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Research Article

Novel Validated Uhplc-Dad Method for Quantification of Leptosperin in New Zealand Mānuka Honey: A Definitive Chemical Marker for Authentication and Quality Control Incorporating Sustainable Practices with Minimal Sample and Plastic Usage for Cost-Effective Analysis

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ABSTRACT

Leptosperin is a unique floral marker compound found exclusively in the Leptospermum genus, with particularly high concentrations in Leptospermum scoparium (mānuka) honey from New Zealand. Due to mānuka honey's global demand and valued therapeutic properties, reliable authentication methods are essential to ensure product quality, traceability, and prevent adulteration.

This study reports the development and full validation of a sensitive and robust Ultra-Performance Liquid Chromatography with Diode Array Detection (UPLC-DAD) method for the quantification of Leptosperin in honey samples. The method employs a straightforward aqueous extraction followed by reverse-phase chromatography, enabling accurate and selective detection of Leptosperin at 282 nm.

Validation was conducted in accordance with international guidelines, encompassing specificity, linearity, precision, accuracy (expressed as recovery), trueness, limit of detection (LOD), and sample stability. The method exhibited excellent linearity ($R^2 > 0.99$) over a concentration range of 5 to 436 mg/kg. Precision studies showed relative standard deviations (%RSD) below 3% for both intra-day and inter-day measurements. Recovery rates ranged between 90% and 94%, demonstrating the method's accuracy in complex honey matrices. The LOD was established at 10 mg/kg, sufficient to detect typical Leptosperin levels in mānuka honey.

Stability testing confirmed that extracted samples remain stable for up to 36 hours' post-preparation under refrigerated conditions, facilitating flexible laboratory workflows. Method performance was further corroborated through comparative analysis with accredited external laboratories, yielding strong concordance with relative standard deviations below 10.1%.

This validated UPLC-DAD method offers a rapid, reliable, and cost-effective analytical tool for mānuka honey authentication and quality control. Its applicability extends to both monofloral and multifloral mānuka honey types, supporting research, regulatory compliance, and commercial quality assurance efforts. In summary, the method provides a scientifically rigorous approach to quantify Leptosperin—a definitive chemical marker of Leptospermum species—thus enabling robust authentication of mānuka honey to maintain consumer confidence and uphold export quality standards.

Keywords: Leptosperin, Mānuka Honey, UPLC-DAD, Honey Authentication, Quality Control, New Zealand, Floral Markers.

INTRODUCTION

Mānuka honey, derived from the nectar of *Leptospermum scoparium* flowers, is internationally acclaimed for its distinctive bioactive and antimicrobial properties. Among its key chemical constituents, Leptosperin has been identified as a definitive floral marker unique to mānuka nectar, serving as a reliable indicator of authenticity and geographic origin [1–3, 8, 9]. Precise quantification of Leptosperin is crucial for regulatory compliance, consumer confidence, and trade certification.

Although various analytical techniques, including LC-MS and immunochemical assays, have been employed for Leptosperin detection [4–7], their complexity and cost restrict routine use in high-throughput quality control settings. This study introduces the development and comprehensive validation of a UPLC-DAD method as a simpler, cost-effective alternative for quantifying Leptosperin in honey. The method was rigorously validated according to AOAC and ICH guidelines and benchmarked against external laboratory standards. Given Leptosperin's exclusive presence in mānuka nectar and its chemical stability, it has emerged as a robust and specific marker capable of differentiating mānuka honey from other floral sources. The objective of this research was to establish a reliable, accurate UPLC-DAD assay for Leptosperin quantification, thereby supporting product authenticity and ensuring accurate labeling.

MATERIALS AND METHODS

Chemicals and Reagents

All reagents used were of HPLC grade. Acetonitrile (≥99.9%, CAS: 75-05-8) and formic acid (≥98%, CAS: 64-18-6) were purchased from Merck. Type 1 water with a conductivity of less than 0.1 µS/cm was produced in-house using a Milli-Q purification system. The Leptosperin standard (purity 99.06%) was obtained from UMFHA-certified suppliers.

Sample Collection

Quality control (QC) and test honey samples were sourced from certified stock maintained by King Honey Limited (refer to Table 1). External reference values for these samples were provided by accredited laboratories, including Hill Laboratories and Analytica NZ.

Sample ID	Sample Reference	Leptosperin (mg/kg)	Location
QC-6	KHL1158	525	KHL Warehouse
QC-7	KHL1166	334	KHL Warehouse
KHL1184	KHL1184	270	KHL Warehouse
KHL1304	KHL1304	187	KHL Warehouse
KHL1554	KHL1554	1010	KHL Warehouse
KHL1504	KHL1504	113	KHL Warehouse
KHL1326	KHL1326	N/A	KHL Warehouse

Table 1. Details of the Sample(S) Used For Method Validation of Leptosperin

Instrumentation

Chromatographic analysis was conducted using a Shimadzu Nexera X2 UPLC system equipped with a diode array detector (DAD) set at 282 nm. Separation was achieved on a Phenomenex Synergi Fusion-RP column (50 mm × 2 mm, 4 µm particle size, 80 Å pore size). Detailed system specifications and gradient parameters are outlined. Additional equipment included analytical balances with sensitivities of 0.1 mg and 0.1 g, vortex mixers, centrifuge tubes, and HPLC vials.

Artificial Honey (Sugar Solution)

To simulate the honey matrix for calibration purposes, artificial honey was prepared by dissolving specific quantities of common sugars in Type 1 water, reflecting the typical carbohydrate composition of natural honey.

Composition (±1 G Unless Otherwise Stated):

- **Glucose:** 34.5 ± 1 g
- **Fructose:** 45.7 ± 1 g
- **Sucrose:** 1.5 ± 0.1 g
- **Type 1 water:** 18.3 ± 1 g

The components were thoroughly mixed until fully dissolved to form a homogenous sugar solution.

Dilution for Use:
For each calibration or test preparation, 1.0 ± 0.1 g of the artificial honey solution was transferred into a 50 mL tube and diluted with 9 mL of Type 1 water. The mixture was vortexed until fully blended.

Storage and Stability:
The diluted artificial honey solution is stable for up to 4 weeks when stored under appropriate conditions.

Extraction and Sample Preparation
Approximately 1.0 ± 0.1 g of honey was accurately weighed and diluted with 9 mL of Type 1 water. The mixture was vortexed thoroughly and subsequently filtered. For each analysis, 0.1 mL of this diluted honey solution was mixed with 0.4 mL of 0.1% formic acid in water, vortexed, and filtered through a 0.2 µm syringe filter directly into HPLC vials. A 1 mL aliquot of the prepared sample was then used for injection.

Calibration Standards and Quality Controls
A five-point calibration curve (C1–C5) was established using Leptosperin standards with concentrations ranging from 5.07 to 436.09 mg/kg. Quality control (QC) samples, QC6 and QC7, were included and analyzed in each batch to ensure assay consistency. The Leptosperin stock solution (S1) was prepared by accurately weighing approximately 22 mg of the pure compound into a 50 mL volumetric flask, then diluting to volume with HPLC-grade water (Table 2 & Table 2a). Calibration standards (C1–C5) were prepared by serial dilution of the stock solution to achieve the target concentration range.

	Leptosperin				
		Purity (%)	99.06%		
		Lot No			
		Prep Date	28.11.19		
		Expiry Date	NA		
		Tech	HS		
	Std (Leptosperin) Vol (ml)	Std Weight (mg) A	Water (ml) B	Standard Weight + Water Weight (g) (A+B)	Calibration Conc (mg/kg)
S1	20	22	50	49.974	436.0908
C1	4	4.057	0	4.057	436.09
C2	3	3.039	1	4.046	327.55
C3	2	2.023	2	4.049	163.66
C4	1	1.008	3	4.04	40.83
C5	0.5	0.5	3.5	4.028	5.07

Table 2. Preparation of Calibration Standards

	C1	C2	C3	C4	C5
Lepto 1mL each	Lepto C1	Lepto C2	Lepto C3	Lepto C4	Lepto C5

Table 2a. Calibration Standard Volumes

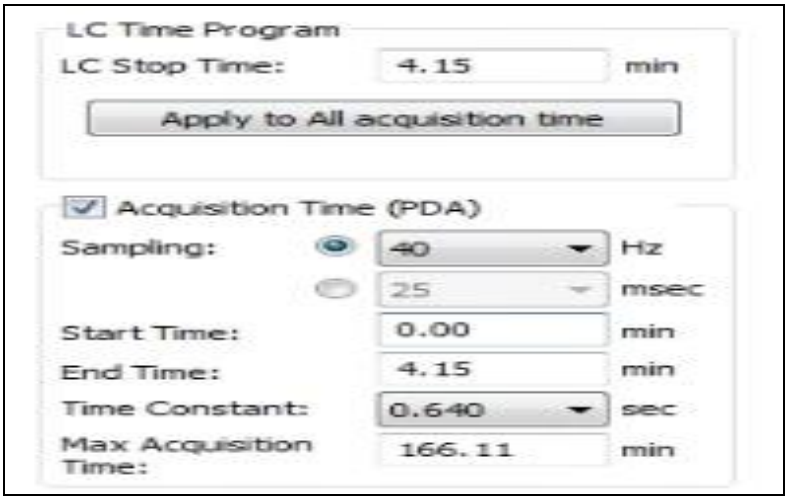
UPLC-DAD Analysis Procedure
Equipment Configuration
System: SHIMADZU Nexera X2
Software: Shimadzu LC Solution
Components:
Pumps: LC-30AD
Autosampler: SIL-30AC
Column Oven: CTO-20AC
Diode Array Detector (DAD): SPD-M20A
Communications Module: CBM-20A
Degasser: DGU-20A5

Chromatographic Conditions
- Mobile Phase A: 1% formic acid in HPLC water (expiry: 2 weeks from preparation)
- Mobile Phase B: 80% acetonitrile + 20% Mobile Phase A (expiry: 2 weeks)
- Column: Phenomenex Synergi Fusion-RP, 4 µm, 80 Å, 50 mm × 2 mm
- Column Temperature: 32°C
- Autosampler Temperature: 20°C
- Flow Rate: 0.5 mL/min

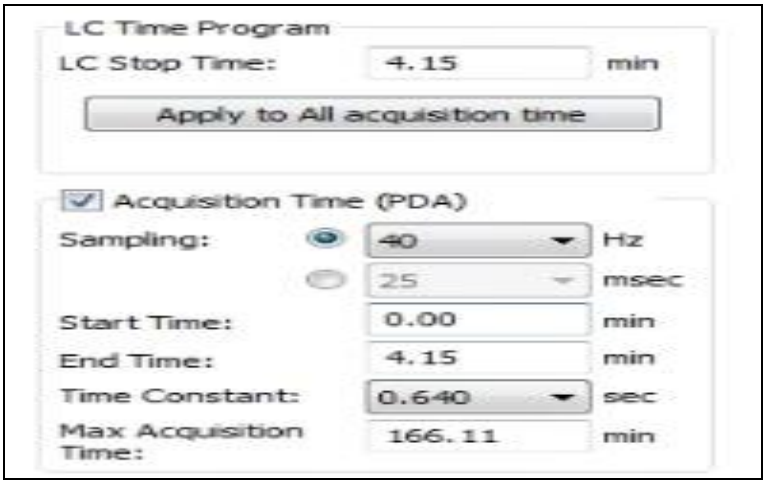
- Injection Volume: [Specify, e.g., 5 µL]
- Detection Wavelength: 282 nm
- Run Time: 4 minutes

Method Setup

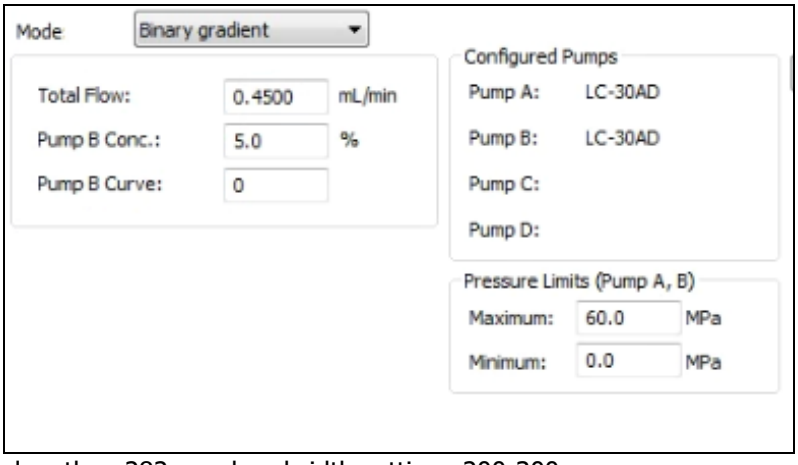
Data Acquisition Time: 4.15min



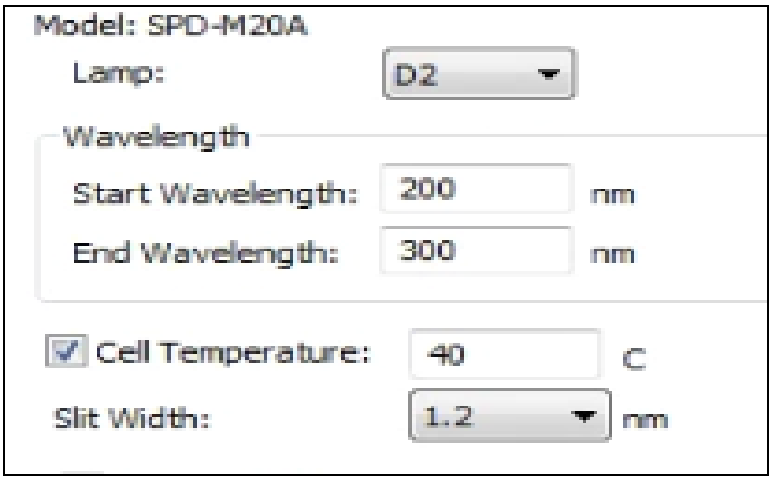
Gradient Profile:



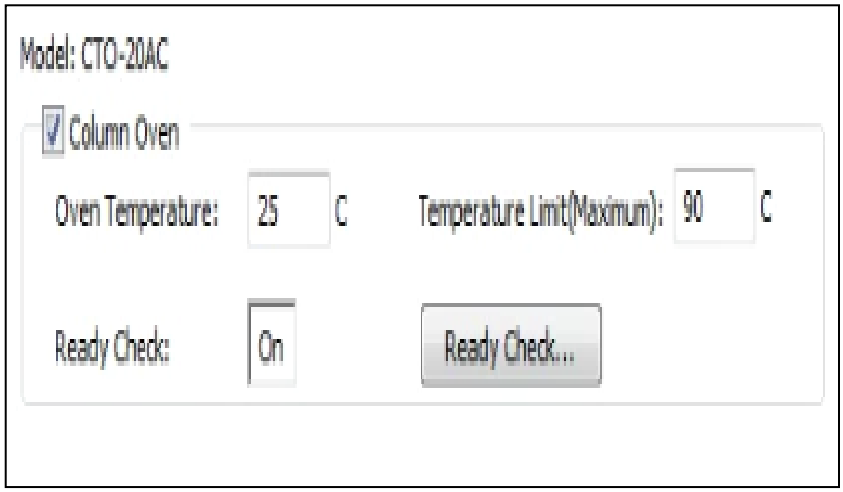
Pumps Configuration:



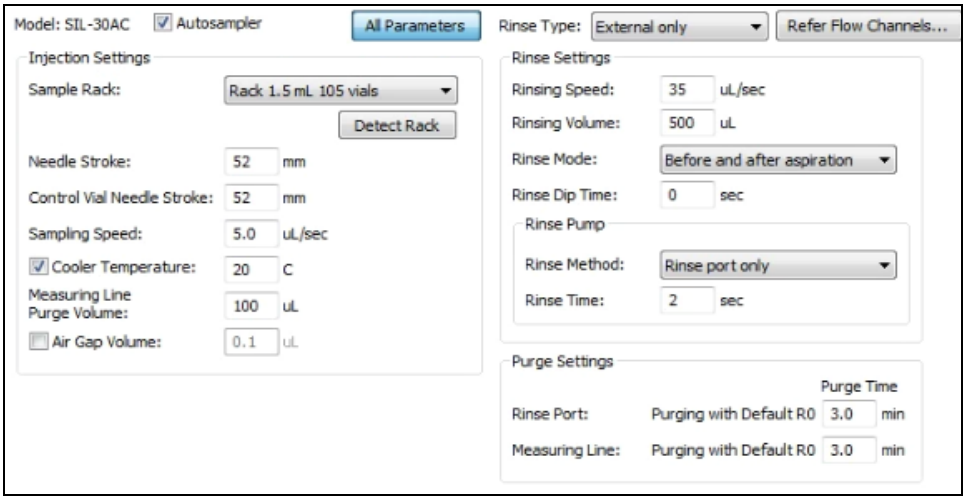
PDA Setup: Wavelength = 282 nm; bandwidth settings 200-300nm.



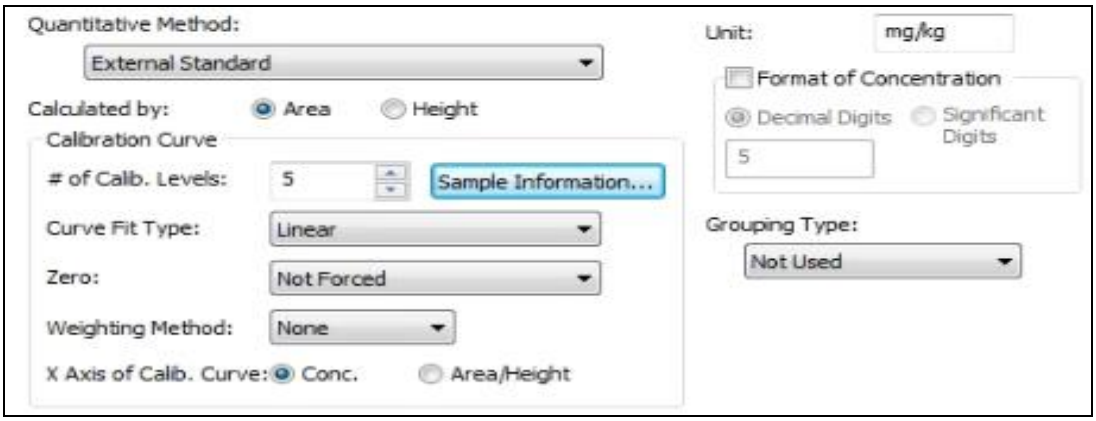
Oven Settings: 32°C



Autosampler Settings: Sample cooler at 20°C

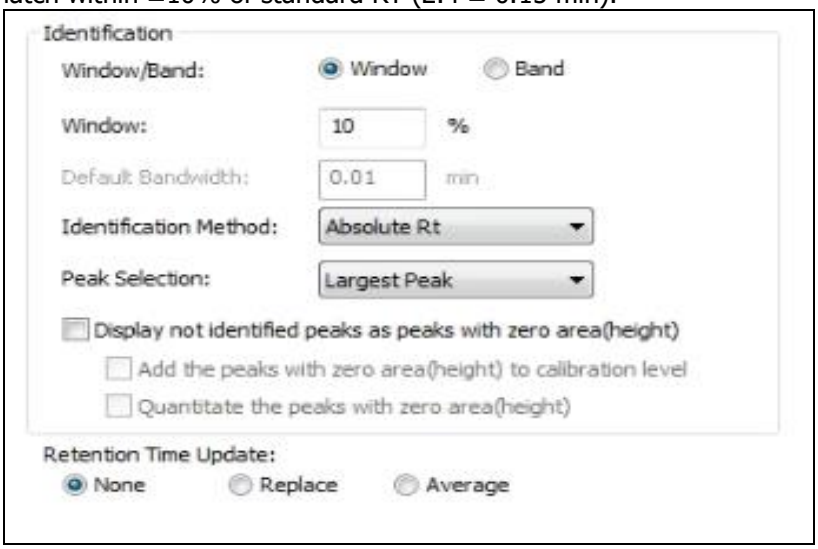


Quantification Settings
Quantitative Analysis:
Based on peak area integration against calibration curve (C1–C5).



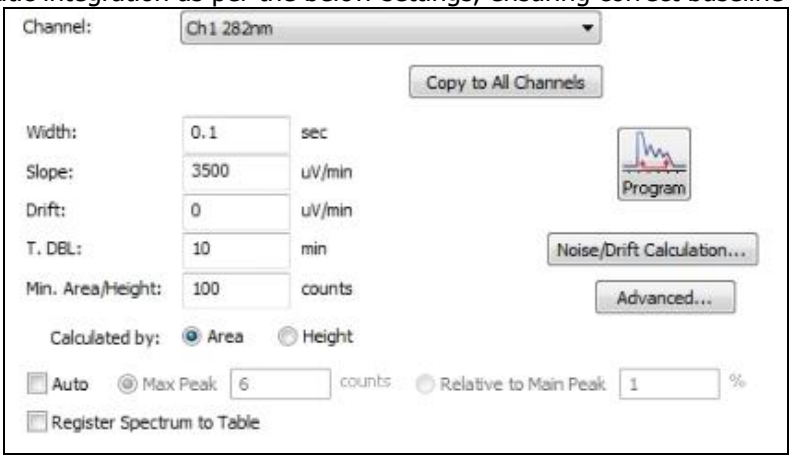
Identification Criteria:

Retention time match within ±10% of standard RT (2.4 ± 0.15 min).



Integration Parameters:

Manual or automatic integration as per the below settings, ensuring correct baseline and peak height.



Pre-Run Instrument Checks

System Pressure: Operating range: 70–120 kgf/cm²

Retention Time and LOD

Analyte	Wavelength	Retention Time (min)	LOD (mg/kg)
Leptosperin	282 nm	2.4 ± 0.15	10

System Suitability Test (C1 Injection)

Inject C1 as a single start analysis. Verify that the retention time is within ±10% of expected. If outside range, do not proceed; initiate a CAPA and investigate.

RESULTS AND VALIDATION

Specificity

No interfering peaks were observed at the Leptosperin retention time (2.4 ± 0.15 minutes) in blank samples, confirming the method's specificity (Figure 1). Calibration standards (C1 and C5) and representative honey samples were chromatographed under the validated conditions to confirm the presence and retention time of Leptosperin. A well-resolved peak was consistently observed at approximately 2.4 minutes with detection at 282 nm (Figures 1a and 1b), demonstrating clear specificity for the analyte of interest.

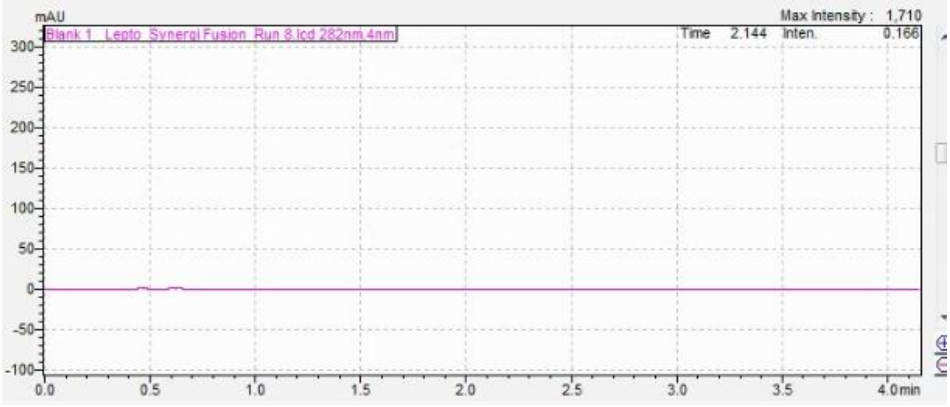


Figure 1. Chromatogram of Blank, 262nm, Lepto Run 8

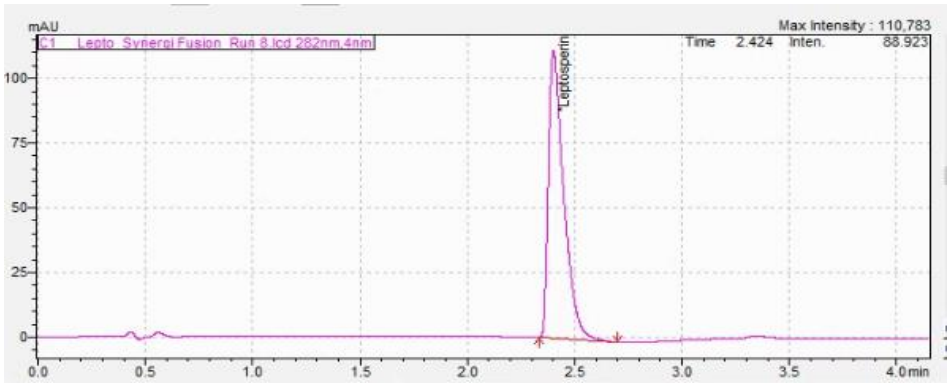


Figure 1a. Chromatogram of C1, 282nm, Lepto Run 8 (Leptosperin 436.090mg/kg)

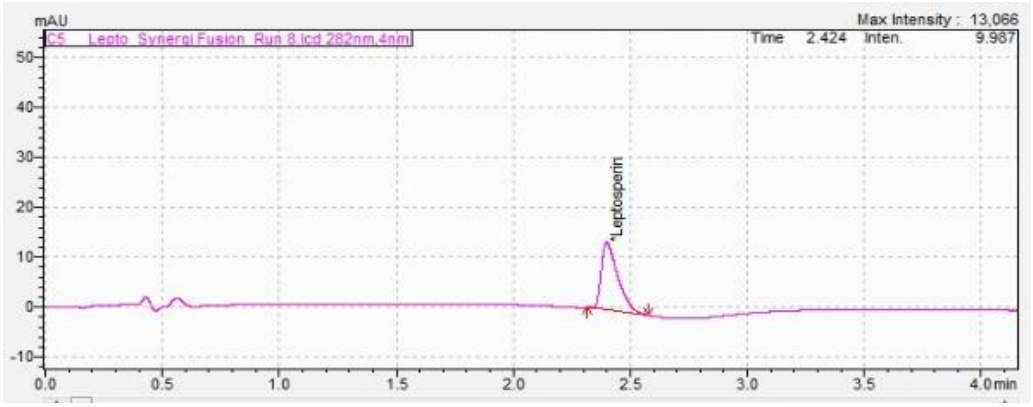


Figure 1b. Chromatogram of C5, 282nm, Lepto Run 8 (53.916mg/kg)

Linearity

A five-point calibration curve (C1–C5) spanning 5.07 to 436.09 mg/kg demonstrated excellent linearity, with a correlation coefficient (R^2) of 0.9992 (Figure 2), meeting established linearity criteria.

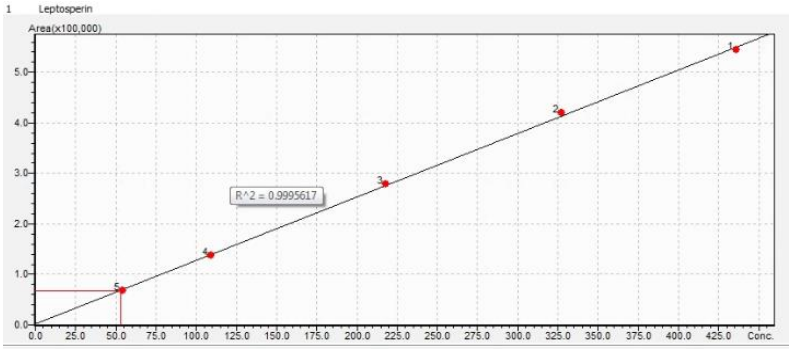


Figure 2. Linearity of the Response Leptosperin Calibration Curve

Precision

System Precision:

The %RSD for six replicate injections of the highest standard (C5) was 0.3% (Table 3), well within the acceptance threshold of <2%.

Calibration Standard	Area (mAU.sec)
	Leptosperin
C5-Injection1	72178
C5-Injection2	72448
C5-Injection3	72507
C5-Injection4	72484
C5-Injection5	72311
C5-Injection6	72052
% RSD	0.3%

Table 3: System Precision Data for Calibration Standard C5, Lepto Run Xx

Intra- and Inter-day Precision:

RSD values for QC samples QC6 and QC7 across five consecutive runs were consistently below 5% (Tables 4–6).

Sample	Leptosperin, mg/kg				
	Run_11	Run_12	Run_14	Run_15	Run_16
QC6 A	512	519	541	533	539
QC6 B	506	515	546	545	533
QC6 C	510	566	548	531	541
QC7 A	371	362	371	365	364
QC7 B	370	380	368	373	360
QC7 C	360	378	365	373	364

Table 4. Leptosperin Concentration, Mg/Kg for Validated Runs

Intra-Day Precision (%RSD)					
QC-6	0.6	5.3	0.7	1.3	0.8
QC-7	1.6	2.6	0.8	1.2	0.6

Table 5. Intra-Day Precision for Leptosperin

Inter-Day Precision			
	Avg	SD	RSD
QC-6	532	13.5	2.5%
QC-7	368	4.0	1.1%

Table 6. Inter-day Precision for Leptosperin

Accuracy (Recovery)

Recovery experiments using spiked rewarewa honey (negative control) samples yielded recoveries between 90% and 94%, demonstrating the method's accuracy (Table 6).

Leptosperin				
Sample ID	**Conc. (mg/kg)**	**Spiked Conc. (mg/kg)**	**Measured Conc. (mg/kg)**	**%Recovery**
Rewarewa Honey	<20	328	295.95	90%
		328	295.95	90%
		328	295.95	90%
		218	203.74	94%
		218	203.74	94%
		218	203.74	94%
		109	100.68	93%
		109	100.68	93%
		109	100.68	93%

Table 6.a. % recovery of standards c2, c3 and c4 of Leptosperin

Trueness

Comparative analysis with external laboratories showed RSD values ranging from 0.3% to 10.1%, all within the acceptable limit of <20% (Table 7).

Sample ID	Leptosperin, mg/kg		
	AgriTesting	**Hill Labs**	**%RSD**
KHL1184	279	270	2.4
KHL1304	212	187	8.7
KHL1554	1014	1010	0.3
KHL1504	130	113	10.1

Table 7. Trueness of Leptosperin

3.6 Stability

Calibration standards and QC samples remained stable for up to 36 hours post-extraction, with area variation below 5% (Table 8).

Sample	DHA Area (mAU.sec)	%RSD
Calibration Standard C1	577690	2.3%
Calibration Standard C1_24Hr	556014	
Calibration Standard C1_36Hr	555031	
Honey QC-6	72500	3.4%
Honey QC-6_24Hr	70115	
Honey QC-6_36Hr	67775	

Table 8. Peak Stability of Leptosperin

3.7 Measurement Uncertainty (MU)

The expanded uncertainty (U, k=2) was calculated as 3.65% for QC6 and 2.2% for QC7, indicating high confidence in the measurement results. (Table 9).

	Measurement Uncertainty for Leptosperin									
	Avg.(mg/kg)	SD	RSD	UCL	LCL	RSD $_{cc}$	RSD $_{cc}^2$	%u_{Rw}^2	U = 2√(%u_{Rw} 2)	Average MU (%)
QC-6	532.4	13.5	2.5	491.7	573.0	2.5	6.5	6.5	5.1	3.65
QC-7	368.3	4.0	1.1	356.5	380.2	1.1	1.2	1.2	2.2	

Table 9. MU of Leptosperin

Limit of Detection and Quantification

LOD: 10 mg/kg

Retention Time (RT): 2.4 ± 0.15 minutes

Detection precision was confirmed by QC sample RSD values below 10% and standard peak area RSDs below 5%.

Quality Control

QC samples (QC6 and QC7), prepared from authentic mānuka honey, consistently met internal quality control criteria (data available upon request or in supplementary figures).

Peak Acceptance Criteria

- Retention time of unknown samples must be within ±10% of calibration standard RT.
- Peaks must be free from:
  - Double peaks
  - Negative peaks
  - Significant peak tailing (Figure 3).

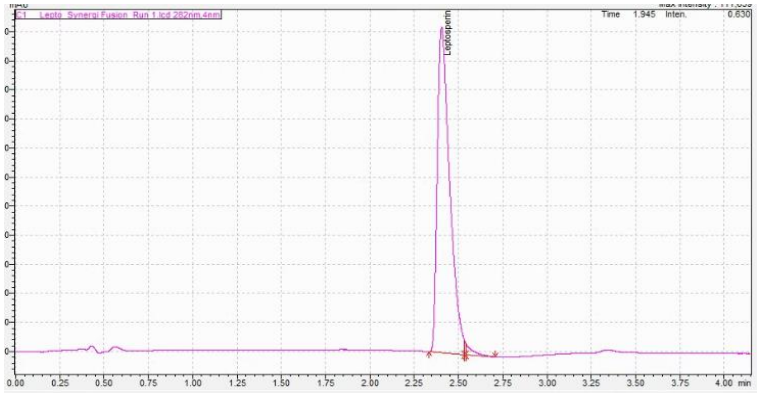


Figure 3. Chromatogram1: Peaks integrated Properly for Leptosperin (2.4 mins), 282nm.

RT Consistency

Average retention time of Leptosperin across C1–C5 and unknowns must be within ±0.15%. Calculated using Lab Solution Browser.

Non-Compliance

If any validation parameter is not met, the run is deemed invalid. A CAPA must be initiated for investigation.

DISCUSSION

The validated UPLC-DAD method provides a high-throughput, cost-effective alternative to more complex techniques such as LC-MS for the quantification of Leptosperin in honey. The method demonstrated strong performance across all validation parameters, meeting or exceeding AOAC standards. Its suitability for both monofloral and multifloral honey types underscores its versatility across a broad range of honey matrices.

Utilizing diode array detection at 282 nm, the method offers a selective yet straightforward detection strategy. Specificity was clearly established, with no interfering peaks observed at the Leptosperin retention time. The calibration curve yielded an excellent correlation coefficient ($R^2 > 0.99$), confirming robust linearity across the tested concentration range. Additionally, cross-validation with

accredited external laboratories supports the method's trueness and enhances its credibility.

The protocol's short run time (4 minutes), high sensitivity, and minimal sample preparation requirements make it especially well-suited for use in commercial and regulatory laboratories. The method consistently demonstrated strong precision, accuracy, and stability, with low RSD values and reliable retention time performance, further confirming its robustness.

Given the growing demand for authenticated mānuka honey, the ability to accurately quantify Leptosperin—an established floral marker—provides an essential tool for industry and regulatory bodies. This method facilitates rapid screening and reliable authentication, reinforcing consumer confidence and supporting rigorous product labeling and classification standards.

CONCLUSION

A robust, sensitive, and validated UPLC-DAD method for the quantification of Leptosperin in honey has been successfully developed and verified across key validation parameters, including specificity, precision, trueness, and accuracy. The method demonstrates excellent sensitivity and is applicable to both monofloral and multifloral mānuka honey samples.

With its short run time, minimal sample preparation, and strong analytical performance, this method is well-suited for routine quality control, product authentication, and regulatory compliance. It provides an effective analytical tool to support traceability, export verification, and quality assurance within the mānuka honey industry.

Acknowledgments

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