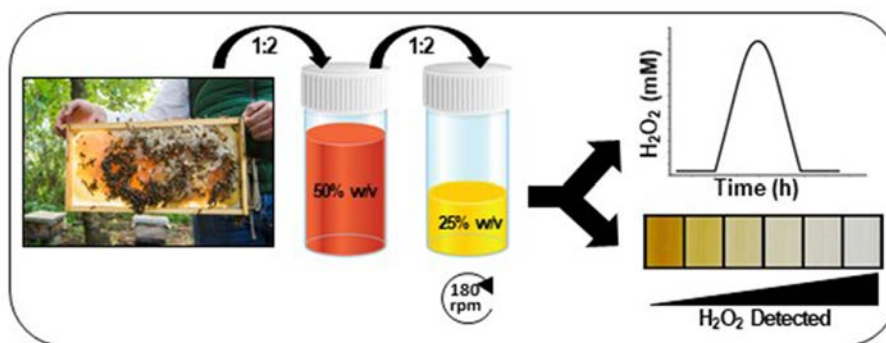


# A cost-effective colourimetric assay for quantifying hydrogen peroxide in honey

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## Graphical abstract

H<sub>2</sub>O<sub>2</sub> is produced in diluted honey by a biochemical reaction and can be easily quantified using the optimized colourimetric assay described here.

## Abstract

Honey is a natural product with many beneficial properties including antimicrobial action. Production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in diluted honey is central to this action. Here, we describe an optimized method for measuring levels of H<sub>2</sub>O<sub>2</sub> in honey. This method is based on established methods, with the level of dilution, the time between dilution and reading the assay, and aeration of the samples during the assay identified as critical points for ensuring reliability and reproducibility. The method is cost-effective and easy to perform using common laboratory equipment. Using this method, we quantified the hydrogen peroxide content of five different, unprocessed polyfloral honeys collected in NC, USA. Our results show that H<sub>2</sub>O<sub>2</sub> production by these honeys varies greatly, with some samples producing negligible levels of H<sub>2</sub>O<sub>2</sub>. We assessed the effect of colour on the assay by measuring the recovery of spiked H<sub>2</sub>O<sub>2</sub> from light and dark honey and from serially diluted dark corn syrup, and found the amount of H<sub>2</sub>O<sub>2</sub> that could be detected was lower in dark corn syrup and darker honey samples.

## INTRODUCTION

Honey is composed of approximately 83% sugar, primarily glucose and fructose, and about 17% water with an average pH of 3.9 [1]. However, it is more than just a supersaturated

sugar solution; honey is a complex mixture composed of sugars, proteins, amino and organic acids, flavonoids, polyphenols, carotenoid-like substances, Maillard reaction products [i.e. 5-hydroxymethyl(fural)], vitamins, minerals and water [2].

Received 18 April 2019; Accepted 17 September 2019; Published 21 October 2019

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**Keywords:** honey; hydrogen peroxide; horse radish peroxidase; glucose oxidase; *o*-dianisidine; colourimetric assay.

**Abbreviations:** CCS, clear corn syrup; DCS, dark corn syrup; dH<sub>2</sub>O, distilled water; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HRP, horseradish peroxidase; H<sub>2</sub>SO<sub>4</sub>, sulfuric acid; MGO, methylglyoxal.

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The actual composition of honey varies and depends on factors including nectar source, pollen content, foraging time of year and other elements of the environment [3]. Due to its unique properties, honey has been utilized as a food source for humans and also has proven useful as a topical treatment for wounds and bacterial infections [4].

Laboratory-based research and a limited number of clinical studies have demonstrated that honey possesses broad-spectrum antimicrobial properties against bacteria, fungi, viral and mycobacterial pathogens [5–14], with maximal effects observed in fresh, unheated honey [15–18]. All honeys possess intrinsic characteristics that, in combination, can inhibit microbial growth and survival. The antimicrobial properties of some honeys are augmented by other compounds introduced by the bees themselves or through their diet, including lysozyme, flavonoids and polyphenols [2]. In addition, phytochemically derived methylglyoxal (MGO) and the antimicrobial peptide bee defensin-1 (i.e. royalisin) were determined to be novel mechanisms of antibacterial action in Manuka honey and RS honey, respectively [6, 19, 20]. Antimicrobial effects also stem from hydrogen peroxide ( $H_2O_2$ ) in honey, which is produced by glucose oxidase, an enzyme introduced into nectar by worker bees [21]. In the presence of certain metals,  $H_2O_2$  decomposes to form reactive oxygen species, which drive lipid peroxidation, ultimately destroying microbes [22].

Glucose oxidase is inactive in fully ripened honey [23]. However, when honey is diluted, glucose oxidase converts  $\beta$ -D-glucose into  $H_2O_2$  and D-Gluconic acid [6]. The amount of  $H_2O_2$  produced is dependent on the type of honey, honey age and storage conditions (i.e. light exposure, temperature and filtration), honey dilution rate, and length of time since dilution [18, 24].  $H_2O_2$  accumulation is greatest in honey samples diluted to 30–50 % strength; due to the low affinity of glucose oxidase for glucose, accumulation decreases when honey is diluted below 30% [23].

Given the high level of interest in the use of honey for wound healing [5, 6, 10, 25–28], characterizing the capacity of honey to produce  $H_2O_2$  is of great interest to medical practitioners, complementary and alternative medicine communities, food chemists and beekeepers. The AmplexRed assay is a reliable test that has been used in a number of studies, however it is relatively expensive and is more suited to medium-to-large throughput studies. A colourimetric assay using horseradish peroxidase (HRP) to catalyse the oxidation of colourless *o*-dianisidine by  $H_2O_2$  to a coloured product has been in use for a number of years [6, 18, 29–32], but detailed optimized methodology is lacking, requiring researchers to perform considerable trouble-shooting. Below, we provide this as an optimized, easy-to-perform and cost-effective protocol for the quantification of  $H_2O_2$  in honey. We demonstrate the utility of this method by examining the  $H_2O_2$  production capacity of five different polyfloral honeys collected in the NC, USA. In addition, we show how the capacity to detect  $H_2O_2$  can

be quenched in darker coloured honey and corn syrup samples.

## METHODS

### Optimized $H_2O_2$ assay

We used a previously developed colourimetric assay to determine the concentration of hydrogen peroxide in honey [6, 18] with modifications that enabled significant improvements in reproducibility and reliability. This assay is based on the fact that upon dilution of honey with water,  $H_2O_2$  production by glucose oxidase is activated, and this is detected by the oxidation of colourless *o*-dianisidine reagent catalysed by HRP, resulting in the formation of a coloured product that is detected spectrophotometrically (Fig. 1).

### Preparation of reagent solutions

In total, 1 M sodium phosphate monohydrate, anhydrous, monobasic, was prepared by dissolving 6.9 g  $NaH_2PO_4 \cdot H_2O$  in a total volume of 50 ml distilled water. Then, 1 M disodium phosphate anhydrous, dibasic was prepared by dissolving 7.1 g  $Na_2HPO_4$  in a total volume of 50 ml distilled water with gentle heating until all solid was visibly dissolved. Next, 10 mM sodium phosphate buffer, pH 6.5, was prepared by adding 3.15 ml  $Na_2HPO_4$  and 6.85 ml  $NaH_2PO_4 \cdot H_2O$  to 990 ml sterile, distilled water and adjusting to pH 6.5 with  $Na_2HPO_4$  (if too acidic) or  $NaH_2PO_4 \cdot H_2O$  (if too basic). All buffers were filter sterilized using a 0.45  $\mu$ m filter and stored at 4 °C.

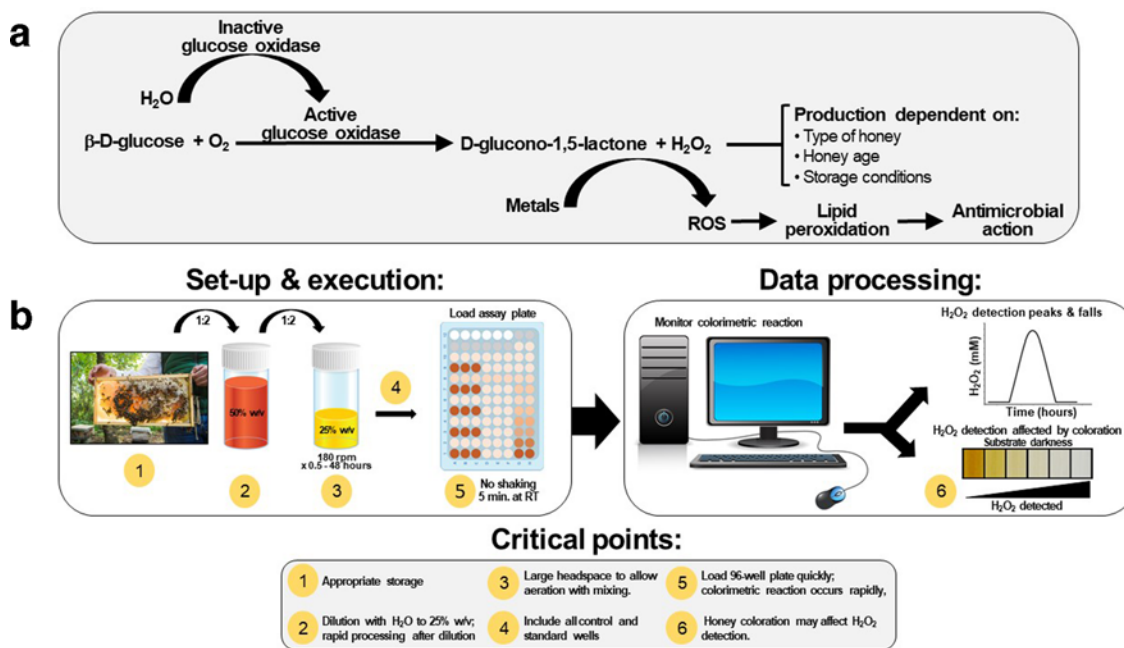
A 5 mg ml<sup>-1</sup> stock solution of *o*-dianisidine was prepared by adding 20 mg of *o*-dianisidine (Sigma Aldrich, Cat. No. D9143) to 4 ml 95% ethanol with gentle mixing until all *o*-dianisidine dissolved. On the day of experimentation, 250  $\mu$ l of this was added to 1 ml of the 10 mM sodium phosphate buffer (pH 6.5) to make a 1 mg ml<sup>-1</sup> working solution.

Then, 10 mg ml<sup>-1</sup> HRP, type II, made fresh on the day of experimentation, was prepared by adding 10 mg HRP, type II (Sigma Aldrich, Cat. No. P8250-25KU) to 1 ml 10 mM sodium phosphate buffer with gentle mixing until the HRP dissolved. Next, 2 mg ml<sup>-1</sup> catalase solution, also made fresh on the day of experimentation, was made by adding 10 mg catalase to 5 ml of 10 mM sodium phosphate buffer with gentle mixing until fully dissolved. Catalase blank solution consisted of 10 mM sodium phosphate buffer only. Then, 6M sulfuric acid was prepared by slowly adding 67 ml of 18 M  $H_2SO_4$  to 50 ml distilled water and adjusting to a final volume of 200 ml with distilled water.

HRP reagent mixture solution was prepared by combining 1 ml of 1 mg ml<sup>-1</sup> *o*-dianisidine working solution and 40  $\mu$ l of 10 mg ml<sup>-1</sup> HRP stock with 18.96 ml of the 10 mM sodium phosphate buffer (this makes enough for 148 wells). This was left at room temperature until used, and any remaining solution was discarded once the assay was completed.

### Preparation of honey samples and $H_2O_2$ standards

All of the following preparation was done on the day of experimentation and was performed under subdued lightning.



**Fig. 1.** H<sub>2</sub>O<sub>2</sub> production mechanism and methodological approach. (a) H<sub>2</sub>O<sub>2</sub> is produced in diluted honey by a reaction involving  $\beta$ -D-glucose, O<sub>2</sub> and activated glucose oxidase. Production of H<sub>2</sub>O<sub>2</sub> is influenced by the indicated parameters. (b) H<sub>2</sub>O<sub>2</sub> production can be easily quantified using the optimized colorimetric assay described here provided attention is paid to by several critical points in the set-up and execution of the assay. ROS=reactive oxygen species.

To prepare the honey samples, 4 g of each honey was added to 4 ml sterilized dH<sub>2</sub>O pre-warmed to 37 °C and incubated at 35 °C protected from light on an orbital shaker (~180 r.p.m.) for 20 min to aid mixing. The resulting 50% (w/v) stock solutions were filter sterilized through a 0.22  $\mu$ m pore filter (Millex). Aliquots (2.5 ml) of the sterilized honey were then transferred to 28 ml McCartney bottles and further diluted to 25% (w/v) using either sterile deionized water, catalase solution or catalase blank solution. The McCartney bottles were protected from light using aluminum foil and incubated at 35 °C in an orbital shaking incubator at 180 r.p.m. for various times to enable a time-course (initially from 0.5 to 48 h and subsequently from 2 to 18 h). Agitating the diluted honey with this large headspace volume enabled thorough aeration of the sample, which proved critical for maximal and reliable H<sub>2</sub>O<sub>2</sub> production. When preparing multiple honey samples, dilutions were done at the same time for all samples.

To prepare the H<sub>2</sub>O<sub>2</sub> standards, 10 ml of 8.8 mM H<sub>2</sub>O<sub>2</sub> was prepared from 0.88 M H<sub>2</sub>O<sub>2</sub> stock using sterile dH<sub>2</sub>O, and this was further diluted to make 1 ml of 2.2 mM H<sub>2</sub>O<sub>2</sub>. From this, 500  $\mu$ l was aliquoted into an amber 1.5 ml tubes, and 250  $\mu$ l of 10 mM sodium phosphate buffer was aliquoted into an additional ten amber tubes. The H<sub>2</sub>O<sub>2</sub> solution was then serially diluted from the first tube and across the remaining ten tubes at a 1:1 ratio to produce 2200, 1100, 550, 275, 137.5, 68.8, 34.4, 17.2, 8.6, 4.3, 2.1  $\mu$ M H<sub>2</sub>O<sub>2</sub> standards. All tubes were vortexed well between dilutions to mix thoroughly. A 0  $\mu$ M H<sub>2</sub>O<sub>2</sub> standard was prepared by

combining 250  $\mu$ l of 10 mM sodium phosphate buffer with 250  $\mu$ l sterile dH<sub>2</sub>O. All H<sub>2</sub>O<sub>2</sub> standards were used within 2 h of preparation.

Working in subdued light, 96-well flat-bottomed microtitre assay plate(s) were loaded in the following manner (see also Fig. 2):

### Standards

*H<sub>2</sub>O<sub>2</sub> standards* – 40  $\mu$ l of the serially diluted H<sub>2</sub>O<sub>2</sub> standards were aliquoted into wells G1-12 and H1 – 12, followed by 135  $\mu$ l of HRP reagent mixture solution.

*Catalase negative control* – 20  $\mu$ l of 550  $\mu$ M H<sub>2</sub>O<sub>2</sub> standard and 20  $\mu$ l of catalase solution were added to wells A11, B11 and C11, followed by 135  $\mu$ l of HRP reagent mixture solution.

*Catalase negative control blank* – 20  $\mu$ l of 550  $\mu$ M H<sub>2</sub>O<sub>2</sub> standard and 20  $\mu$ l of 10 mM sodium phosphate buffer were added to wells D11, E11 and F11, followed by 135  $\mu$ l of HRP reagent mixture solution.

### Honey samples

*Honey sample no. 1 test* – 40  $\mu$ l of the first honey sample (diluted 25% w/v in dH<sub>2</sub>O) was added to wells A1, B1 and C1, followed by 135  $\mu$ l of HRP reagent mixture solution.

*Honey sample no. 1 blank* – 40  $\mu$ l of the first honey sample (diluted 25% w/v in dH<sub>2</sub>O) was added to wells D1, E1 and F1, followed by 135  $\mu$ l of 10 mM sodium phosphate buffer.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Honey sample 1	Honey sample 1 w/ catalase	Honey sample 2	Honey sample 3	Honey sample 4	Honey sample 5	Catalase negative control	Catalase negative control blank	Catalase negative control	Catalase negative control blank	Catalase negative control	Catalase negative control blank
B												
C												
D	Honey sample 1 blank	Honey sample 1 w/ catalase blank	Honey sample 2	Honey sample 3	Honey sample 4	Honey sample 5	Catalase negative control	Catalase negative control blank	Catalase negative control	Catalase negative control blank	Catalase negative control	Catalase negative control blank
E												
F												
G	2,200 $\mu\text{M}$ $\text{H}_2\text{O}_2$	1,100 $\mu\text{M}$ $\text{H}_2\text{O}_2$	550 $\mu\text{M}$ $\text{H}_2\text{O}_2$	275 $\mu\text{M}$ $\text{H}_2\text{O}_2$	138.5 $\mu\text{M}$ $\text{H}_2\text{O}_2$	67.8 $\mu\text{M}$ $\text{H}_2\text{O}_2$	34.4 $\mu\text{M}$ $\text{H}_2\text{O}_2$	17.8 $\mu\text{M}$ $\text{H}_2\text{O}_2$	8.6 $\mu\text{M}$ $\text{H}_2\text{O}_2$	4.3 $\mu\text{M}$ $\text{H}_2\text{O}_2$	2.1 $\mu\text{M}$ $\text{H}_2\text{O}_2$	0 $\mu\text{M}$ $\text{H}_2\text{O}_2$
H												

**Fig. 2.** The 96-well microplate lay-out for quantification of hydrogen peroxide in honey samples. Each honey sample requires 12 wells divided into quadrants with three replicates in each quadrant. Quadrants consist of honey alone and three honey-containing controls. This scheme allows for up to five honey samples and catalase controls to be assayed (in triplicate) on a single plate. The diagonal line in column 12 indicates empty assay plate wells.

*Honey sample no. 1 with catalase* – 40  $\mu\text{l}$  of the first honey sample (diluted 25% w/v with catalase solution) was added to wells A2, B2 and C2, followed by 135  $\mu\text{l}$  of HRP reagent mixture solution.

*Honey sample no. 1 with catalase blank* – 40  $\mu\text{l}$  of the first honey sample (diluted 25% w/v with catalase blank solution) was added to wells D2, E2 and F2, followed by 135  $\mu\text{l}$  of 10 mM sodium phosphate buffer.

The above was repeated for each additional honey sample, allowing a total of five samples to be tested per plate. Once fully loaded, the plate was covered with a lid and tinfoil to protect from light, tapped gently on the side to mix and incubated for 5 min at room temperature (no shaking). To stop the reaction, 120  $\mu\text{l}$  6M  $\text{H}_2\text{SO}_4$  was then added to all wells and mixed by gently tapping the side of the plate. The foil and plate lid were then removed and absorbance at  $\text{OD}_{560}$  was read using a spectrophotometer. This assay produces a quantifiable  $\text{H}_2\text{O}_2$  range of 0 to 550  $\mu\text{M}$ .

### Data analysis

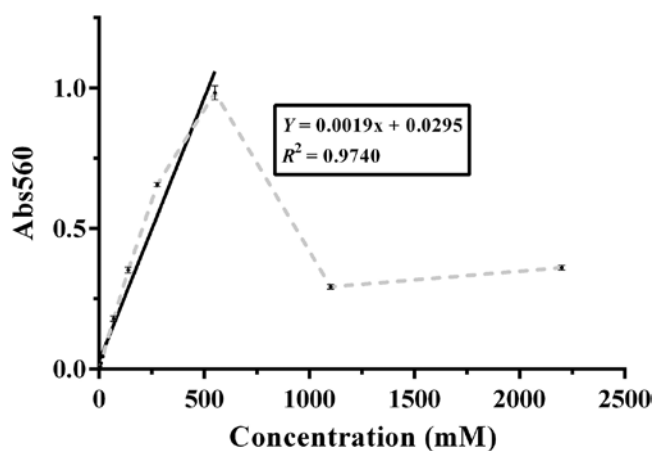
*Plotting the standard curve* – Blank-corrected  $\text{H}_2\text{O}_2$  standards were calculated by subtracting the mean absorbance of the 0  $\mu\text{M}$   $\text{H}_2\text{O}_2$  standard from the mean absorbance value for each  $\text{H}_2\text{O}_2$  standard. These data were then used to generate a standard curve in GraphPad Prism, with concentration on the  $x$ -axis and mean absorbance on the  $y$ -axis. As absorbance increases until  $\text{H}_2\text{O}_2$  reaches 550  $\mu\text{M}$  and then declines, the first portion of the graph (0–550  $\mu\text{M}$ ) was used to fit a linear trend line with the equation  $y=mx+b$ , where  $m$ =slope of the line and  $b$ =the  $y$  intercept.

*Determining the concentration of  $\text{H}_2\text{O}_2$  in the honey samples* – The intensity of the coloured reaction produced in the honey samples is directly related to their level of  $\text{H}_2\text{O}_2$  production. The latter is therefore calculated by comparing the absorbance value with the standards and reading the concentration from the standard curve (Fig. 3). Since honey is a coloured product that can vary considerably according to floral source and age, it is important to subtract the honey blank (no HRP reagent

mixture solution) from the honey test. Catalase-treated honey samples were included to confirm that the observed values were in fact the result of  $\text{H}_2\text{O}_2$  production; if this was the case the absorbance of the honey sample with catalase should be the same as that of the same honey sample without catalase. As the assay is critically dependent on incubation time and temperature, separate standard curves should be calculated from each assay plate to ensure standardization across tests.

### Application of the assay to freshly collected honey samples

North Carolina beekeepers collected honey directly from honeycomb into sterile, tightly capped, black 50 ml polypropylene tubes. Colonies were not receiving supplemental feed during the time of honey collection. Within 4 h of field collection, honey samples were assigned a sample number, passed through a 100  $\mu\text{m}$  filter



**Fig. 3.** Typical standard curve for quantification of  $\text{H}_2\text{O}_2$ . Mean absorbance values were plotted for blank-corrected  $\text{H}_2\text{O}_2$  standards ranging from 0 to 2200  $\mu\text{M}$  (grey broken line). The solid black line indicates the linear portion of the curve used for calculations. Data shown  $n=3$ ,  $\pm\text{SD}$ . For some points, error bars are very small (0.1–0.25) and therefore difficult to see.

to remove debris, partitioned into 2.5 g single-use aliquots and transferred to cold storage at 4 °C protected from light. The pH of each honey sample was assessed following dilution to 10% (w/v) in Milli-Q water. Honey moisture content was determined using a Misco BKPR-1 (Solon, OH, USA) refractometer and following the manufacturer's instructions. Testing was undertaken as outlined above.

### Assessment of the effect of honey colour on H<sub>2</sub>O<sub>2</sub> detection

Honey samples can vary considerably in colour, and this may affect the assay readings. To test this, clear corn syrup (CCS; a sugar solution with no glucose oxidase), dark corn syrup (DCS; CCS with added caramel colour and molasses), buckwheat honey (which is very dark in colour) and clover honey (which is very pale) were heated at 70 °C for 1 h to eliminate glucose oxidase and catalase activities [33]. Samples were then diluted (to 50% w/v with water for CCS and the honey samples, to 50% w/v with water for DCS and then in doubling dilutions to 1.56% w/v in CCS), and 250 µl was added to the microwell plates and spiked with an equal volume of 1 mM H<sub>2</sub>O<sub>2</sub> to achieve a final concentration of 25% CCS, DCS or honey and 500 µM H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> levels were assayed immediately according to the optimized protocol, with incubation for 5 min.

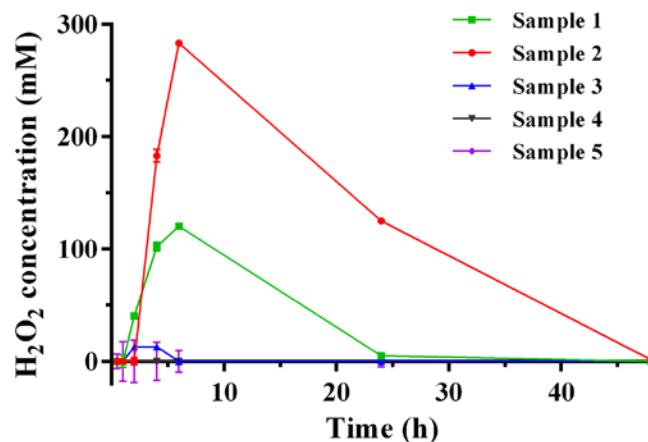
## RESULTS AND DISCUSSION

### Optimization of the HRP-*o*-dianisidine colourimetric method for detecting H<sub>2</sub>O<sub>2</sub> production in honey

Hydrogen peroxide, well known as an antimicrobial agent, is responsible for the antimicrobial activity of the majority of honey types. Peroxide activity can be determined via biological testing that compares killing of a test strain of bacterial pathogen *Staphylococcus aureus* by honey in the presence and absence of catalase using a well diffusion assay [31]. If all activity is abolished by catalase it is assumed to be due to H<sub>2</sub>O<sub>2</sub>, and this can be quantified by comparison to established phenol standards. While on the surface a simple test, the well diffusion assay is difficult to standardize, highly dependent on culture conditions including the quality and quantity of agar, quality of the tester strain, and incubation temperature and time, requiring considerable time, a skilled operator, a laboratory certified for handling bacterial pathogens and a high level of attention to detail. In addition, the biological assay is an indirect measure of H<sub>2</sub>O<sub>2</sub> as an assessment of toxicity to bacterial cells. Chemical tests could by-pass these issues, enabling a rapid, high-throughput assay that is simple and cost-effective to perform.

At the outset of this study, we found the HRP-*o*-dianisidine colourimetric method to be variable and difficult to standardize. The following parameters were identified as being critical to the successful deployment of this method:

(1) Honey dilution – H<sub>2</sub>O<sub>2</sub> accumulation is known to be highest when honey is diluted to 30–50% strength; below 30% the low-affinity glucose oxidase becomes limiting,



**Fig. 4.** Time course for H<sub>2</sub>O<sub>2</sub> production in five different freshly collected honey samples. Following dilution, the concentration of H<sub>2</sub>O<sub>2</sub> in honey (25% w/v) was assayed at various time points. Although collected at the same time and from the same region, the different samples varied substantially in H<sub>2</sub>O<sub>2</sub> production levels. *n*=3, error bars show  $\pm$ SD. Error bars ranged from 0.4 to 19 and were particularly small at the higher H<sub>2</sub>O<sub>2</sub> concentrations, making these difficult to see.

and above 50% there is too little free water for H<sub>2</sub>O<sub>2</sub> production [23]. However, here (and in previous studies) 25% honey was used, which provided an optimal trade-off between maximal H<sub>2</sub>O<sub>2</sub> production and simplicity of the assay.

- (2) Dilution time – The kinetics of H<sub>2</sub>O<sub>2</sub> production and degradation varied among honey samples such that the time of incubation for maximum production occurred anywhere between 3–6 h, depending on the sample (Fig. 4). Generally, samples with higher levels of H<sub>2</sub>O<sub>2</sub> had later peaks in production, and the overall kinetics are in good agreement with other published reports [21, 24, 34]. It is recommended that at least two time-points (4 and 6 h) be tested for each sample to maximize the chance of seeing peak production.
- (3) Aeration – Incubation with a large headspace volume was found to be critical for maximal H<sub>2</sub>O<sub>2</sub> production. Here we found using 5 ml honey in 28 ml McCartney bottles with shaking at 180 r.p.m. provided ideal aeration and greatly improved the assay.
- (4) Rapid workflow – As the assay components degrade quickly over time it is essential to work quickly, ensuring all reagents are made just prior to use and that the plates are loaded in the shortest time possible. We recommend doing no more than two plates at one time.

This optimized protocol is expected to be broadly applicable based on published ranges for H<sub>2</sub>O<sub>2</sub> in honey [6, 18, 21, 24, 35]. Our protocol provides sufficient detail to allow adoption by new users. If an investigator needs to customize the assay, the protocol can be easily modified using the detailed reagent formulae and the rationale for assay design and execution provided here.

**Table 1.** Moisture content, pH, and H<sub>2</sub>O<sub>2</sub> production of honey samples collected in NC, USA

Honey sample	pH		Moisture content (%)		Maximum H <sub>2</sub> O <sub>2</sub> production (μM)	
	Mean	SD	Mean	SD	Mean	SD
1*	4.1	0.1	17.3	0.6	120.4	3.0
2*	4.3	0.2	18.8	0.6	283.2	0.4
3	3.8	0.1	16.8	0.4	13.2	1.2
4	4.6	0.2	19.3	0.4	0	0.7
5	5.0	0.2	16.4	0.6	0	9.6

\*Peak H<sub>2</sub>O<sub>2</sub> production occurred at 6 h for samples 1 and 2 and 4 h for sample 3. Mean derived from three replicates. SD=standard deviation.

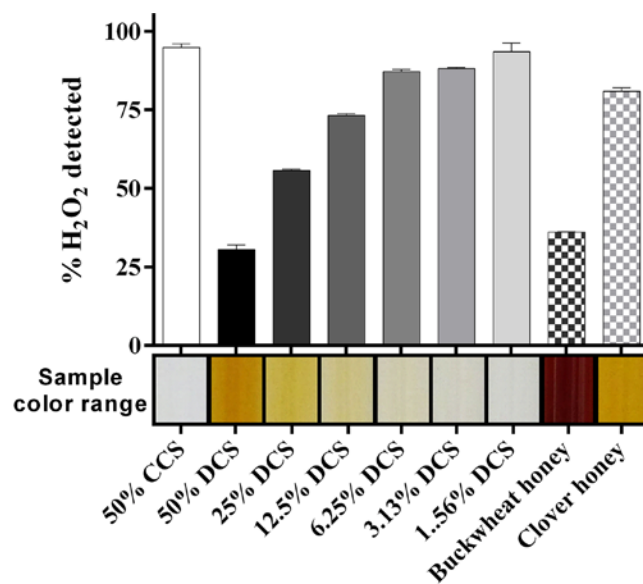
### Application of the optimized method for quantification of H<sub>2</sub>O<sub>2</sub> in honey samples

H<sub>2</sub>O<sub>2</sub> production capacity was evaluated for five unprocessed polyfloral honey samples using our optimized method. All samples had typical pH and moisture content (Table 1) [2]. Each honey sample was diluted to a final concentration of 25% (w/v) and evaluated for production of H<sub>2</sub>O<sub>2</sub> over a multipoint time course (Fig. 4). For honey samples 1 and 2, H<sub>2</sub>O<sub>2</sub> production peaked 6 h post-dilution to 120.4±3.0 μM and 283.2±0.4 μM, respectively, and returned to zero by 48 h (Table 1 and Fig. 4). Sample 3 produced low (13.2±1.2 μM), but detectable H<sub>2</sub>O<sub>2</sub> levels. H<sub>2</sub>O<sub>2</sub> was not detected in samples 4 and 5. In all instances, assay responses were eliminated by incubation with catalase, indicating specificity of the assay for H<sub>2</sub>O<sub>2</sub>.

Differences in H<sub>2</sub>O<sub>2</sub> accumulation capacity of different honeys are well-known [18, 35] and have been attributed to floral source [9, 25, 36], foraging time of year, honey age, storage conditions [37] and processing [18]. Polyfloral honeys were used in this work, and all were collected during a 1 week period in the same general geographical area before being stored and processed under the same conditions. Given that glucose oxidase is derived from bees one might expect similar levels in minimally processed honey. This high level of variability may reflect differences in bee and hive health, or environmental conditions such as temperature and humidity. Other components present in the honey might also augment or suppress glucose oxidase activity; for example, catalase can be introduced into honey with pollen grains [21, 38, 39]. As noted below, colour may affect the H<sub>2</sub>O<sub>2</sub> readout, however all of the North Carolina honeys used for this study were of a similar hue (Pfund colour range >35 to 50 mm).

### Honey colour can influence the capacity to detect H<sub>2</sub>O<sub>2</sub> by the HRP-*o*-dianisidine assay

Honey samples vary considerably in colour due to the presence of phenolics, flavonoids and maillard reaction products that are produced when sugars and other components age. To



**Fig. 5.** Sample colour and H<sub>2</sub>O<sub>2</sub> detection. Clear corn syrup (CCS), dark corn syrup (DCS), and dark- and light-coloured store-bought honey samples were heated to eliminate glucose oxidase and catalase, diluted and spiked to a final concentration of 500 μM H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> levels were assayed immediately after spiking with H<sub>2</sub>O<sub>2</sub>, according to our optimized protocol. The coloured bar on the horizontal axis shows the approximate colour of each sample. DCS reduced H<sub>2</sub>O<sub>2</sub> detection in a dose-dependent manner, and substantial quenching was also seen in the dark buckwheat honey sample. *n*=3, error bars show ±SD, which ranged from 0.22 to 2.8.

test the influence of colour we spiked heat-treated clear corn syrup (CCS) and dark corn syrup (DCS; from 1.56–50% w/v) and a light and dark honey sample with a known amount of H<sub>2</sub>O<sub>2</sub> and looked for recovery in the assay. The lightest matrix tested, 50% (w/v) CCS, was the only vehicle where the H<sub>2</sub>O<sub>2</sub> was fully recovered (Fig. 5). In contrast, 50% (w/v) DCS reduced detectable H<sub>2</sub>O<sub>2</sub> to 33.9±0.4% of the original level. Diluting the DCS with CCS increased the detection of H<sub>2</sub>O<sub>2</sub> in a dose-dependent manner. Similarly, buckwheat honey, which is very dark, yielded only 36.1±0.2% of the spiked H<sub>2</sub>O<sub>2</sub> whereas 80.9±1.1% was detected in the much paler clover honey (Fig. 5). Our data are the first to suggest that H<sub>2</sub>O<sub>2</sub> levels may be underestimated when using this colourimetric assay with darker honeys.

### Concluding comments

This report details an optimized procedure for quantifying H<sub>2</sub>O<sub>2</sub> in honey and presents a strategy for evaluating the H<sub>2</sub>O<sub>2</sub> production capacity of honeys. Prior to this work, a detailed protocol and strategy for quantification of H<sub>2</sub>O<sub>2</sub> in honey based on the oxidation of *o*-dianisidine was not available. Our optimized protocol is expected to be broadly applicable based on published ranges for H<sub>2</sub>O<sub>2</sub> in honey. The assay is cost-effective and easily performed using common laboratory equipment. We highlight critical steps in the protocol that enable maximal and reliable H<sub>2</sub>O<sub>2</sub> production. Additional

research is required to better understand the potential impact of colour and other honey components on H<sub>2</sub>O<sub>2</sub> quantification. As various factors within honey may act to suppress or augment H<sub>2</sub>O<sub>2</sub> levels or interfere with their detection, our work suggests that this method is best-suited for studies tracking changes in H<sub>2</sub>O<sub>2</sub> production capacity over time or in response to processing and storage conditions in a single sample and may not be so well-suited for comparing H<sub>2</sub>O<sub>2</sub> levels in honey samples, particularly if these are of significantly different hues.

## Disclaimer

This article has been reviewed by the U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency or of the US Federal Government, nor does the mention of trade names or commercial products constitute endorsement or recommendations for use of those products. The authors report no financial or other conflicts of interest. The authors alone are responsible for the content and writing of this article.

## Funding information

This work was funded by AgriFutures Australia (<https://www.agrifutures.com.au/>) (formerly the Australian Rural Industries Research and Development Corporation) under the Honey Bee and Pollination program.

## Acknowledgements

The authors thank Drs G. M. Lehmann and R. Koethe for their thoughtful and critical review of this manuscript. We are also grateful to Dr A. A. Camp for assisting with photography and the North Carolina beekeepers who generously donated the honey samples used to support this work.

## Author contributions

Conceptualization and methodology (D.C., D.L., K.K., A.H., M.B.), validation, formal analysis, investigation (D.L., K.K., A.H.), resources (D.C., N.C., E.H.); writing – original draft (D.L., D.C., K.K., M.B.); writing – review and editing (D.L., D.C., K.K., N.C., E.H.); visualization (D.L., D.C.); supervision (D.C., E.H.); project administration (D.L., D.C., E.H., N.C.); funding (D.C., E.H.).

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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