Original Article

Development and Validation of UV Spectrophotometric Method for Estimation of Tea Tree Oil in Bulk and Cosmeceutical Creams

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ABSTRACT

Background: Tea tree oil, also known as *Melaleuca alternifolia* essential oil is widely used in skin care cosmeceuticals for its antibacterial, antifungal, analgesic and anti-inflammatory effect. The complex and variable composition of the oil poses many challenges to the analytical chemist and a recent survey of analytical methods indicated only a few simple and validated methods for estimation of the oil. **Aim:** A quality by design approach has therefore been adopted to develop a simple and novel UV spectrophotometric method for estimation of tea tree oil in bulk and cosmeceutical creams. **Materials and Methods:** UV spectrophotometric method was developed for estimation of Tea tree oil using dichloromethane- methanol (54 % v/v) solvent system. The method was then validated under optimized conditions for accuracy, precision and ruggedness, limit of detection and limit of quantification as per ICH: Q2 (R^1) guidelines. **Results:** A characteristic spectral peak was observed at 267 nm and linearity was established over a concentration range of 10-160 mcg/ml with R^2 value of 0.9961. Accuracy based on recovery studies, 100.5-113.6 %, precision and ruggedness based on % RSD values less than 2 %, LOD, 0.1277mcg/ml and LOQ and 4.195 mcg/ml indicated that the method is sensitive enough to be used for routine estimation. **Conclusion:** The method is robust and has been used to determine the essential oil content of two cosmeceutical creams.

Key words: Tea tree oil, UV spectrophotometry, Box Behnken design, Optimization, Routine determination, Cosmeceutical cream.

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INTRODUCTION

TTO, an essential oil obtained by steam distillation of aerial parts of *Melaleuca alternifolia* is widely used in skin care cosmeceuticals for its antibacterial, antifungal, analgesic and anti-inflammatory effect.[1] TTO is included in European Pharmacopoeia, Merck Index and in Indian Pharmacopoeia Addendum 2016. The chemical composition of tea tree oil is defined by international standard ISO 4730: 2017 and the identical Australian standard AS 2782-2017 which specifies levels for 15 of more than 113 components found in the pure oil including physical parameters like relative density, refractive index and optical rotation. Terpinen-4-ol is the principal constituent of the oil (35%- 48%) followed by γ-terpinene (14% -28%), α-terpinene (6%-12%) and 1, 8-cineole also known as eucalyptol (≤15%), all of which have potential antimicrobial activity.[2] The physical and chemical properties specified in the document published by the Health Canada Pest Management Regulatory Agency states that the oil is not expected to show a λ_{max} beyond 300nm. Hence the obtained λ_{max} at 267nm cannot be attributed to any particular constituent and the oil used in the study conforms to the limits mentioned in ISO4730:2017 and AS 2782:2017 standards. The complex and variable composition of TTO poses many challenges to the analytical chemist and as indicated from a

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literature survey, only a few simple validated methods for estimation of the oil have been reported. However, UV spectrophotometric method for analysis of TTO has not been reported till date. Biju SS *et al.* 2005 has reported a validated HPTLC method for determination of TTO content of cosmeceutical formulations by estimating the principal constituent, terpinen-4-ol.[3] Likewise Sonia K and Anupama D. 2011 have determined the terpinen-4-ol content for 5% TTO micro emulsion.[4] Gulati N *et al.* 2012 developed a gas chromatographic method using flame ionization detector for assay of α-pinene in TTO formulations.[5] Venugopal V. 2016 has analysed ethosomes loaded with TTO for oil content by HPTLC method using terpinen-4-ol as reference standard.[6] An in–house validated reverse phase HPLC method was developed to determine the eucalyptol content of TTO in bulk and cosmeceutical formulations since higher levels of eucalyptol, a putative skin irritant is generally associated with lower levels of terpinen-4-ol, the main antimicrobial component of the oil.[2,7] Estimation of eucalyptol content may throw some light on the safety and dermal irritancy of the pure oil as well as TTO based cosmeceuticals.[8]

In spite of all these developments, there is still an absolute need for a simple and robust UV spectrophotometric method suitable for routine determination of TTO in bulk and cosmeceutical formulations. The present research is an attempt in this direction. As described earlier, the method has as an advantage that there is no need for the use of a standard or a specific marker compound since TTO complies with the specifications.

MATERIALS

Tea tree oil was purchased from Messrs. Falcon Essential Oils, Bengaluru. Dichloromethane (SD Fine Chem) extra pure and methanol (Qualigens) HPLC grade were used for method development. DXN Tea Tree Cream (DXN Cream) and Healthvit Bath and Body Tea Tree Cream (HVT Cream) were purchased online from Amazon.in. Product information of the cosmeceutical creams are given in Table 1.

Instruments

Shimadzu UV 1800 Spectrophotometer and Shimadzu analytical balance were used for the study.

METHODS

Screening Study

Preliminary studies were carried out to identify a suitable solvent system for preparing standard solution of TTO. The solubility of TTO was tested using various solvents such as n-hexane, cyclohexane, toluene, DCM and Me OH; binary solvent systems like chloroform-methanol (2:1v/v) and DCM-Me OH (3:2v/v) were also chosen for the study. DCM-Me OH (3:2v/v) was found to be suitable for carrying out analytical work.

Preparation of standard solution of TTO

0.025g TTO was weighed accurately into a 25ml volumetric flask, dissolved using DCM-Me OH (3:2v/v) solvent system and volume made up to the mark using the same. The concentration of the resulting solution was 1.0mg/ml of TTO.

UV spectrum of TTO standard solution

Standard solution of TTO was used for a spectral scan of TTO between 400-250nm using the solvent system as blank.

Preparation of calibration curve of TTO

Aliquots of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6 ml of standard TTO solution were transferred into separate 10 ml volumetric flasks and the volume made up with the solvent system. The absorbance of the resulting solutions was read at 267 nm against a solvent blank.

Analytical method development based on design of experiments

Design expert software trial version 11 was chosen for analytical method development. Box-Behnken design was most appropriate for the study. Factors chosen were percentage of dichloromethane, time of measurement of absorbance and concentration range corresponding to A, B and C respectively. Factors and their coded levels are summarized in Table 2.

Table 1: Description of cosmeceutical creams.

DXN Tea Tree Cream	Healthvit Bath and Body Tea Tree Cream
Net weight:30g	Net weight:50g
Ingredients: Tea tree oil, camphor, menthol, cetomacrogol, emulsifying wax, water	Ingredients: Tea tree oil, light liquid paraffin, white soft paraffin, cetostearyl alcohol, stearic acid, glycerin, phenoxy ethanol, perfume, tartrazine, sunset yellow, brilliant blue, light green, amaranth, water
Batch No:71C009	Batch No:WCHV0132
Best before:02/2020	Expiry date:01/2019
Registration Certificate No:COS-778/15	Mfg Lic No: GC/974
Mfg by: DXN Pharmaceutical, Malaysia.	Mfg by: West Coast Pharmaceutical Works Ltd, Gota, Ahmedabad.

Table 2: Factors and their coded levels.

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	DCM	%	Numeric	50.0	60.0	-1 ↔ 50.0	+1 ↔ 60.0	55.0	3.78
B	TIME	min	Numeric	0.0	60.0	-1 ↔ 0.0	+1 ↔ 60.0	30.0	22.68
C	CONCENTRATION	mcg/ml	Numeric	10.0	200.0	-1 ↔ 10.0	+1 ↔ 200.0	105.0	71.81

The design suggested 15 runs and the response chosen was measurement of absorbance at 267nm, details of which are summarized in Table 3.

All the runs were performed in triplicate and average values of the response was analysed by ANOVA at 0.05 level. F-test was used to assess each parameter and response by subjecting it to multiple regression analysis to generate polynomial equations. Numerical optimization as well as graphical optimization was carried out to arrive at the selected solution. The analysis of the oil was carried out for the selected solution and observed responses were compared with predicted responses.

Table 3: Runs suggested by Box -Behnken design.

Std	Run	Factor 1 A: DCM %	Factor 2 B:Time (min)	Factor 3 C:Concentration mcg/ml
15	1	55	30	105
12	2	55	60	200
11	3	55	0	200
2	4	60	0	105
9	5	55	0	10
4	6	60	60	105
8	7	60	30	200
5	8	50	30	10
10	9	55	60	10
6	10	60	30	10
3	11	50	60	105
1	12	50	0	105
13	13	55	30	105
7	14	50	30	200
14	15	55	30	105

Validation of the method under optimized conditions as per ICH guidelines

The method was then validated as per ICH guidelines for accuracy, precision, linearity, LOD, LOQ for the method under optimized conditions.

Accuracy of the method was carried out by adding known amount of standard solution, 80%, 100% and 120% to a fixed concentration of oil and then determining the percent recovery.

The precision of the method was established at two levels- repeatability and intermediate precision or ruggedness. Repeatability was based on intraday measurements at different times of the day and ruggedness by using different instruments and inter-day measurements for three consecutive days respectively.

Linearity represents the concentration range for which a linear relation exists between concentration and the response. This was confirmed from the equation of best fit and R^2 value, a statistical measure of goodness of fit. An R^2 value> 0.99 is desirable for establishing linearity.

LOD and LOQ were determined from standard deviation of the response at low concentration and slope of the calibration curve.

$$LOD = 3.3\ \sigma/Slope$$

$$LOQ = 10\ \sigma/Slope$$

Where: σ = the standard deviation of the response at low concentration.

Robustness is a measure of the resistance of accuracy and precision of the method to small variations in the method. A robust method has been developed by carrying out optimization by which deliberate changes are done for the three independent variables (factors) viz. percentage of dichloromethane and time of measurement and concentration of TTO.

Determination of TTO content in cosmeceutical cream

0.5g of the cosmeceutical cream was weighed accurately into a 50 ml volumetric flask and dissolved in the solvent system comprising of 54% v/v DCM in Me OH. 1.0ml of the solution was transferred into a 10ml volumetric flask and diluted to the mark with the same solvent system. The absorbance of the resulting solution was read at 267 nm. All determinations were done in triplicate and average absorbance was used for calculating the content of TTO.

TTO content of the cream was calculated using the following formula:

TTO Content %w/v = Absorbance x dilution factor x100 / (0.5×10^6)

Where dilution factor = 500

RESULTS AND DISCUSSION

The composition of TTO is governed by ISO 4730:2017. TTO is known to contain more than 113 components of which minimum and maximum levels of occurrence in the pure oil are specified for 15 different components. Few important constituents and their minimum and maximum concentrations are indicated in the chromatographic profile of the oil. Terpinen-4-ol is the principal constituent of the oil (35%- 48%) followed by γ-Terpinene (14% -28%) and α-Terpinene (6%-12%) and 1, 8-Cineole or eucalyptol (traces -15%).[9] Hence an attempt has been made to develop a simple and robust UV spectrophotometric method for determination of TTO, based on sound principles of statistical design of experiments and validate the method as per ICH guidelines.

Preliminary studies were carried out to identify a suitable solvent system for preparing standard solution of TTO. The oil was found to be miscible with less than two volumes of 85 % v/v ethanol at 20°C as per the specifications provided by the supplier. However the solubility of TTO was tested using various solvents such as n-hexane, cyclohexane, toluene, dichloromethane and methanol; binary solvent systems like chloroform-methanol (2:1v/v) and dichloromethane–methanol (3:2v/v) were also chosen for the study. This was done with an intention of finding a common solvent for both TTO and Neem seed oil since simultaneous estimation of both oils by UV spectrophotometry was planned as part of an extended work. Dichloromethane - methanol (3:2v/v) was found to be suitable for carrying out analytical work since both the oils were miscible with this solvent system. A spectral scan from 400-250 nm was obtained for standard solution of TTO which exhibited a sharp peak at 267nm (Figure 1).

Preliminary screening studies were carried out to identify the factors which could affect the method and also to ascertain the concentration range for establishing the linearity. Accordingly three factors which could affect the response viz. absorbance at 267 nm were identified as percentage composition of DCM in the solvent system, time of measurement of absorbance and concentration of TTO.

The next stage of development began with selection of a design using Design Expert software, Version 11. Box Behnken design, a response surface design was chosen for the study since it is most suitable for determining the best factor settings or operating conditions for optimizing the response and to develop a polynomial model with less number of runs or experiments.

Box Behnken design was generated using three factors A, B and C corresponding to percentage of DCM, time of measurement and concentration of TTO respectively and their minimum and maximum levels were fixed as shown in the Table 2. Absorbance at 267 nm was chosen as the response. The design generated 15 runs which were performed and the response was measured in triplicate. The response was then analysed and optimized by numerical and graphical method (Figure 2-4).

Optimization of response (absorbance at 267 nm)

A linear factorial model was used for the analysis of response, the model F-value (*p*-value) of 38.92(0.0001) implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. *P*-values less than 0.0500 indicate model terms are significant. In this case C (concentration) is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. The lack of fit F-value of 0.0386 implies the lack of fit is not significant relative to the pure error (Table 4). Non-significant lack of fit is good - we want the model to fit. The predicted R^2 of 0.8247 is in reasonable agreement with the adjusted R^2 of 0.8904; i.e. the difference is less than 0.2. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 15.675 indicates

Table 4: ANOVA for Linear model.

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value
Model	3.69	3	1.23	38.92	< 0.0001*
A-DCM	0.0139	1	0.0139	0.4390	0.5212 NS
B-TIME	0.0002	1	0.0002	0.0060	0.9395 NS
C-CONCENTRATION	3.67	1	3.67	116.31	< 0.0001*
Residual	0.3473	11	0.0316		
Lack of Fit	0.3473	9	0.0386		
Pure Error	0.0000	2	0.0000		
Cor Total	4.03	14			

*-significant NS-not significant

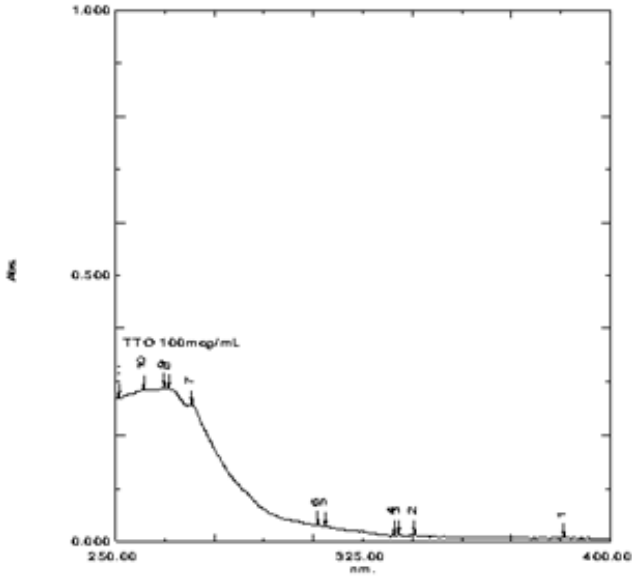

Figure 1: UV spectrum of TTO.

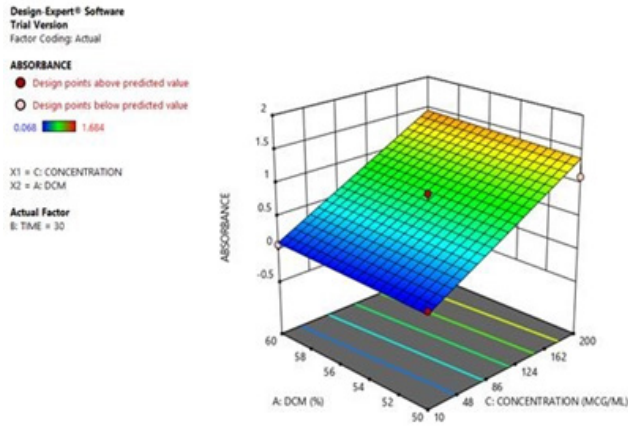

Figure 2: Response surface graph of absorbance.

Table 5: Optimized conditions for estimation.			
Response	**DCM %**	**Time (min)**	**Concentration mcg/ml)**
Absorbance at 267 nm	54	5 min	60

an adequate signal. Hence the model can be used to navigate the design space.

Absorbance = 0.472566 + 0.008325* DCM + 0.000163* Time + 0.007132 *Concentration.... (Eqn5)

The above equation clearly indicates that all the three factors viz. DCM, time and concentration have a positive impact on absorbance.

Analysis of TTO under optimized conditions

The predicted versus actual value of the response was found to be in close agreement with each other which indicated the suitability of the method for estimation of TTO

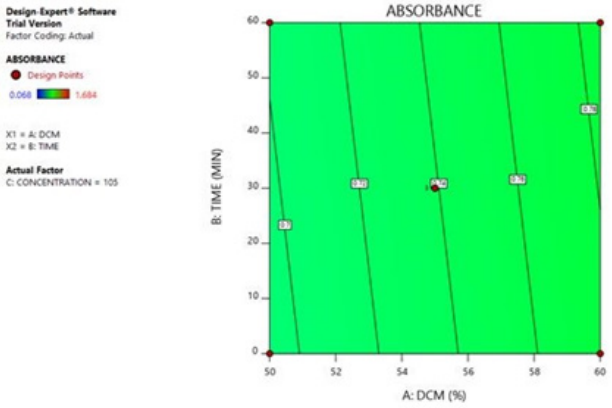

Figure 3: Contour plot of absorbance.

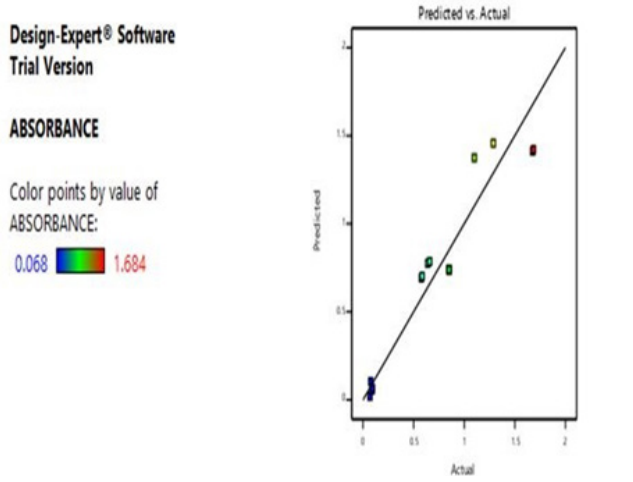

Figure 4: Predicted vs Actual values of response.

Table 6: Predicted and actual values of response for 60 mcg/ml of TTO.		
Response	**Predicted value**	**Actual value**
Absorbance at 267 nm	0.409	0.404

Desirability approach was used to optimize the response, desirability value (di) for all factors and responses was found to be 1. The optimized conditions of the method have a desirability of 1, which indicates that the response is also optimum (Figure 5) (Table 5 and 6).

The optimized conditions indicated for analysis of response are 54% DCM and time of measurement 5 min. These conditions were maintained for validating the method as per ICH guidelines for accuracy, precision (repeatability, intermediate precision and ruggedness), concentration range and linearity, LOD and LOQ. The method showed linearity in the range of 10-160mcg/ml with R^2 value of 0.9961 (Figure 6). LOD and LOQ values of 0.1277 mcg/ml and 4.195 mcg/ml

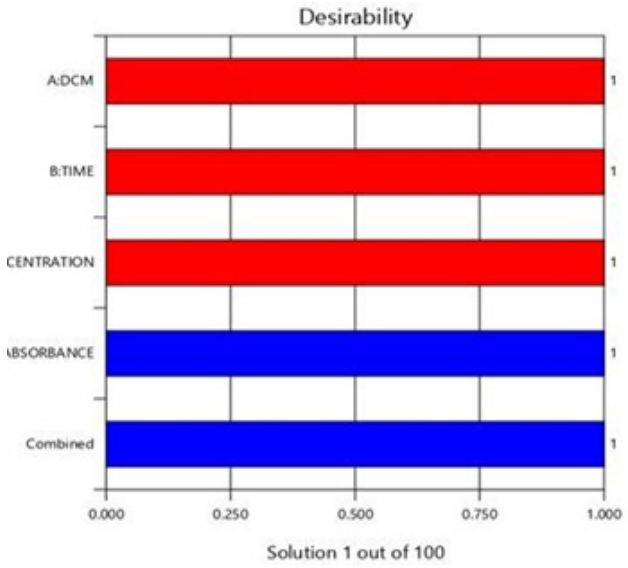

Figure 5: Desirability graph.

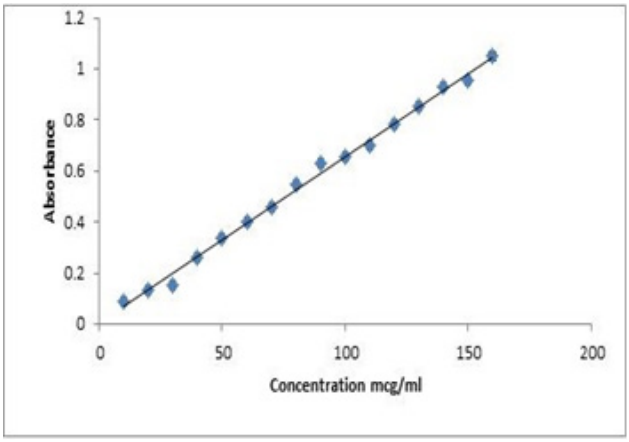

Figure 6: Calibration curve of Tea Tree Oil.

were low indicating that the method is quite sensitive for estimation of TTO (Table 7 and 8). Accuracy of the method was established from % recovered being more than 100 for all the levels (Table 9) and precision of the method confirmed by % RSD values < 2% for repeatability, intermediate precision and ruggedness (Table 10 and 11).

The content of TTO in two marketed creams was determined by the developed method viz., DXN Cream, 0.34%w/v and HVT Cream, 0.45%w/v respectively (Table 12). Though, it is not possible to verify the label claim owing to the current labelling practices adopted for cosmeceuticals, still it is possible to conclude that the content of TTO in the creams is well within the acceptable limit of 1-5%; however the composition of TTO is not mentioned on the label It is our observation, based on a market survey of various cosmetic and personal care products that 1-5% Tea tree

Table 7: Calibration curve of Tea Tree Oil.

Concentration mcg/ml	Absorbance			Average absorbance ± SD
	Trial I	Trial II	Trial III	
10.0	0.086	0.089	0.085	0.087±0.002
20.0	0.134	0.131	0.131	0.132±0.002
30.0	0.153	0.153	0.156	0.154±0.002
40.0	0.256	0.263	0.261	0.260±0.002
50.0	0.337	0.337	0.336	0.337±0.004
60.0	0.401	0.402	0.404	0.402±0.001
70.0	0.453	0.461	0.465	0.460±0.001
80.0	0.543	0.548	0.546	0.546±0.006
90.0	0.628	0.629	0.632	0.630±0.003
100.0	0.654	0.659	0.656	0.656±0.002
110.0	0.702	0.701	0.704	0.702±0.003
120.0	0.782	0.785	0.790	0.786±0.002
130.0	0.851	0.856	0.855	0.854±0.004
140.0	0.927	0.927	0.931	0.928±0.003
150.0	0.948	0.962	0.962	0.957±0.008
160.0	1.038	1.042	1.067	1.049±0.016

Table 8: Linearity parameters for estimation of Tea Tree Oil, LOD and LOQ.

Parameter	Value
Equation	y = 0.0065x + 0.0046
R^2 value	0.996
LOD mcg/ml	0.1277
LOQ mcg/ml	4.195

Table 9: Results of accuracy determination.

Response*	Amount taken %	Actual amount mcg/ml	Average amount recovered ±SD mcg/ml	Percentage recovered
Absorbance at 267 nm	80	40	42.6±1.19	106.5
	100	60	60.3±0.50	100.5
	120	80	90.9±0.47	113.6

*n=3

Table 10: Results of method precision.

Response*	Precision	Amount mcg/ml	Amount recovered mcg/ml ±SD	Percent RSD (SD/ Average) *100
Absorbance at 267 nm	Intraday	80	90.9±0.47	0.52
	Inter-day	80	90.9±0.47	0.52

*n=3

Table 11: Ruggedness (Intermediate precision).

Response*	Instrument	Amount mcg/ml	Amount recovered mcg/ml ±SD	Percent RSD (SD/ Average)* 100
Absorbance at 267 nm	Instrument 1	60	57.4±0.82	1.43
	Instrument 2	60	61.2±0.27	0.44

Table 12: Content of TTO in the cosmeceutical cream.

Name of the cream	TTO Content %w/v
DXN Cream	0.34
HVT Cream	0.45

oil, an active cosmetic ingredient of many products is included in ointments, mouthwashes, lotions and skin care creams. TTO is also available as pure essential oil. Most commonly, it is available in 5% concentration, but higher concentrations are also available. It is also known from several studies and reports that TTO, though a potent antimicrobial is also a potential skin irritant and must be used with caution.[10-12]

CONCLUSION

A simple and robust, cost effective UV spectrophotometric method was developed and validated for routine estimation of TTO in bulk and cosmeceutical creams. The developed method, though non-specific, considering the highly complex and varied composition of the oil, may be put to use for defining standards for TTO based cosmeceuticals at a time when none is existent. This study may be of significance to various regulatory bodies that are in the pursuit of maintaining highest possible quality standards for essential oils based cosmeceuticals.

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CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest involved in the study.

ABBREVIATIONS

Abs: Absorbance; **ANOVA:** Analysis of Variance; **DCM:** Dichloromethane; **HPTLC:** High performance thin layer chromatography; **ICH:** International Conference on Harmonization; **ISO:** International Organization for Standardization; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **Me OH:** Methanol; **QbD:** Quality by design; **RP-HPLC:** Reverse phase high performance liquid chromatography; **RSD:** Relative standard deviation; **TTO:** Tea tree oil; **UV:** Ultra violet.

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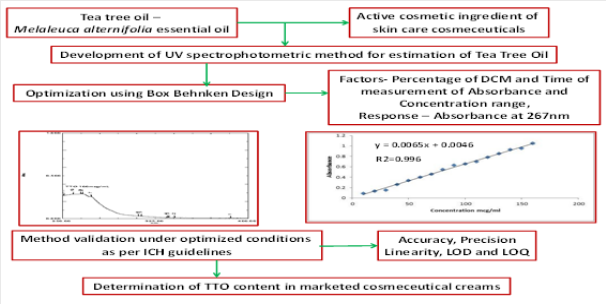
PICTORIAL ABSTRACT



SUMMARY

TTO has a complex and variable composition consisting of more than 113 components and therefore poses many challenges to the analytical chemist for method development. A quality by design approach has been employed to develop a simple and novel UV spectrophotometric method for estimation of tea tree oil in bulk and cosmeceutical creams. The method was validated as per ICH Q2:R(1) and is suitable for routine determination of TTO in bulk and cosmeceutical creams. The analytical method is significant in the light of scant quality standards available for standardization of creams containing TTO, an essential oil having both beneficial as well as skin irritant properties.

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