 100 drops per minute till all the yellow colour disappeared. Enough ethanol was then eluted

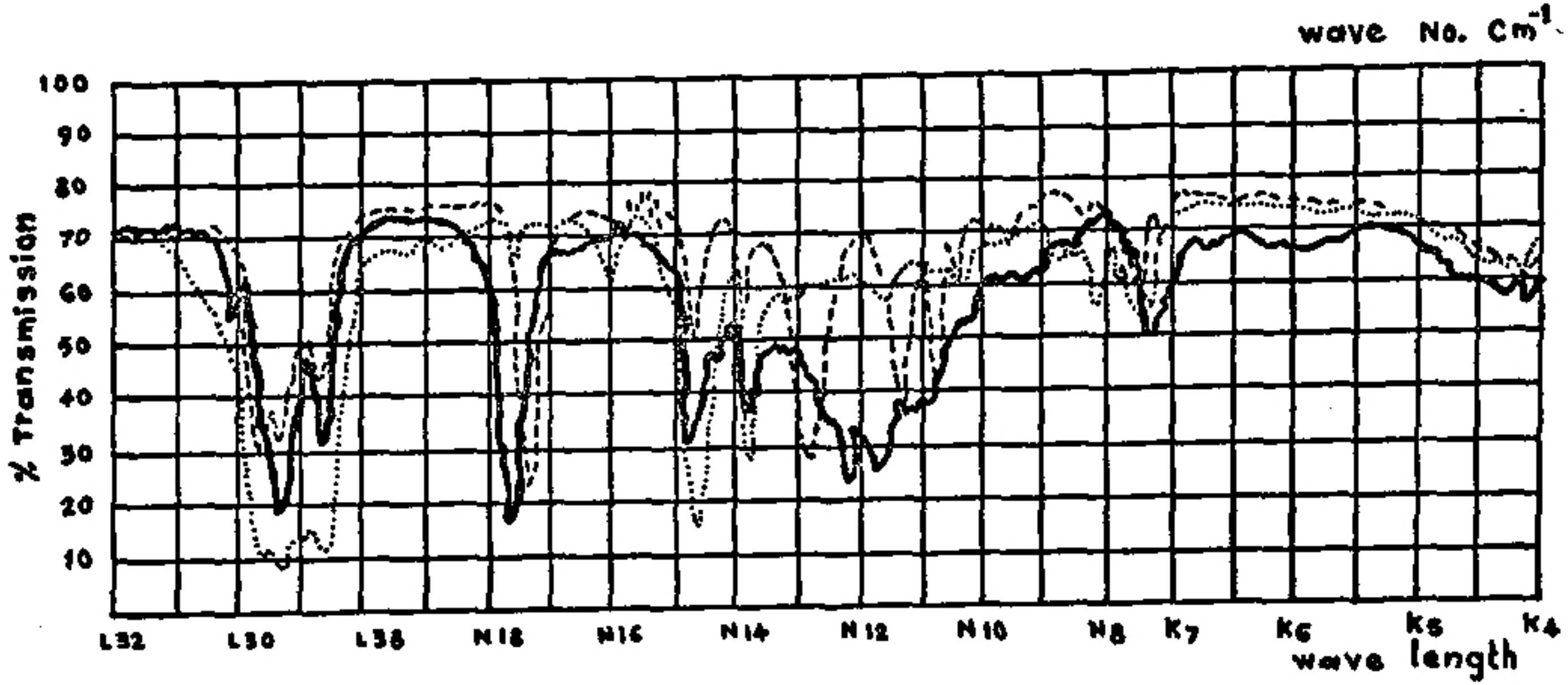
Fig. 1. IR spectra tracing for stigmasterols (on IR—10).



K = Potassium bromide prism.
N = Sodium bromide prism.
L = Lithium fluoride prism.
——= Reference standard stigmasterol (Calbiochem Lab. U.S.A.)
- - - -= Reference standard β sitosterol (Calbiochem Lab. U.S.A.)
. . . .= Stigmasterol isolated from Leptadenia reticulata.

The stigmasterols were used as 0.5% solution in carbon tetrachloride to charge the KBr Cell.

Fig. 2. IR *spectra* tracings for tocopherols (on IR—10).



K = Potassium bromide prism.

N = *Sodium* bromide prism.

L = Lithium fluoride prism.

——= Vitamin E oil reference standard (Glaxo Viteoline capsule. Tocopherol—100 mg)

..... Vitamin E oil H_2SO_4 purified.*

----- Vitamin E oil cotton column purified.*)

(*=*Isolated from Leptadenia reticulata*)

The oils were applied as wet film on KBr prism.

through the column, till it picked up all the absorbed oil. The benzene fraction contained carotenoids and the ethanolic fraction contained tocopherols. Ethanol was slowly evaporated and tocopherol oil was recovered.

The stigmasterol and tocopherols were typed and characterised by studying the melting points (for sterols) and by Infra-red radiation spectral analysis on IR 10 (Zies Ikon) apparatus. The stigmasterol and beta-sitosterol (from *Calbiochem Lab. USA*) and Veteoline (Vit. E oil of Glaxo Lab.) for tocopherols, were used as reference standard.

The IR method for stigmasterol was followed after Jhonson *et al* (1957) and for tocopherols after Harris and Wool (1962).

RESULTS AND DISCUSSION

Comparative yield of stigmasterol by different methods and the melting points are given in Table *1*. The yield of tocopherols have also been given.

TABLE—1

Comparative yield and melting point of stigmasterol and tocopherols from *Lr*.

	Substance	*Yield* %	*M.P.*	*Remarks*
SS	(Hydrated) from *Lr* reported after the method of Murthy and Sheshadhari (1944).	0.04	140°C	Purified
SS	(Hydrated) from *Lr* after-short-cut method.	0.035	139°C	Purified
SS	*(Hydrated) from Lr* after chromatographic method.	*0.029*	*120°C*	*remained crude*
SS	(Hydrated) from *Lr* green leaves after short cut method.	0.04	129°C	remained crude
Ref.	standard SS (hydrated) M.P. reported after Ikan (1969).	—	138°C to 144°C	
Ref.	standard SS (dehydrated) Calbiochem.	—	165°C	
Tocopherols after sulphuric acid method.		0.15	—	Yield of tocopherols from *Lr* has not been reported by any previous work.
Tocopherols after cotton column method (short cut).		0.20	—	

Lr—Leptadenia reticulata
M.P.—Melting point
SS—*Stigmasterol*

The comparative Infra-red spectral analysis curves are shown in Fig. 1, for sterols and in Fig. 2 for tocopherols.

On comparision of melting points and the IR analysis with the reference standard and according to various other works referred previously, the sterols and tocopherols could be characterised. The *Lr* yielded 0.035 per cent pure stigmasterol, and 0.2 per cent tocopherols out of which alpha tocopherol seemed to be the major component.

The short-cut-method for isolation of the non-saponifiable principles from *Lr* especially the stigmasterol and the tocopherols could be employed with ease and gave encouraging results particularly for the yield of stigmasterols in pure form. The process is simple, short, less time consuming and involves minimal use of solvents, which could be recovered to a good extent. The method could also be used for isolation of non-saponifiable material and phytosterols directly from the green plant. With the isolation of pure sterols, and on processing the remaining non-saponifiable oil, it was seen that cotton column method was very useful and gave a better yield (0.2 %) of tocopherols than sulphuric acid method (0.15 %).

It remains to be seen whether the present short cut method can be applied to other phytosterogenic plants for the separation of non-saponifiable principles and especially the phytosterols. If this be possible it may be a good addition to the processes available with analytical chemists and indigenous drug pharmacologists.

REFERENCES

Anjaria, J. V. and I. Gupta, 1967. Studies on lactogenic property of *Leptadenia reticulata* and Leptaden tablet in goats, sheep, cows and buffaloes. *Indian Vet. J.* **44**: 967.

Anjaria, J. V. and I. Gupta, 1970. Preliminary chemical investigations and extraction of *Leptadenia reticulata*. *Gujvet* **4**: 81.

Harris, R. S. and I.G. Wool, 1962. Vitamins and hormone, Academic Press, New York, and London. **20**: 412

Ikan, R., 1969. Natural Products. A laboratory guide; Academic Press, New York, 1st Ed. p. 113.

Johnson, Z.L., F. Marvin, and A.O. Jenson, 1967. Determination of stigmasterol in soyabean sterol mixture by IR Analytical chem. **29** : 468.

Mukherjee, and Shrivastava, 1951. A dictionery of Indian raw materials and Industrial products, CSIR New Delhi, India. *J. Sci. Indust. Res.* **10**: 3221. (c.f. The Wealth of India 1962. **6**: LN 73).

Murthy, P.B.R. and T.R. Sheshadhari, 1944. Wax and resin components of *Leptadenia reticulata*. Proc. *Indian Acad. Sci.* **20**: 261.

Parker, W.E. and W.D. Mc Farlane, 1940. A proposed modification of iron dipyridin method for determining tocopherol content of oils. *Canad J. Res.* **18** : 405. (c.f. Chem. Abst. **35** : 2351).

Paech, K. and M.V. Tracy, 1955. Modern methods of plant analysis, Springer Verlag, Berlin Gottinger—Heidelberg. **3**: p. 153.

Vil'yams, V. V. and N.K. Semenova, 1968. Distribution of vitamin E in plants. Izv. Timiryazer. Selskokhoz Akad., **9** (6) : 36-47 (c.f. Chem. Abst. 66361).