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ORIGINAL ARTICLE

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Development and Validation of a Simple HPLC PDA Method for the Simultaneous Analysis of 13-Docosenamide, Squalene and n-Tetracosanol-1 from the Leaf Extracts of *Wagatea spicata*

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Wagatea spicata (Dalzell) Wight [*Moullava spicata* (Dalzell) Nicolson] exhibits a diverse concentration of biologically active constituents such as Lupeol, Bergenin, Stigmasterol, Friedelin, n-Hexadecanoic acid, Palmitic acid, Gamma sitosterol,13docosenamide, squalene and n-Tetracosanol-1.

Present work focuses on development and validation of a precise, accurate and reproducible HPLC method for simultaneous quantification of three pharmacologically active phytochemical markers 13docosenamide, squalene and n-Tetracosanol-1 from the aerial parts of *Wagatea spicata*.

Key words: HPLC, 13-docosenamide, squalene, n-Tetracosanol-1, simultaneous, method development, method validation

Traditional knowledge of India is gaining increasing acceptance all over the world for its therapeutic efficacy and safety (Chaudhary and Singh, 2011). However, only a small percentage of the entire treasure has been explored and there is a lot more to be put forth. To meet this objective, it is essential that the ethnomedicinal plants of Indian origin are scientifically studied for their known medicinal benefits and documented. Development of a formulation from such plants will also require extensive standardization for establishing the quality and/or efficacy of individual plant components as well as raw material or as a final product component. (Nandini *et al.*, 2017)

With the above objective in mind, the isolation of the compounds 13docosenamide, squalene and n-Tetracosanol-1 has been previously performed using HPTLC and the structural elucidation was performed with the help of FTIR, NMR and GCMS techniques (Surange *and* Deokule, 1986; Nandini *and* Vikas, 2019). Further, it was felt worthwhile to develop simultaneous methods for the quantitative analysis of the isolated components.

Thus, the present work explains the development of a simple, sensitive and accurate high-performance liquid chromatographic method for the simultaneous determination of 13docosenamide, squalene and n-Tetracosanol-1 from the leaf extracts of the plant *Wagatea spicata*.

MATERIALS AND METHODS

Chemicals and materials

HPLC grade Methanol, Ethanol was procured from E. Merck, Mumbai, India. Reference standards of 13 Docosenamide (Purity>95%), Squalene (Purity>95%), and n-Tetracosanol-1 also called as Lignoceryl alcohol (Purity>95%) were purchased from Sigma-Aldrich (Aldrich Division; Steinheim, Germany).

Plant Material

Wagatea spicata fresh plant was collected from the field area of Kankeshwar, Alibaug, District- Raigad, and Maharashtra, India in the month of November 2014; and the herbarium specimens were identified and authenticated by Botanical Survey of India, Pune.

Sample preparation

1 g of fine leaf powder in 10ml of ethanol was subjected to accelerated maceration by ultrasonication for 30 minutes, followed by overnight steady state extraction. After filtration using syringe filter (pore size 0.2 microns), the clear extract was used for HPLC analysis.

Preparation of standard solution(s)

Individual stock solutions of n-Tetracosanol-1, 13 docosenamide and Squalene (1000 µg/ mL) were prepared in ethanol. 1000 µg/mL stock solution of standard mixture was also prepared in ethanol.

RESULTS AND DISCUSSION

Optimization of the Chromatography

Initial trial experiments were conducted to select a suitable mobile phase for accurate analysis of the standards and HPLC grade acetonitrile containing formic acid (0.1%) in isocratic mode was finalized as the best for the separation of the three analytes 13 docosenamide, squalene and n-tetracosanol-1.

The separation of analytes of interest was also evaluated at different flow rates (0.6-1mL/min). Finally, the flow rate was optimized to 1 mL/min to avoid the interference of solvent peaks. Considering the complexity of herbal samples, the total run time for the method was determined to be 35 mins.

The wavelength of 202 nm was selected for the detection of analytes eluting out from the column as phytoconstituents under study demonstrated the maximum absorption at the specified wavelength.

The optimized chromatographic conditions, chromatograms and spectra of individual standards, standard mixture and sample extract as obtained under the same chromatographic conditions are depicted in Table 1 and Figures 1-7 respectively.

Method Validation

The developed method was validated as per tripartite ICH guidelines (ICH Harmonised Tripartite Guideline, 2005).

Selectivity*:* As shown in figures 7A and 7B, there was no interference observed from diluent blank (ethanol) and mobile phase overlapping with the

retention times of 13 docosenamide, squalene and n-tetracosanol-1. Hence the method is selective.

***System suitability**:* The RSD values for area and retention time of 13 docosenamide, squalene and n-tetracosanol-1 and were found to be <2% indicating that the system was suitable to carry out further analysis. Squalene, 13 docosenamide and n-Tetracosanol-1 were detected at 15.298, 8.963 and 32.054 minutes respectively.

***Linearity**:* The method was found to be linear from 50-300 µg/mL for 13 docosenamide, 100-300 µg/mL for squalene, 5-300 µg/ml for n-Tetracosanol-1. The correlation coefficient was found to be ≥0.99 for all the three components.

Sensitivity: Sensitivity of the method was affirmed in terms of LOD and LOQ for 13 docosenamide, squalene and n-Tetracosanol-1.

The value of limit of detection was found to be 50 µg/mL for squalene and 13 docosenamide and while it was 5 µg/mL for n-tetracosanol-1, whereas the limit of quantification was found to be 100 µg/mL for squalene, 50 µg/mL for 13 docosenamide and 5 µg/mL for n-Tetracosanol-1 (Griffiths, 2003; Vuppugalla *et al*., 2003, Lu *et al.*, 2004; Wichitnithad *et al*, 2009; Le *et al.*, 2019).

***Precision**:* In the repeatability study, intra-day and inter-day precision of the HPLC method were investigated using replicate injection (*n*=3) of quality control samples of all the three standards. The developed method was found to be precise with RSD<2%.

Robustness: As inferred from the %RSD values of robustness testing results, it can be said that the proposed method was not influenced by slight change in the wavelength of analysis. Minor change in flow rate and mobile phase composition affected the separation of the three analytes. However, the simplicity of the method increases the robustness by minimizing mobile phase alterations.

Stability: Stability studies showed that the components were found stable in the mixture for at least 24.0 h at room temperature and 48 hours at 2-8°C of storage condition. The individual stock solutions, however, were stable for up to 72 hours under both the conditions.

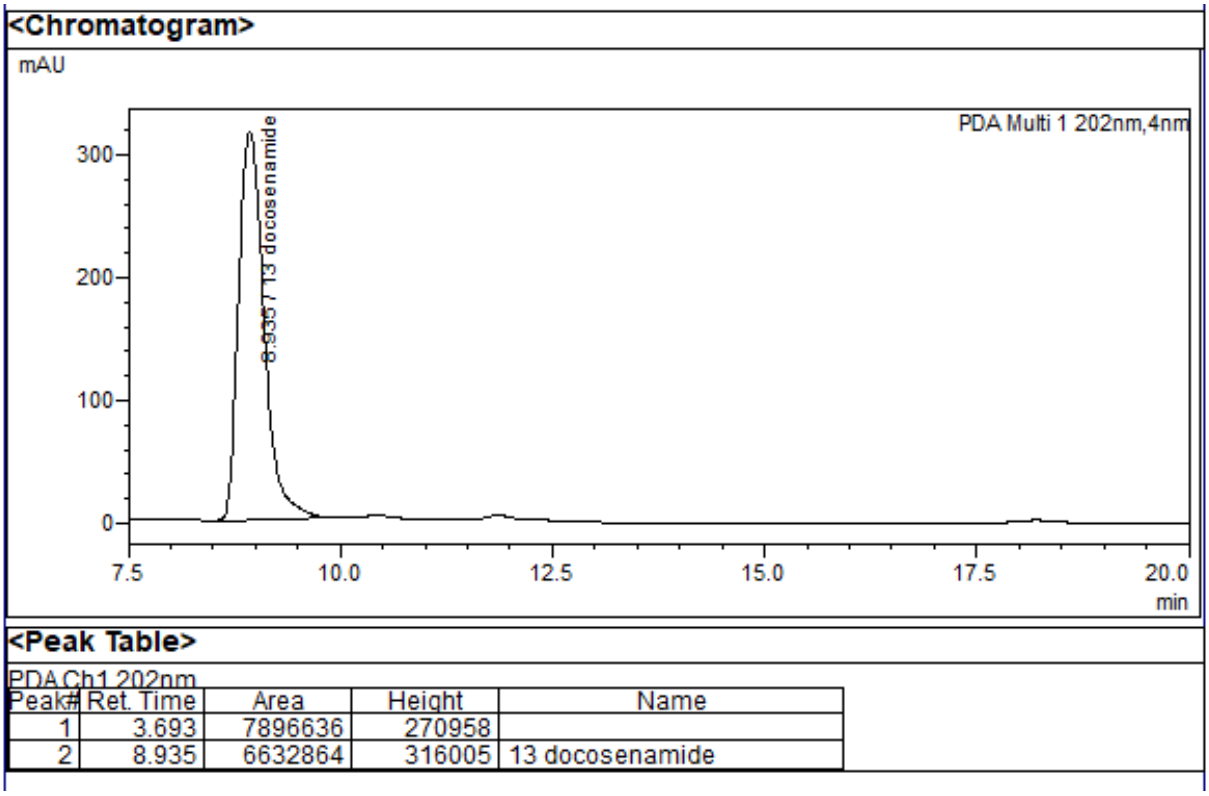
Assay: The percentage of n-tetracosanol-1, 13 docosenamide and Squalene in the leaf extracts of *Wagatea spicata* extract was found to be 0.009%, 2.54% and 1.07% respectively. The method is sensitive and selective for the aforementioned phytoconstituents in presence of other phytochemicals present in the extract.

Recovery: The recovery values for all the three components were within acceptable limits (80.0 to 120.0%). This indicated that the method was reliable and accurate.

Thus, the proposed HPLC method was found to be suitable for qualitative and simultaneous quantitative analysis of 13 docosenamide, squalene and n-Tetracosanol-1 in the ethanolic extract of *Wagatea spicata*. The summary of validation is charted in Table 2.

Table 1: Optimised Chromatographic Conditions.

Parameter	Description
Instrument	HPLC- Prominence-i, LC-2030C 3D Plus Liquid chromatograph
Pump	LC 2030 pump
Injector	LC 2030, Autosampler
Injection volume	50ul
Column oven	LC 2030 oven,40^{o}C
Column	Shimadzu, C18 250mm X 4.6 mm, 5µm
Mobile Phase	HPLC grade acetonitrile containing formic acid (0.1%)
Flow Rate	1mL/min
Detector	LC 2030/2040 PDA
Detection Wavelength	202nm

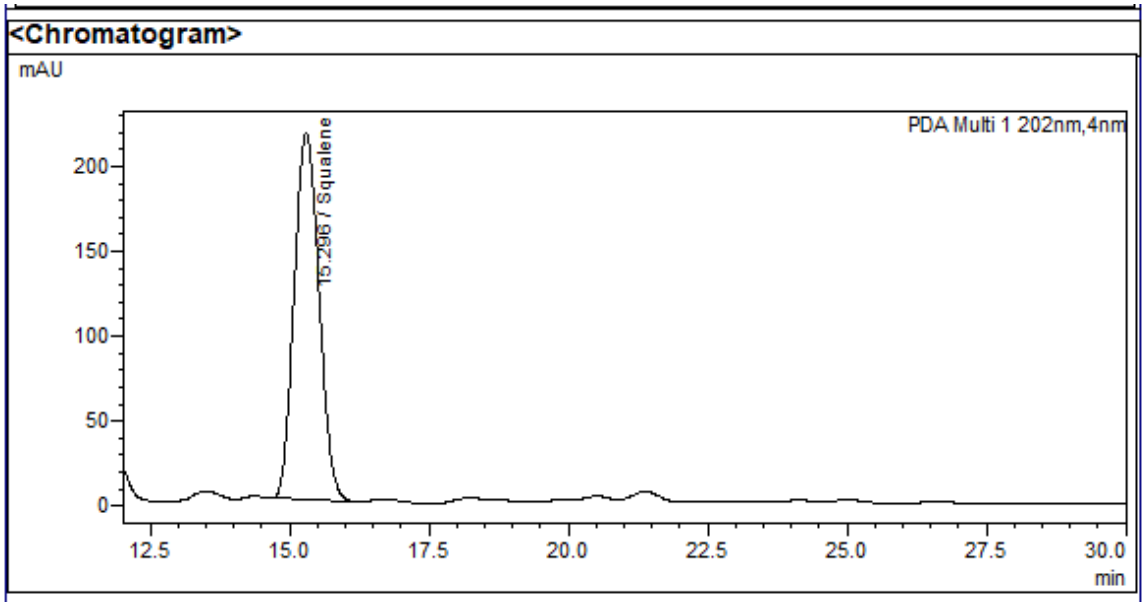


<Peak Table>

PDA Ch1 202nm

Peak#	Ret. Time	Area	Height	Name
1	3.693	7896636	270958	
2	8.935	6632864	316005	13 docosenamide

A



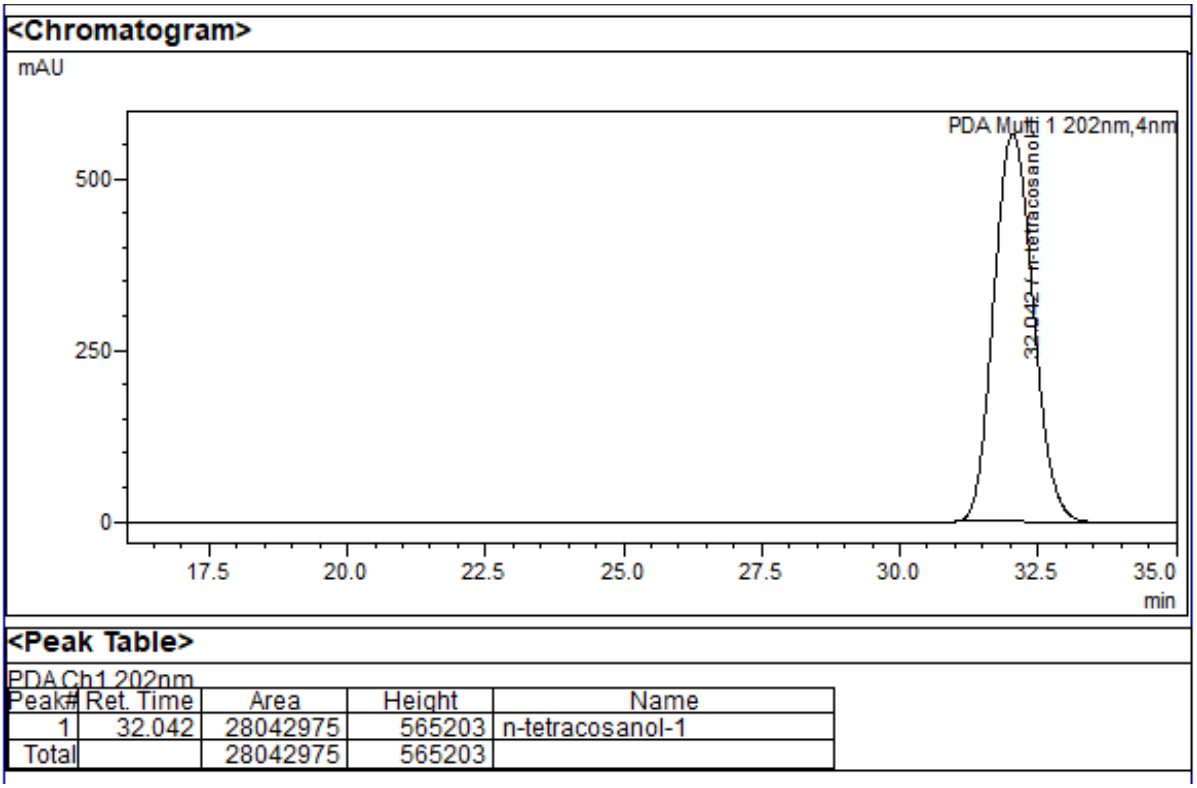
<Peak Table>

PDA Ch1 202nm

Peak#	Ret. Time	Area	Height	Name
1	2.837	2954943	372324	
2	3.004	6410179	346734	
3	3.696	4516941	179659	
4	15.296	6679980	215758	Squalene

B

Figure 1: A - HPLC chromatogram of standard 13 docosenamide; **B -** HPLC chromatogram of standard squalene;

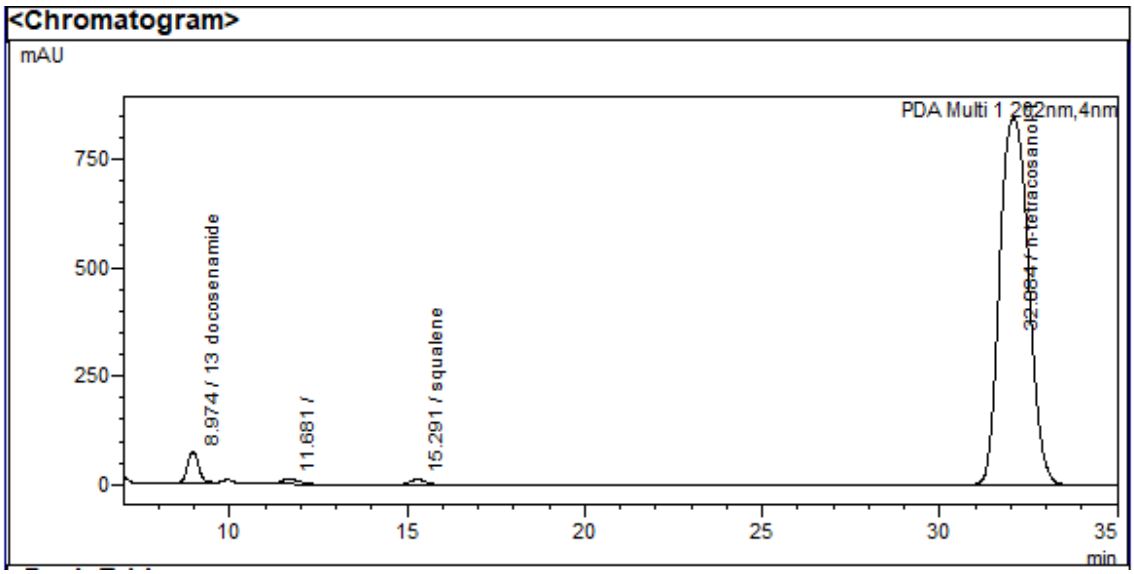


<Peak Table>

PDA Ch1 202nm

Peak#	Ret. Time	Area	Height	Name
1	32.042	28042975	565203	n-tetracosanol-1
Total		28042975	565203	

A



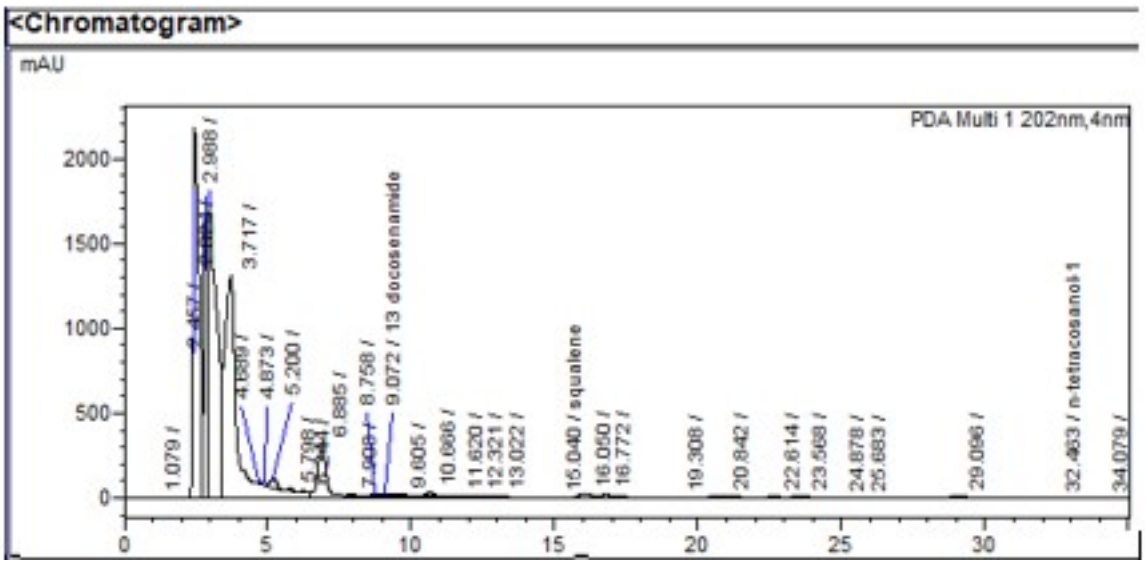
<Peak Table>

PDA Ch1 202nm

Peak#	Ret. Time	Area	Height	Name
1	8.974	1684717	72330	13 docosenamide
2	11.681	438963	11816	
3	15.291	394912	12731	squalene
4	32.084	47666422	844539	n-tetracosanol-1
Total		50185014	941416	

B

Figure 2: **A** - HPLC chromatogram of standard n-tetracosanol-1 ; **B** - HPLC chromatogram of standard mixture

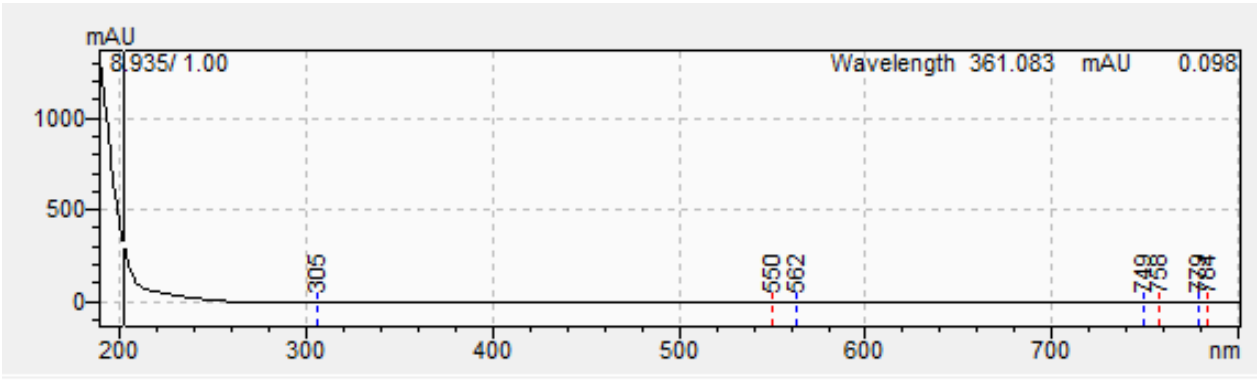


Peak Table

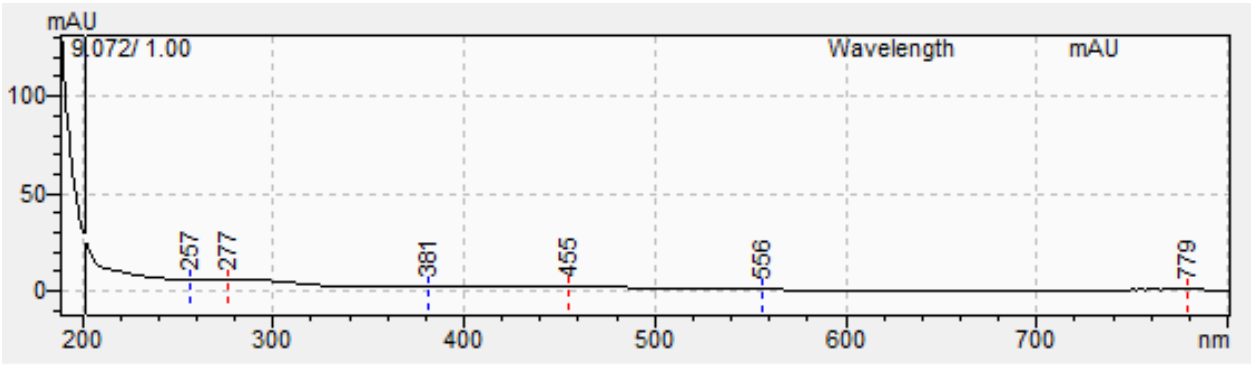
PDA Ch1 202nm

Peak#	Ret. Time	Area	Height	Name
1	1.079	8888	352	
2	2.457	31047203	2180566	
3	2.824	11646167	1776267	
4	2.988	39405061	1725598	
5	3.717	42304879	1304481	
6	4.689	85499	10424	
7	4.873	140786	14624	
8	5.200	859633	53472	
9	5.798	189010	13690	
10	6.244	85337	7458	
11	6.885	8803191	317681	
12	7.908	66163	5545	
13	8.758	114570	7648	
14	9.072	279125	12858	13 docosenamide
15	9.605	410607	17034	
16	10.666	486386	22043	
17	11.620	215841	5937	
18	12.321	46645	1428	
19	13.022	63122	2286	
20	15.040	6917	333	squalene
21	16.050	632275	24601	
22	16.772	615166	14356	
23	19.308	27150	745	
24	20.842	497251	10234	
25	22.614	128474	4120	
26	23.568	298015	8711	
27	24.878	54385	1532	
28	25.683	64404	1441	
29	29.096	295818	6958	
30	32.463	47626	1039	n-tetracosanol-1
31	34.079	171376	3805	
Total		139096971	7557268	

Figure 3: HPLC chromatogram of leaf extract of *Wagatea spicata*

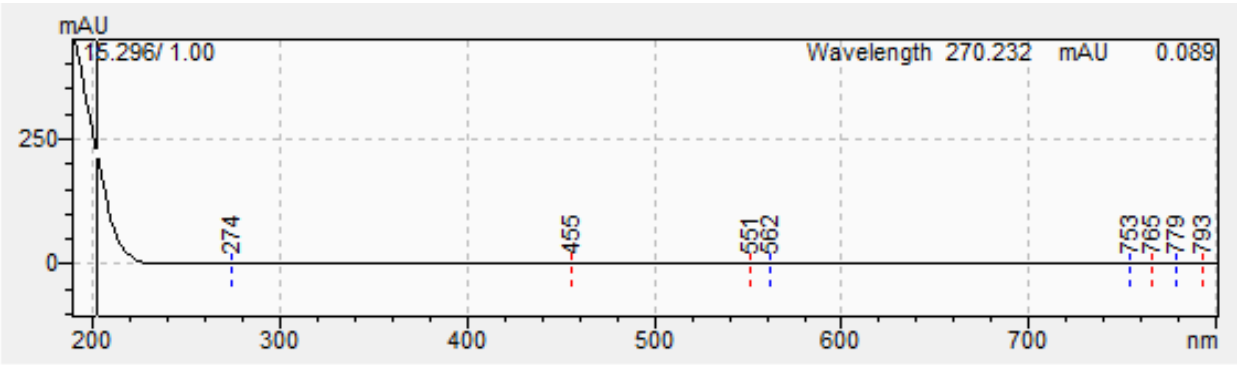


A

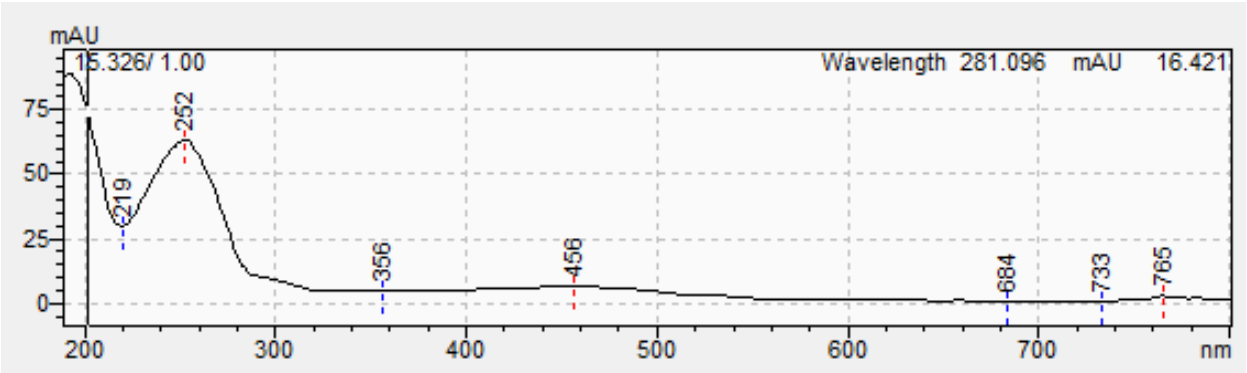


B

Figure 4: A - Spectrum of 13 docosenamide standard; B - Spectrum of 13 docosenamide from sample.



A



B

Figure 5: A - Spectrum of squalene standard; B - Spectrum of squalene from sample.

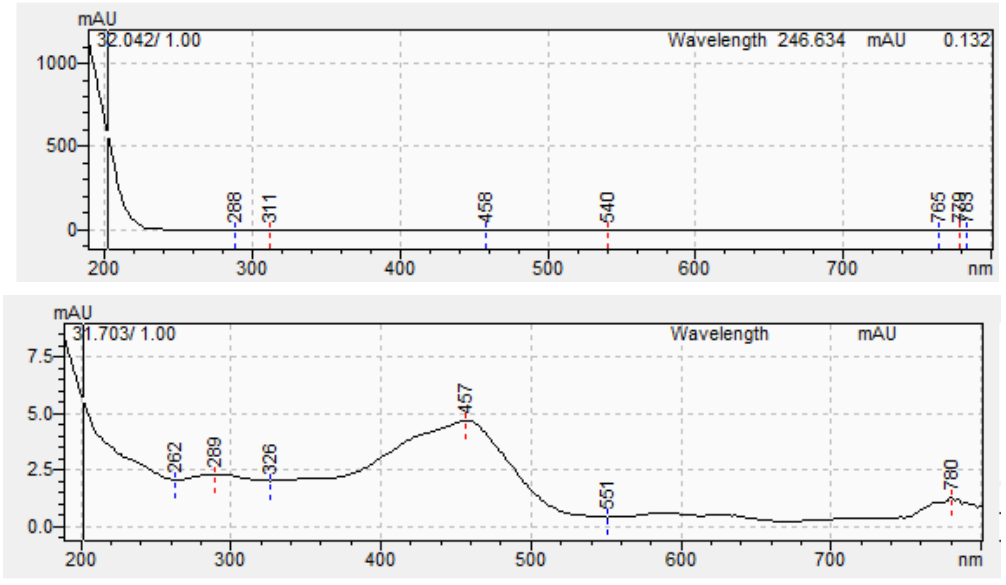
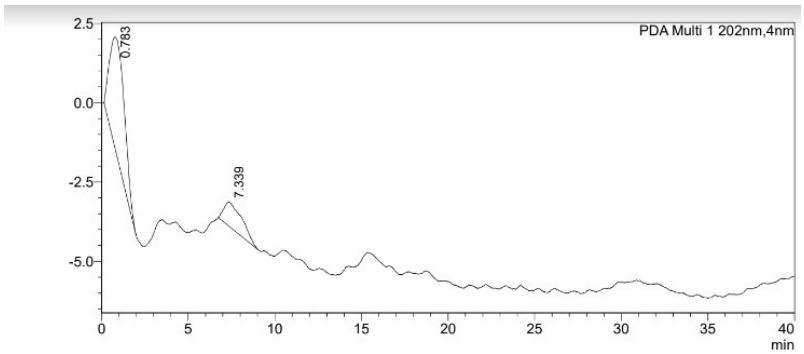


Figure 6: A - Spectrum of n-tetracosanol-1 standard; B - Spectrum of n-tetracosanol-1 from sample.

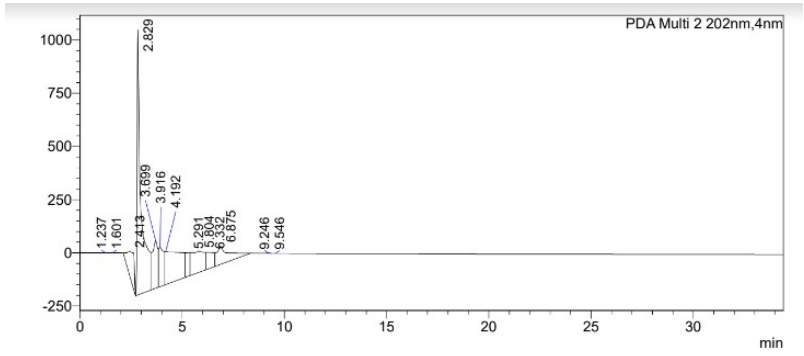


<Peak Table>

PDA Ch1 202nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	0.783	217163	3525	78.461			
2	7.339	59615	755	21.539			
Total		276779	4280				

A



<Peak Table>

PDA Ch2 202nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	1.237	1264	46	0.003			
2	1.601	1214	198	0.003		V	
3	2.413	3132303	109685	6.697			
4	2.829	16676098	1243806	35.656			
5	3.699	3882022	223511	8.300		V	
6	3.916	3293884	184211	7.043		V	
7	4.192	8065380	155756	17.245		V	
8	5.291	1575381	110875	3.368		V	
9	5.804	4536933	99339	9.701		V	
10	6.332	1867379	74910	3.993		V	
11	6.875	3732673	84721	7.981		V	
12	9.246	2603	237	0.006			
13	9.546	1987	224	0.004			
Total		46769121	2287520				

B

Figure 7: A - HPLC chromatogram of mobile phase; B - HPLC chromatogram of ethanol blank .

Table 2: Summary of Validation

Parameter	13 docosenamide	Squalene	n-Tetracosanol-1
System Suitability(%RSD)	Area = 0.696 Rt = 0.52	Area=1.476 Rt = 0.589	Area = 0.393 Rt = 0.585
Specificity and Robustness	Specific and Robust	Specific and Robust	Specific and Robust
Precision(%RSD) Intraday,200(µg/ml)	0.779	0.255	1.558
Precision(%RSD) Interday, 200(µg/ml)	0.094	0.709	0.914
LOD	50 µg/ml	50 µg/ml	5 µg/ml
LOQ	50 µg/ml	100 µg/ml	5 µg/ml
Linearity	50-300 µg/ml	100-300 µg/ml	5-300 µg/ml
Assay	2.54%	1.075%	0.009%
Recovery	100.894%	97.550%	100.787%

CONCLUSION

The current work provides a simple, precise, accurate and reproducible method for the qualitative and quantitative analysis of 13 docosenamide, squalene and n-Tetracosanol-1 from *Wagatea spicata*. The developed method can be used as a tool to asses phytochemical variation caused due to diverse geographical, climatic, genotypic factors. It can also be used as a quality control method for the plant and formulations containing *Wagatea spicata*.

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