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Isolation and identification of stigmasterol *in vivo* and *in vitro* from *Sesamum indicum*

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Abstract

In the present investigation, isolation and identification of stigmasterol has been carried out *in vivo* and *in vitro* from *Sesamum indicum* L. of pedaliaceae family. Quantification data revealed that the total sterol content was higher in callus than leaves. The *in vitro* studies showed that the maximum amount of total sterol was found in 6 weeks old tissue and minimum in 2 weeks old callus culture. *In vivo* studies showed higher content of sterol was in leaf and lower was in stem. The present investigation was aimed to investigate stigma sterol present in *P. murex.* Stigma sterol has been isolated from various plant parts and callus cultures of *S. indicum* and was identified using TLC, GC-MS, mp, IR, and UV studies, which was comparable to that of the standard stigmasterol .

INTRODUCTION

Sterols comprise several major groups of steroids characterised by having a hydroxyl group at C-3, normally in the β-configuration and branching side chains from eight to ten or more carbon atoms at C-17. They occur widely throughout the animal and particularly the plant kingdoms. They have both structural roles, as membrane constituents, and a key place in the biosynthetic sequences which lead to the steroid hormones and other biologically active steroidal species. The sterols are the starting material for the biosynthesis of plant steroids [1]. A rapid method for quantification of sterols after thin layer chromatography has also been established [2].The common phytosterols reported from the plants are β-sitosterol, stigmasterol. Sterols have been isolated from large number of plant species and probably occur in all the angiosperms and gymnosperms, but so far no work has been done on the extraction of sterols from *S. Indicum.*

S. indicum (til) is an erect tropical annual plant growing up to 100 cm tall. Today, it is mostly grown in India and the Far East (China, Korea), but its origin is probably tropic Africa although some other sources seem to favour an Indian origin. In the present investigation, a protocol has been developed for callus establishment as well as isolation and identification of stigmasterol.

MATERIALS AND METHODS

Plant Material

All plant parts were collected from field conditions at Jaipur. Plant parts were cleaned and oven dried at 35ºC for 30 min and then at 25°C till constant weight was achieved and then powdered. The voucher specimen of experimental plant was deposited in Herbarium at Department of Botany, University of Rajasthan, Jaipur (RUBL NO. 20628).

Tissue Culture

Cotyledonary leaves (derived from mature seed germination) were surface sterilized with mercuric chloride ($HgCl_2$) solution (0.1; w/v) for 2 min and subsequently rinsed thrice with sterile distilled water and treated with antibiotic (ciprofloxacin, 250mgL^{-1}) prior to inoculation in order to remove any kind of microbial interactions. Surface sterilization was done in a horizontal Laminar flow hood fitted with ultraviolet light and cotyledonary leaves were inoculated in the flasks containing culture medium aseptically supplemented with NAA+BAP (5.0+0.5 mg/l). Cultured flasks were incubated in culture camber. The cultures were observed and examined every week and final morphogenetic data were recorded.

Growth indices

The maintained calli were harvested regularly at the transfer age of 2, 4, 6, 8 weeks. Each of the callus samples was harvested and their growth indices (GI) calculated on fresh weight basis.

$$GI = \frac{\text{Final wt. of the tissue} \quad \text{Initial wt. of the tissue}}{\text{Initial wt. of the tissue}}$$

Sterol Extraction

Dried and powdered samples were extracted with petroleum ether (150ml) in Soxhlet's apparatus for 24 hrs. on water bath. The residual mass was refluxed with 15% ethanolic HCl for 4 hrs. on water bath [3] The filtrate was then extracted with ethyl acetate in separating funnel. Ethyl acetate extraction was subjected to neutrality by repeated washing with distilled water and passed ones to sodium sulphate for 1 hour to remove traces of moisture. All ethyl acetate extracts were collected together and dried in vacuum. Choloroform was added to the residue to dissolve it and used for further analysis and spectral studies [4].

Thin layer chromatography

To examine phytosterols, thin layer chromatography was performed using silica gel G coated plates (20X20 cm; wet thickness 0.2-0.4 mm) which were dried at room temperature , activated at ~ 100°C in an oven for 30 min and brought to room temperature before use. Each of the EtoAc fractions of plant

Abbreviations :

MS	: Murashige and Skoog's medium	TLC	: Thin Layer Chromatography
IR	: Infra- Red Spectrosocopy	GC-MS	: Gas Chromatography Mass Spectroscopy

parts were analyzed using CO-TLC with standard reference markers (stigmasterol). All fractions were applied 1 cm above the edge of the activated TLC plates, developed in a number of solvent systems *viz.*, system A-hexane: acetone; 8:2 and system B- benzene: ethyl acetate; 85:15 [5].

PTLC (Preparative thin layer chromatography)

Preparative thin layer chromatography of isolated stigmasterol samples was also carried out as above on thick Silica Gel G coated plates (500 µg thick) by spotting extract along with standard. The developed plates were compared with authentic samples under UV light. Spot coinciding with standard compound was marked and collected by scrapping silica gel G, dried, weighed (mg/g.dw) and crystallized in methanol and water. Crystals were subjected to Mp, GC-MS and IR studies.

Gas Chromatography and Mass Spectroscopy (GC-MS)

The Gas Chromatography-Mass Spectrometry (GCMS) data of *P. murex* and *S. indicum* were obtained on the GCMS-QP 2010 Plus of SHIMADZU company, using a Rtx-5MS column (60 m × 0.25 mm i.d., film thickness 0.25 µm). Both plants sample (methanolic extract of Leaf, stem and calli) of 0.2 ml was injected

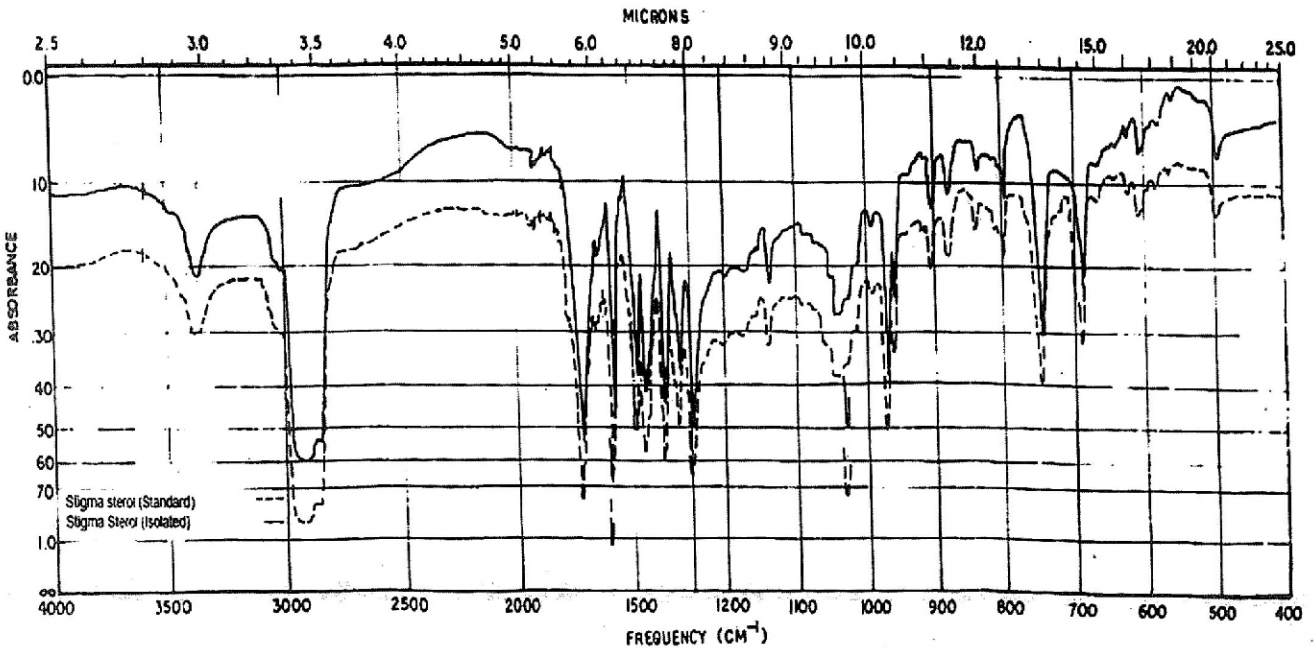

Figure 1 : Infrared spectra of standard and isolated Stigmasterol.

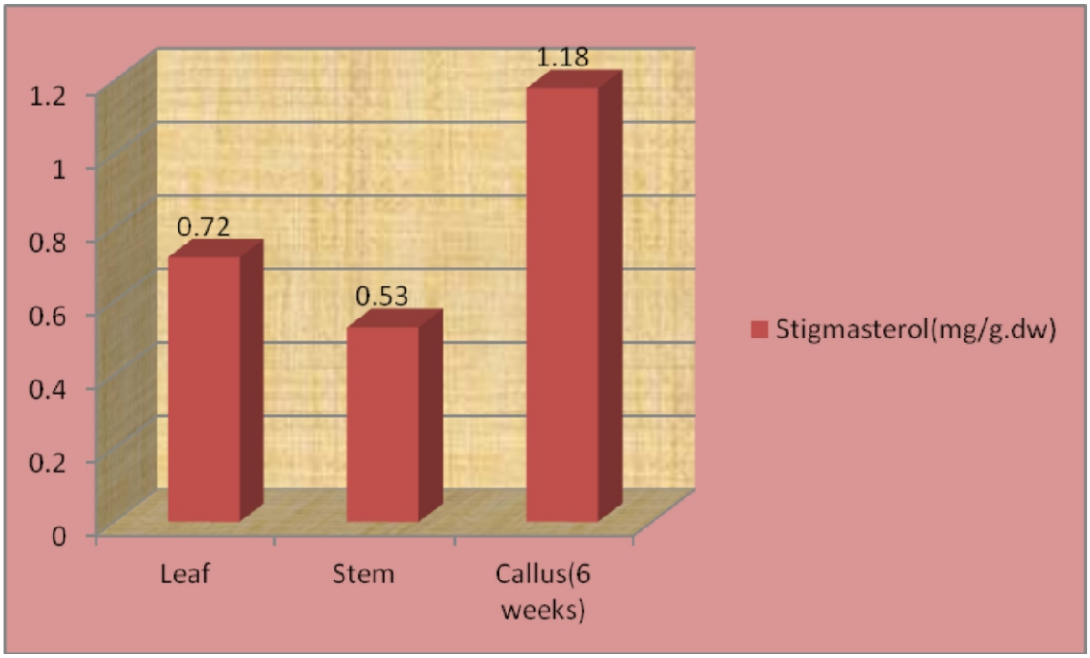

Figure 2 : Stigmasterol Content (mg/gdw) in Various Plant Parts and Callus Cultures of S. indicum

into column where carrier gas was helium. Temperature programming was 2 min at 100 °C, rising at 5°C/min to 220 °C, with a hold time of 20 min at 220 °C; split, 1:50. The compounds were identified by comparing their retention indices of peaks on the Rtx-5MS column with literature values, computer matching against the library spectra build up using pure substances and components of known sterols. Retention indices were determined by using retention times of *samples* that were injected under the same chromatographic conditions. The components of the standard and plant samples were identified by comparison of their mass spectra and retention time with those given in literature and by comparison with the mass spectra of the library or with the published mass spectra.

RESULTS AND DISCUSSION

Tissue culture

Cotyledonary leaves explants of *S. indicum* L. was tried with MS medium supplemented with NAA and BAP individually and integratively for direct regeneration. The callus formation was observed in all the concentration of growth hormones tried but maximum callus growth was observed when MS medium was supplemented with NAA+ BAP(5.0+0.5mg/l). The GI was calculated at various time intervals of 2, 4, 6 and 8 weeks. The GI was found to be minimum in 2 weeks (0.72) and maximum in 6 weeks old tissue (2.23).

Stigmasterol

The qualitative and quantitative analysis of stigmasterol from plant parts and callus cultures of *S. indicum* was carried out. Among various plant parts maximum stigmasterol content was observed in leaves (0.72 mg/gdw) followed by stem (0.53 mg/gdw). Callus cultures had higher (1.18 mg/gdw) content than leaves and stem (Fig. 2).

GC-MS

The GC-MS studied showed that the retention time and peaks of the isolated stigmasterol was comparable with that of standard. Many new compounds were identified in plant samples as shown (Table-2and Fig.3 and 4)

CONCLUSION

In the present investigation callus was raised from cotyledonary leaves on each of the combination hormonal doses, the best response was observed in combination doses of

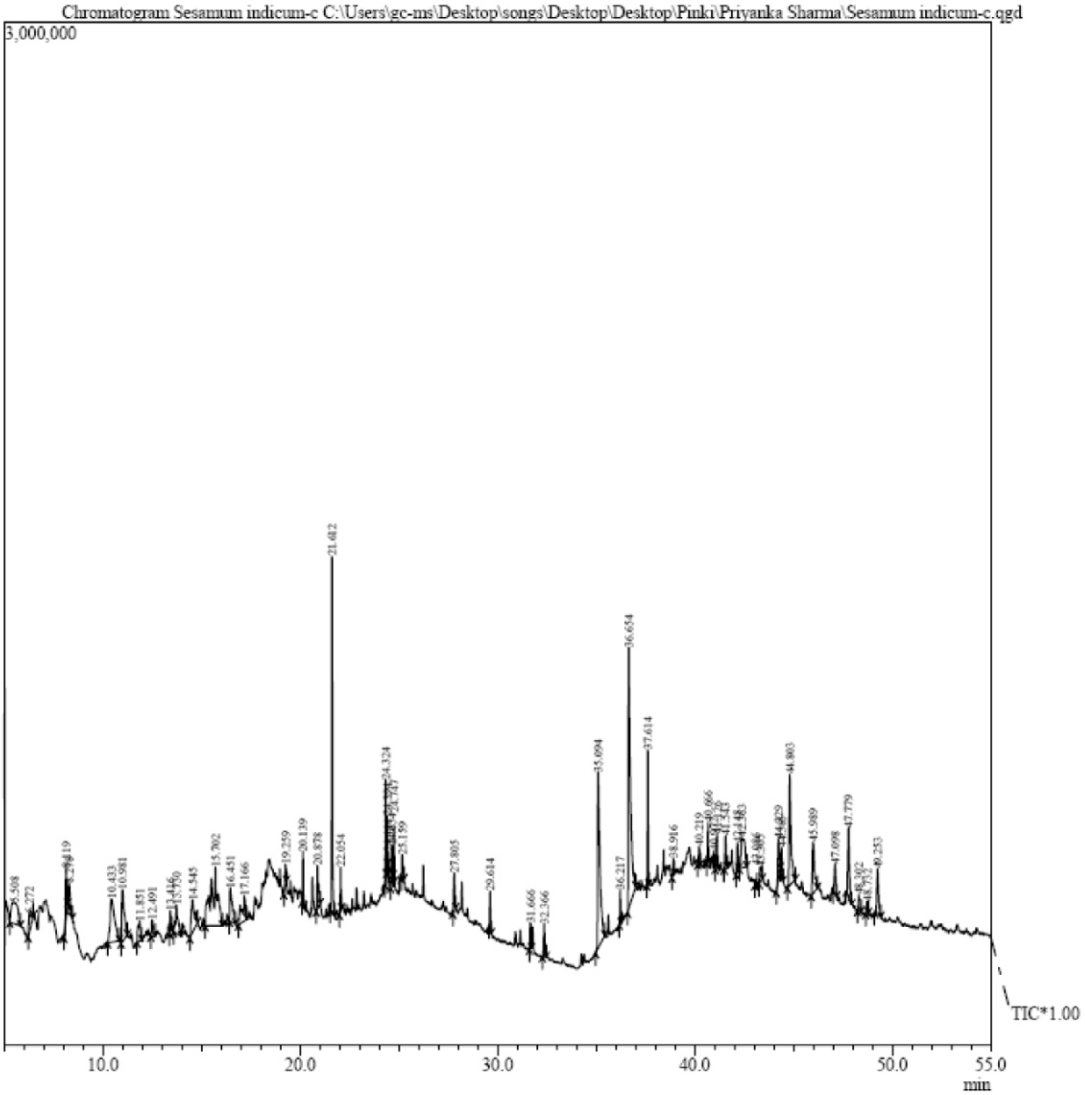

Figure 3 : GC-MS Spectra of Compounds identified from isolated sterol extract from S.indicum

Table 1 : Yield of sterols isolated (mg/g. dw) from plant parts and tissue culture of *S. Indicum*

Plant Parts & Callus	Stigmasterol(mg/g.dw)
Leaf	0.72
Stem	0.53
Callus(6 weeks)	1.18

NAA+BAP(5.0+0.5mg/l). In the present study presence of stigmasterol in *S. indicum* was reported. The maximum content was found in 6 weeks old callus (1.18mg/gdw) as compared to other plant parts. The presence of stigmasterol in *S. indicum* under the present investigation was confirmed by IR and GC-MS analysis.

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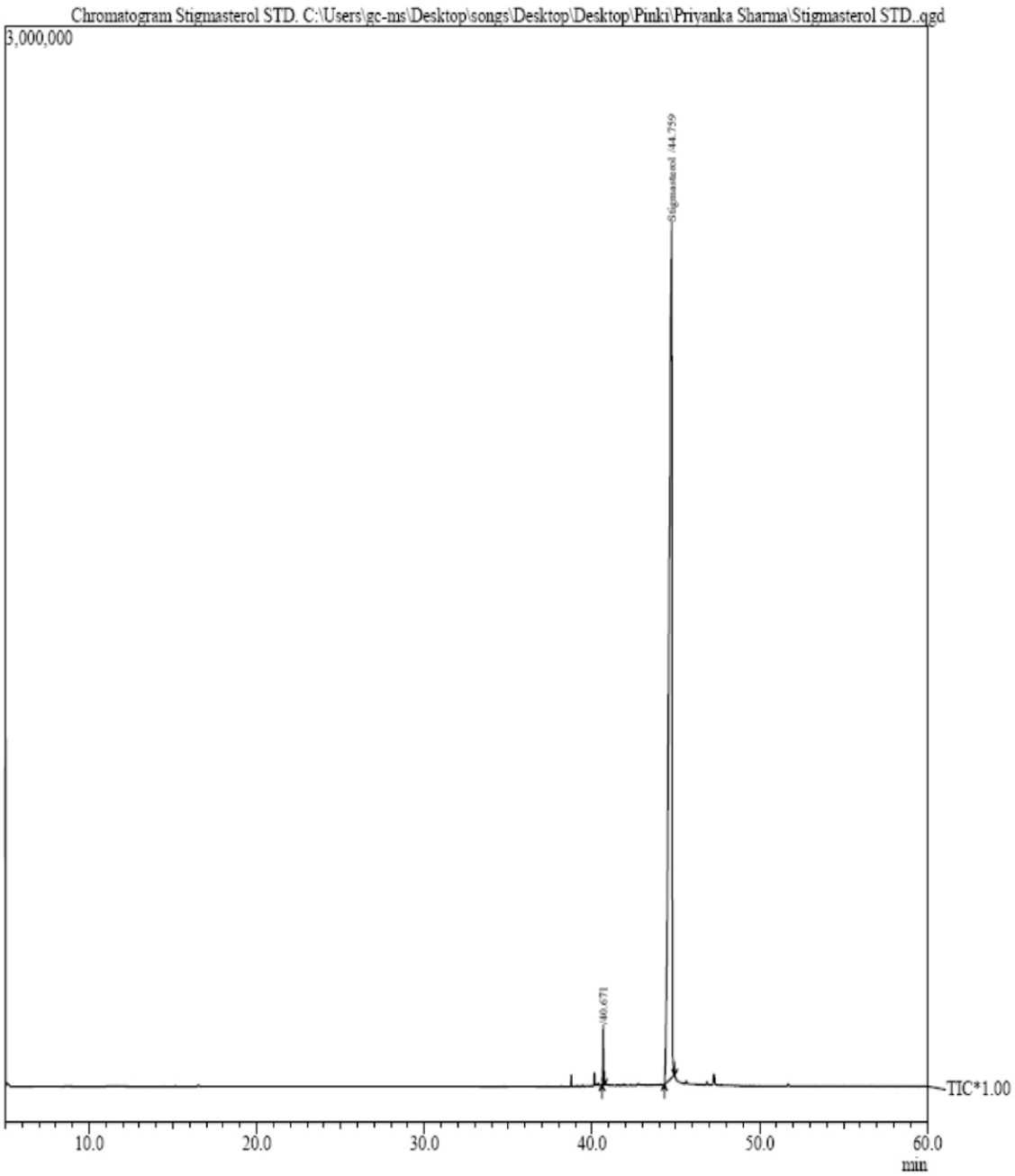

Figure 4 : GC-MS spectra of standard Sterol (Stigmasterol)

Table 2 : Compounds identified from methanolic extract of *Sesamum indicum* callus by GC-MS

Peak#	R.Time	Area	Area%	Name
1	5.508	1486826	3.34	Anone
2	6.272	455669	1.02	1-PROPENOL-3
3	8.119	511845	1.15	Furaneol
4	8.278	443585	1	1-Ethyldecyl acetate
5	10.433	2108917	4.73	(-)-cis-Myrtanol
6	10.981	1373015	3.08	3-Acetylanisole
7	11.851	529934	1.19	3-Chloropropionic acid, tridecyl ester
8	12.491	305018	0.68	Methyl 6-deoxyhexopyranoside
9	13.416	216251	0.49	5,7-Octadien-2-one, 3-acetyl
10	13.73	800106	1.79	Mint furanone
11	14.545	1074860	2.41	Guanosine
12	15.702	3967899	8.9	Ketopinic acid
13	16.451	896117	2.01	Oxiranecarboxylic acid, 3-methyl-3-phenyl-,
14	17.166	931050	2.09	Methyl 3-cyclohexenecarboxylate
15	19.259	200822	0.45	Alloaromadendrene oxide
16	20.139	333550	0.75	Phytol
17	20.878	542923	1.22	(-)-Alloaromadendrene
18	21.612	2797246	6.27	Hexadecanoic acid, methyl ester
19	22.054	317544	0.71	Benzenepropanoic acid, 3,5-bis(1,1-dimethy
20	24.324	625424	1.4	1-Octadecanol
21	24.394	286067	0.64	Oleic acid, methyl ester
22	24.444	66141	0.15	Linolenic acid, methyl ester
23	24.634	397934	0.89	1-Heptatriacontanol
24	24.747	332899	0.75	Stearic acid, methyl ester
25	25.159	282513	0.63	Kauren-18-ol, acetate, (4.beta.)-
26	27.805	487275	1.09	Palmitaldehyde
27	29.614	307563	0.69	n-Eicosylcyclohexane
28	31.666	231814	0.52	Tetratriacontane
29	32.366	358702	0.8	1,2-Benzenedicarboxylic acid, mono(2-ethy
30	35.094	4394717	9.86	Anthraquinone, 2-methyl
31	36.217	247304	0.55	Kemester
32	36.654	5864673	13.16	Flavonol
33	37.614	908233	2.04	Squalene
34	38.916	131812	0.3	Cerotic acid, methyl ester
35	40.219	205841	0.46	Cholesterol, chloroformate
36	40.666	332881	0.75	Stigmasta-4,7,22-trien-3.alpha.-ol
37	40.972	45959	0.1	(-)-Cholesterol
38	41.126	379642	0.85	Dotriacontane
39	41.543	358387	0.8	Cholesta-4,6-dien-3-ol, (3.beta.)
40	42.148	286753	0.64	Methyl triacontanoate
41	42.363	1276330	2.86	Lupenyl acetate
42	43.086	56638	0.13	Cyclononasiloxane, octadecamethyl
43	43.305	251830	0.56	Sesamin
44	44.229	662164	1.49	Nonacosane
45	44.385	264901	0.59	n-Hexatriacontane
46	44.803	2149300	4.82	**Stigmasterol**
47	45.989	1179733	2.65	Beta-Sitosterol
48	47.098	475815	1.07	Cholestenone
49	47.779	1195494	2.68	Stigmasterol, 3,4-dedihydro-, acetate
50	48.302	238376	0.53	Stigmasta-3,5-dien-7-one
51	48.732	157279	0.35	Tetrapentacontane
52	49.253	846185	1.9	Sitostenone
		44579756	100.00	

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