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ORIGINAL RESEARCH PAPER

Validation of a high-performance thin-layer chromatography method for the quantitative determination of trehalulose

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Abstract
A novel, simple and reliable high-performance thin-layer chromatography (HPTLC) method was developed and validated for the quantification of trehalulose in stingless bee honey. The chromatographic separation was performed using silica gel 60 F_{254} HPTLC plates and 1-butanol−2-propanol−aqueous boric acid solution (5 mg/mL) (30:50:10, *V/V*) as the mobile phase. The retardation factor (R_F) for trehalulose was found to be 0.045. The method showed linearity over the concentration range of 100–800 ng per band with a coefficient of correlation (*R*) of 0.9996. The limit of detection and limit of quantification for trehalulose were found to be 20.04 ng per band and 60.72 ng per band, respectively and the mean per cent recovery of trehalulose was 101.8%. The method has been validated for its specificity, linearity, sensitivity, precision, accuracy, repeatability and robustness following the International Council for Harmonisation Q2 (R1), and it has been successfully applied in the determination of trehalulose in stingless bee honey.

Keywords High-performance thin-layer chromatography (HPTLC) · Trehalulose · Quantitative analysis · Validation · ICH guidelines · Stingless bee honey

1 Introduction

Trehalulose (1-*O*-α-D-glucopyranosyl-D-fructose) is a structural isomer of sucrose (2-*O*-α-D-glucopyranosyl-D-fructose) and has a different glycosidic linkage (Fig. 1) [1, 2]. This disaccharide is naturally found in honeys and honeydew secreted by insects such as whiteflies and aphids [3, 4]. It was previously believed that honeys contained only small amounts of trehalulose, but a recent study demonstrated it to be the major sugar in honey produced by stingless bees [5].

Trehalulose is highly soluble in water and exhibits high antioxidant activity [6, 7]. In terms of sweetness, trehalulose is approximately 60% as sweet as sucrose, but it is non-cariogenic and releases monosaccharides into the blood at a slower rate [2, 6]. It has been used in human food and animal feed to extend shelf life, in diets of patients with diabetes to help manage diabetes mellitus, in sports drinks to help control body weight and in dental products for its non-cariogenic properties [3].

Several analytical methods have been developed for the determination of trehalulose, including high-performance liquid chromatography (HPLC) [8, 9], nuclear magnetic resonance spectroscopy (NMR) [1, 5] and ion chromatography [10, 11]. However, besides their high cost, a number of other challenges are also associated with these analytical approaches, including complex sample preparation methods (e.g. solid phase extraction) preceding HPLC analysis and the intrinsic insensitivity of NMR analysis [12, 13]. Therefore, a simple, convenient, accurate and cost-effective method for determining trehalulose content in natural products and foods is still required. Furthermore, a method that can efficiently and confidently determine the concentration of trehalulose in stingless bee honey across a variety of species and geographical regions could be a vital tool in the development of quality standards for this type of honey [14].

High-performance thin-layer chromatography (HPTLC) has in recent years emerged as a convenient alternative tool to other more complex analytical techniques for sugar analysis, including HPLC, NMR and ion chromatography. Researchers have, for example, developed and validated a method to quantify glucose, fructose, maltose and sucrose in honeys using HPTLC [13, 15]. However, to the best of

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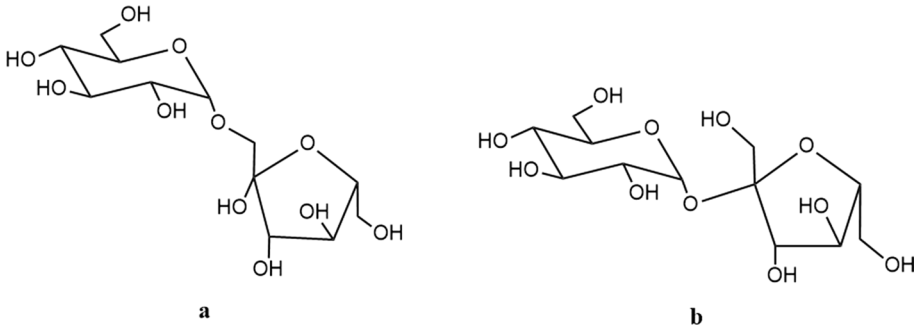

Fig. 1 The chemical structures of trehalulose (**a**) and sucrose (**b**)

the authors' knowledge, no HPTLC-based method for the quantitative analysis of trehalulose has yet been reported.

HPTLC is a sophisticated analytical technique based on thin-layer chromatography (TLC) principles but provides superior separation efficiency for qualitative and quantitative analysis. The use of high-performance adsorbent plates and semi-automated instrumentation for sample application, chromatographic development, derivatisation and chromatogram evaluation makes HPTLC a very popular technique [16, 17]. The advantages of HPTLC over other traditional separation methods include its low running cost, minimal solvent and reagent input, the ability to analyse multiple samples simultaneously, and ease of use. Thus, HPTLC presents itself as a potential new analytical method for the quantification of trehalulose in natural products and food items.

2 Experimental

2.1 Materials, chemicals and reagents

The chemicals and reagents were sourced from: trehalulose (purity > 90% as specified by the supplier) (Biosynth Carbosynth, Staad, Switzerland); sucrose, fructose and 1-butanol (Chem-Supply Pty Ltd., Gillman, Australia); maltose (Glentham Life Sciences, Corsham, UK); glucose and phosphoric acid (Ajax Finechem Pvt Ltds., Cheltenham, Australia); diphenylamine (The British Drug Houses Ltd., London, UK); boric acid (Pharma Scope, Welshpool, Australia); methanol and 2-propanol (Merck KGaA, Darmstadt, Germany) and aniline (Fluka AG, Buchs, Switzerland). Silica gel 60 F_{254} HPTLC glass plates (20 cm × 10 cm) were obtained from Merck KGaA (Darmstadt, Germany). Stingless bee honey produced by the bee species *Tetragonula carbonaria* was purchased from a beekeeper in Queensland, Australia. The honey sample was stored in a refrigerator (4 °C) until its analysis.

2.2 Sample preparation

2.2.1 Standard and reagent preparation

Standard solutions of trehalulose, glucose, fructose, maltose and sucrose (200 µg/mL) were prepared by transferring 10 mg of each sugar into a 50 mL volumetric flask and dissolving it in 50% aqueous methanol. After sonication for 10 min, the solutions were made up to their final volume with 50% aqueous methanol. The mobile phase used was composed of 1-butanol−2-propanol−aqueous boric acid (5 mg/mL) at a ratio of 30:50:10 (*V/V*) [13]. To prepare the derivatisation reagent, 2 g of diphenylamine and 2 mL of aniline were dissolved in 80 mL of methanol, followed by the addition of 10 mL of phosphoric acid (85%). The solution was made up to 100 mL using methanol.

2.2.2 Preparation of honey solution

Artificial honey was prepared as described previously [18] by dissolving 1.5 g sucrose, 7.5 g maltose, 40.5 g fructose and 33.5 g glucose in 17 mL of distilled water. Artificial honey samples for analysis were prepared by dissolving 20 mg of artificial honey in 100 mL of 50% aqueous methanol.

To prepare the stingless bee honey solution (1 mg/mL), an amount of 100 mg of the honey was dissolved in 100 mL of 50% aqueous methanol.

2.3 Instrumentation and HPTLC method

2.3.1 Sample application

A semi-automated HPTLC application device (Linomat 5; CAMAG, Muttenz, Switzerland) was used to apply the standard trehalulose solution (application volume 0.5–4.0 µL) at a rate of 40 nL/s at 8.0 mm from the bottom

and 20.2 mm from the side edges of the HPTLC plate with 8.0 mm band length and 11.4 mm of distance between bands, producing a standard curve ranging from 100 to 800 ng of trehalulose per band.

2.3.2 Development and derivatisation

HPTLC plates were developed with the mobile phase in a saturated (33% relative humidity) automated development chamber (ADC2, CAMAG) at room temperature. The development chamber was saturated for 60 min, the plates were pre-conditioned with the mobile phase for 5 min, and 10 mL of mobile phase were used for development at room temperature to a migration distance of 85 mm. After development, the plates were dried for 5 min. A HPTLC imaging device (TLC Visualiser 2, CAMAG) was used to document the chromatographic results under white light. The HPTLC software (visionCATS v3.1, CAMAG) was used to analyse the documented images.

After development, the plate was derivatised with 2 mL of aniline−diphenylamine−phosphoric acid reagent using a TLC derivatiser (CAMAG Derivatiser, yellow nozzle). Afterwards, the plate was heated for 10 min at 115 °C using a CAMAG TLC Plate Heater 3. After cooling the plate to room temperature, the HPTLC imaging device was again used to analyse the plates under white light. Analyses were conducted using three sets of images: remission white (R white), transmission white (T white), and remission–transmission white (RT white).

2.4 Method validation

The developed method for the quantification of trehalulose was validated for specificity, linearity, sensitivity, precision, accuracy, repeatability and robustness following current International Conference on Harmonisation (ICH) guidelines [19].

2.4.1 Specificity

Specificity refers to the ability of a method to measure the analyte of interest in the presence of potentially interfering substances or closely related compounds that may be present in the sample matrix. In the case of HPTLC analysis, specificity is assured by complete separation of peak(s) of analyte(s) from other peaks originated from the sample matrix and distinct retardation factor (R_F) values on the chromatographic plate. In this study, specificity evaluation was carried out by testing the ability of the method to separate common sugars as they might occur in a honey's matrix. For this, 2 µL of each standard sugar solution (trehalulose, glucose, fructose, maltose and sucrose) was applied on a HPTLC plate. The plate was developed with the mobile phase, derivatised and analysed under white light to evaluate the R_F for each sugar separately. In addition, 2 µL of artificial honey solution was overspotted with increasing volumes of trehalulose standard solution for analysis. Similarly, 1 µL of stingless bee honey solution was also overspotted with increasing volumes of trehalulose standard solution for analysis.

Furthermore, to confirm that the trehalulose band did not overlap with any other band in the stingless bee honey sample, the honey sample was double developed (2D analysis) in the mobile phase and the trehalulose band closely evaluated for potential co-elution. For this, 5 µL of the stingless bee honey solution (1 mg/mL) was placed as 8.0 mm band on a 10 × 10 cm HPTLC plate at 8.0 mm from the lower edge and 15.0 mm from the left edge of the plate. The HPTLC plate was first developed with the mobile phase to a solvent migration distance of 85 mm. After drying, the plate was rotated by 90° counter clockwise and developed again with the same mobile phase to a solvent migration distance of 85 mm. Following the second development, the plate was derivatised and analysed under white light using the HPTLC software.

2.4.2 Linearity and range

Linearity is the ability of the analytical procedure to obtain results that are directly proportional to the concentration of the analyte in the sample. To evaluate the linearity of the proposed method, three calibration curves, each consisting of five data points, were constructed by analysing a series of standard trehalulose solutions in a concentration range of 100–800 ng per band. The peak areas and heights were plotted against the corresponding concentrations of the standard. The resulting calibration curves were evaluated for their linearity using linear and polynomial regression analysis, and by determining the coefficient of correlation (R), slope (m), y-intercept (c), uncertainty of the slope and y-intercept, and standard deviation (SD).

Additionally, to ensure that the method has an appropriate level of accuracy and precision at the extremes of its specified range (100–800 ng per band), trehalulose solutions at low (100 ng per band) and high (800 ng per band) concentration were analysed in triplicate. Results were expressed as % recovery and standard deviation.

2.4.3 Sensitivity

The sensitivity of the method refers to its ability to detect and quantify small amounts of the analyte in the sample. To evaluate sensitivity, the limit of detection (LOD) and limit of quantification (LOQ) were determined. LOD is the lowest amount or concentration of analyte in a sample that can be detected with sufficient degree of confidence. LOQ is the lowest amount or concentration of analyte in a sample

that can be quantified with acceptable repeatability, accuracy and precision.

On the basis of three calibration curves, LOQ and LOD were calculated using the following equations:

$$\mathrm{LOD} = 3.3 \times \sigma / S$$

$$\mathrm{LOQ} = 10 \times \sigma / S$$

where σ is the standard deviation of the regression line and S is the slope of the calibration curve.

2.4.4 Precision

Method validation requires testing for intra-day and inter-day precision, which are important measures of the reliability and consistency of the analytical method. Intra-day precision refers to variation in results obtained from multiple measurements of the same sample within the same day, while inter-day precision refers to the variation in results obtained from multiple measurements of the same sample over different days.

To test for intra-day precision, three different concentrations of trehalulose (300, 400 and 500 ng per band) were analysed, in triplicate, on the same day using the developed method. Similarly, to test for inter-day precision, trehalulose standard solutions (300, 400 and 500 ng per band) were analysed, in triplicate, on different days. The level of precision was expressed as the percentage relative standard deviation (*%RSD*) of the results obtained from peak area.

2.4.5 Accuracy

The accuracy of an analytical method refers to its ability to produce results that are close to the true or expected value of the samples being analysed. The accuracy of the current method was tested by applying 1 µL of trehalulose solutions (200 µg/mL) on a HPTLC plate and spiking the sample bands with known quantities of trehalulose in the range of 300, 400 and 500 ng per band. The % recovery and *%RSD* of the method were used to determine its accuracy. All experiments were conducted in triplicate.

2.4.6 Repeatability

The repeatability of an analytical method is determined by evaluating the consistency of results obtained after multiple measurements of the same sample under the same conditions. The repeatability of the proposed method was assessed by analysing trehalulose standard solutions at a concentration of 400 ng per band five times. Results are expressed as *%RSD* of standards.

2.4.7 Robustness

The robustness of a method is its ability to produce reliable and reproducible results regardless of small variations in experimental conditions. The robustness of the method was tested by analysing three trehalulose standards at concentrations of 300, 400 and 500 ng per band using slight changes in the volume of the mobile phase (± 2 mL), in the ratios of the mobile phase composition (1-butanol−2-propanol−aqueous boric acid; 30:52:10 and 32:50:10, *V/V*) and in chamber saturation time (± 5 min). The results were analysed for robustness based on obtained trehalulose R_F values and its % recovery.

2.4.8 Quantification of trehalulose in stingless bee honey

To determine the concentration of trehalulose in the stingless bee honey, 2.5 µL of the honey solution (1 mg/mL) were applied on a HPTLC plate and subjected to chromatography using the prescribed conditions. By comparing the peak area of the sample band with the corresponding values obtained from the standard curve, the amount of trehalulose present in the honey was calculated. The results were expressed in g/100 g based on three replicate analyses ($n = 3$).

3 Results and discussion

3.1 Mobile phase selection

This study aimed to develop a method for quantifying trehalulose in stingless bee honey. Since this type of honey is known to contain various sugars, including glucose, fructose and sucrose [5, 20], it is crucial to develop a method that can accurately quantify trehalulose in the presence of other sugars.

To achieve this goal, the mobile phase 1-butanol−2-propanol−aqueous boric acid (30:50:10, *V/V*) was chosen for the analysis. This mobile phase has been used in a previously published and validated HPTLC method for the quantification of glucose, fructose and sucrose in common honey [13].

Using the selected mobile phase, the R_F value for trehalulose was found to be 0.045 ± 0.002, which is below the ideal range for R_F values in complex mixtures that is generally considered to be between 0.1 and 0.8. To ensure that no other compounds co-eluted with trehalulose at this low R_F, a double development analysis was performed.

Despite its relatively low R_F value, the method demonstrated good separation between trehalulose and the other major sugars present in stingless bee honey sample. Furthermore, the use of a single mobile phase for the analysis of multiple sugars, including trehalulose, was deemed more commercially feasible than using a separate mobile

phase developed exclusively for trehalulose analysis. This approach simplifies the analytical procedure and reduces associated costs and analysis times.

3.2 Selection of detection wavelength and absorbance peak intensity

The analysis involved three sets of images, namely R white, T white and RT white. Each set of images was evaluated using visionCATS v3.1 software, and T white was determined to provide the most accurate data across the entire concentration range of the calibration curve, providing better % recovery and *R* values.

For quantitative analysis, two graphs were plotted against the concentration of the corresponding sugar standard curves: absorbance peak areas and absorbance peak heights. It was found that across the entire calibration range the peak areas *versus* concentration approach produced more consistent and accurate analysis results compared to peak heights *versus* concentration approach. Therefore, the validation was carried out using peak area for trehalulose quantification.

3.3 Chromatographic results

After the derivatisation process with aniline−diphenylamine−phosphoric acid reagent, the trehalulose band was identified by an orange-light brown coloured band at R_F value 0.045.

Moreover, the HPTLC analysis successfully separated trehalulose from fructose, maltose, sucrose and glucose with each of them showing different R_F values (0.11, 0.18, 0.23, 0.29, respectively) (Fig. 2). Thus, the method allows for the quantification of trehalulose in the presence of these other sugars that are also commonly found in honey.

Furthermore, the purity of the trehalulose band in native bee honey was confirmed by 2D chromatography analysis. The lack of additional bands overlapping with the trehalulose band indicated that it did not co-elude with any other compounds in the stingless bee honey sample (Fig. 3).

3.4 Method validation

To evaluate the specificity of the analytical method, artificial honey and stingless bee honey sample solutions were overspotted with trehalulose solutions (Figs. 4 and 5). In both analyses, a standard curve (100–800 ng per band) was built to quantify trehalulose. In the artificial honey analysis,

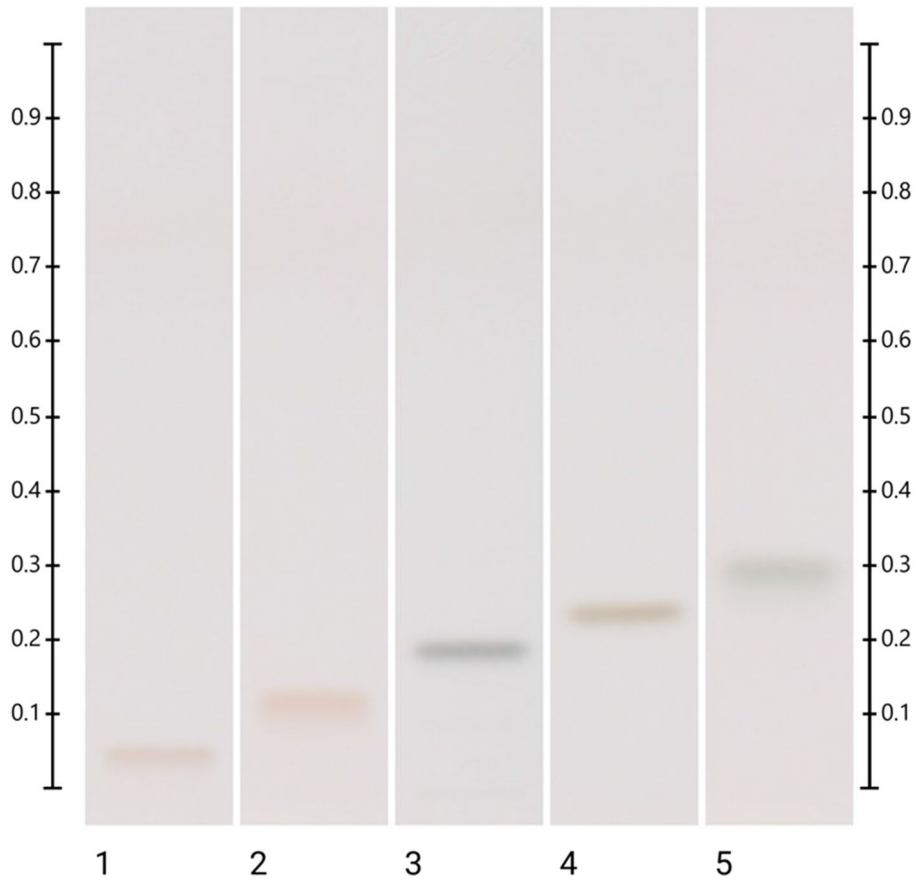

Fig. 2 Image of HPTLC plate taken after derivatisation. Track 1: trehalulose; track 2: fructose; track 3: maltose; track 4: sucrose; track 5: glucose

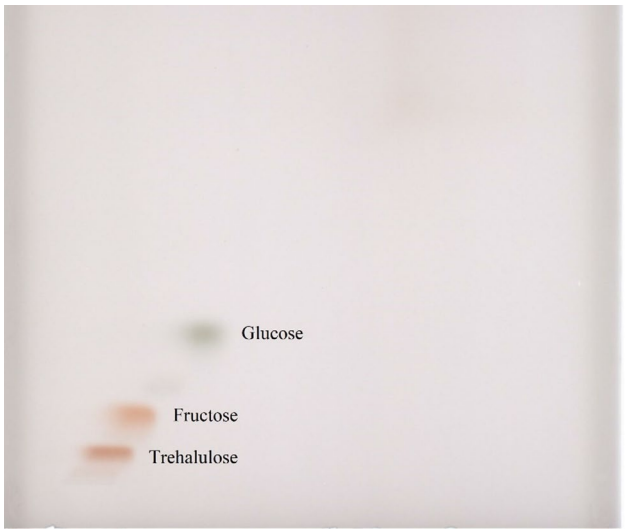

Fig. 3 2D chromatography separation of stingless bee honey sample

trehalulose recovery ranged from 96.63% to 102.6%. In the stingless bee honey analysis, it was not possible to calculate the trehalulose recovery on the basis of the theoretical concentration since the honey sample also contains a certain amount of trehalulose, however, a linear relationship was observed in the calibration curve with an *R* value of 0.9991. A high degree of specificity for trehalulose is evident from this result.

For quantitative analysis, standard trehalulose solution was applied on a HPTLC plate (0.5, 1.0, 2.0, 3.0, 4.0 µL) and the resulting peak areas plotted against the respective concentrations. The five-point calibration curve was found to be linear over the concentration range of 100 to 800 ng per band. The linearity of the method was validated by using linear regression and coefficient of correlation (*R*) of the standard curves. The *R* values ranged from 0.9993 to 0.9998. The uncertainty values of the slope and *y*-intercept were also calculated and are presented in Table 1.

To determine the sensitivity of the method, the trend line equation of three calibration curves was used to calculate the LOD and LOQ. LOD and LOQ of the method were found to be 20.04 and 60.72 ng per band, respectively (Table 1).

The validation of the method's range showed that it produced acceptable results at the extreme edges of the specified range, as evidenced by a % recovery of $101.2 \pm 1.474\%$ and $100.1 \pm 5.997\%$ for the trehalulose concentrations of 100 ng per band and 800 ng per band, respectively.

The precision of the method was assessed for both intra- and inter-day analyses, utilising linear regression data from the calibration curves. The precision study involved repeatedly analysing three different concentrations of trehalulose solutions (300, 400 and 500 ng per band). The obtained *%RSD* values were found to be in a range of 0.2986% and 2.699% (Tables 2 and 3) and within an acceptable limit. This suggests that the method exhibits a high degree of precision.

Repeatability is a key parameter in determining the precision of an analytical method and it was expressed in terms of SD and *%RSD*. As shown in Table 4, the *%RSD* values obtained to assess the repeatability of the method were within acceptable limits.

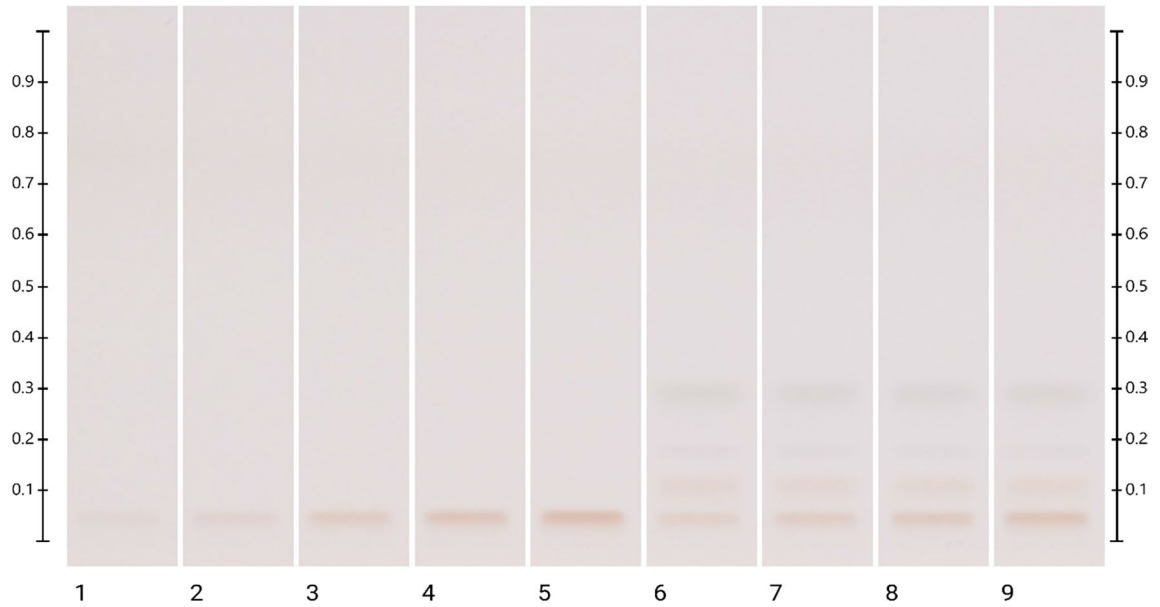

Fig. 4 Image of HPTLC plate taken after derivatisation. Tracks 1−5: trehalulose standards (0.5, 1.0, 2.0, 3.0, 4.0 µL); tracks 6–9: 2 µL of artificial honey overspotted with 1.5, 2.0, 2.5 and 3.0 µL of trehalulose, respectively

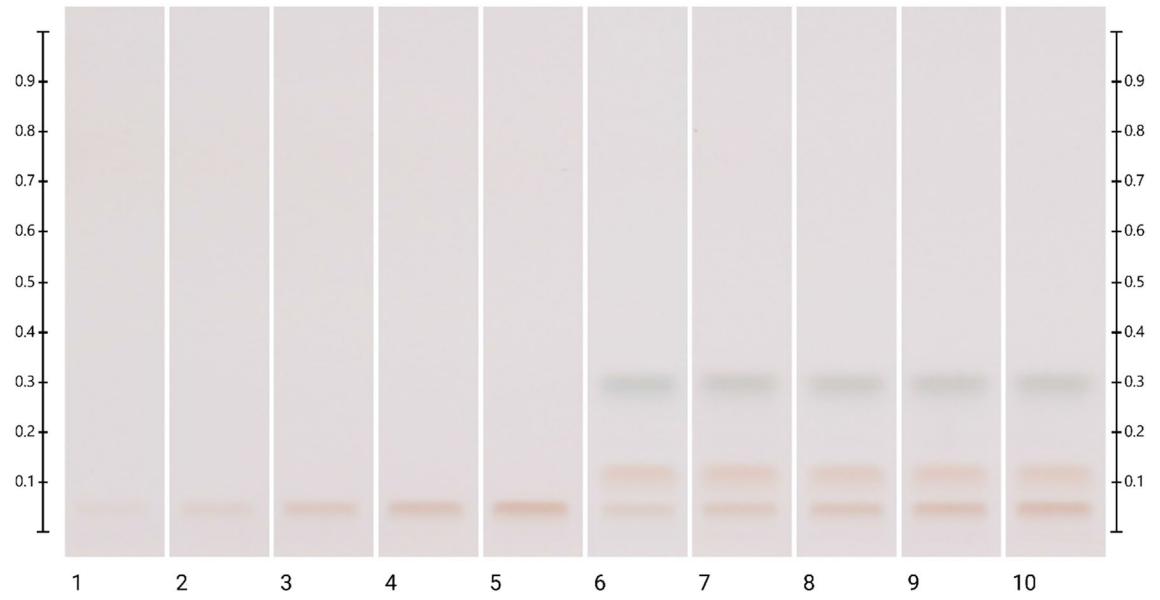

Fig. 5 Image of HPTLC plate taken after derivatisation. Tracks 1−5: trehalulose standards (0.5, 1.0, 2.0, 3.0, 4.0 µL); tracks 6−10: 1 µL of stingless bee honey overspotted with 1.0, 1.5, 2.0, 2.5 and 3.0 µL of trehalulose, respectively

Table 1 Linear regression data for the calibration curves of trehalulose

Run	Linearity range (ng per band)	Regression equation	Coefficient of correlation (*R*)	Slope uncertainty value	*Y*-intercept uncertainty value	LOD (ng)	LOQ (ng)
1	100–800	$y = 6 \times 10^{-6}x + 0.0003$	0.9998	6.936×10^{-8}	3.412×10^{-5}	19.15	58.02
2	100–800	$y = 6 \times 10^{-6}x + 0.0003$	0.9993	1.197×10^{-7}	5.887×10^{-5}	20.88	63.26
3	100–800	$y = 7 \times 10^{-6}x + 0.0003$	0.9998	7.716×10^{-8}	3.796×10^{-5}	20.10	60.89
Mean			0.9996	8.873×10^{-8}	4.365×10^{-5}	20.04	60.72

Table 2 Precision study: intra-day precision

Trehalulose concentration (ng per band)	Run 1 amount found (ng per band)	Run 2 amount found (ng per band)	Run 3 amount found (ng per band)	Mean	SD	*%RSD*
300	290.3	305.2	293.8	296.4	5.971	2.014
400	408.1	401.3	405.3	404.9	2.173	0.5366
500	489.7	498.9	500.8	496.5	2.182	0.4396

Table 3 Precision study: inter-day precision

Trehalulose concentration (ng per band)	Run 1 amount found (ng per band)	Run 2 amount found (ng per band)	Run 3 amount found (ng per band)	Mean	SD	*%RSD*
300	313.6	299.2	305.2	306.0	3.720	1.216
400	430.8	422.7	401.3	418.3	11.29	2.699
500	501.4	501.9	498.9	500.7	1.495	0.2986

Table 4 Repeatability study

Trehalulose concentration (ng per band)	Amount found (ng per band)	Mean	SD	*%RSD*
400	412.30	400.1	8.425	2.106
400	389.40			
400	402.80			
400	396.50			
400	399.50			

Table 7 Robustness study: changes in saturation time

Saturation time (min)	Trehalulose concentration (ng per band)	% Recovery	R_F (mean ± SD)
55	300	99.93	
	400	99.03	0.049 ± 0.002
	500	97.87	
65	300	99.03	
	400	97.94	0.042 ± 0.001
	500	98.72	

To determine the accuracy of the method in terms of sample recovery, the % mean recovery of trehalulose was calculated using the standard addition method. This method involves adding a known amount of the analyte to the sample and then quantifying the amount recovered. The results of the analysis, presented in Table 5, indicate that the method exhibits a high level of accuracy, with recovery rates ranging from 99.74% to 104.4%, and with a % mean recovery of 101.8%. These findings suggest that the method can be used with confidence to provide accurate and reliable analytical results.

A robustness test was conducted by intentionally varying parameters of the method, such as saturation time, mobile phase volume and composition. It was found that variations in saturation time and mobile phase composition had minimal influence on the quantification of trehalulose and its R_F value. On the other hand, variations in the mobile phase volume had more impact on the R_F value, but did not significantly affect % recovery ($p < 0.05$). Therefore, the proposed method can be considered robust. The summary of the robustness study is presented in Tables 6, 7 and 8.

The trehalulose content in stingless bee honey was determined using the newly developed and validated method (Table 9). Overall, the results demonstrate the suitability of the method for accurate and reliable analysis of trehalulose in honey samples.

4 Conclusion

A simple, cost-effective and selective HPTLC method has been developed for the quantification of trehalulose in stingless bee honey samples. The method was successfully validated according to ICH guidelines for its sensitivity, specificity, linearity, precision, accuracy and robustness. Thus, this newly developed method can be used as a standard quality control method for analysing stingless bee honey samples and can potentially be used to quantify trehalulose in other natural products and food items.

Table 5 Accuracy study

Trehalulose concentration (ng per band)	Run 1			Run 2			Run 3		
	Recovery (ng)	% Recovery	% Mean recovery	Recovery (ng)	% Recovery	%Mean recovery	Recovery (ng)	% Recovery	% Mean recovery
300	302.4	100.8	101.7	304.5	101.5	101.3	302.9	101.0	102.3
400	417.8	104.4		409.6	102.4		410.5	102.6	
500	498.7	99.74		500.4	100.1		517.1	103.4	

Table 6 Robustness study: changes in mobile phase composition

Mobile phase composition	Trehalulose concentration (ng per band)	% Recovery	R_F (mean ± SD)
1-Butanol−2-propanol−boric acid (30:52:10, *V/V*)	300	105.6	0.042 ± 0.001
	400	96.80	
	500	102.9	
1-Butanol−2-propanol−boric acid (32:50:10, *V/V*)	300	94.20	0.044 ± 0.002
	400	98.43	
	500	95.33	

Table 8 Robustness study: changes in mobile phase volume

Mobile phase volume (mL)	Trehalulose concentration (ng per band)	% Recovery	R_F (mean ± SD)
8	300	93.43	
	400	98.32	0.054 ± 0.002
	500	98.50	
12	300	96.84	
	400	98.28	0.063 ± 0.002
	500	98.13	

Table 9 Trehalulose content of stingless bee honey

	Trehalulose concentration (g/100 g)	Mean	SD
Run 1	10.13	10.17	0.04924
Run 2	10.16		
Run 3	10.22		

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Declarations

Conflict of interest The authors declare that they do not have any conflict of interest.

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