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A MODIFIED METHOD TO DETERMINE HYDROGEN PEROXIDE ACTIVITY AS A CRITERION FOR BEE HONEY QUALITY

By

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Summary

Honey contains glucose oxidase which converts glucose to gluconic acid and H_2O_2. This enzyme is active in unripe or dilute honey. Produced H_2O_2 is unstable and is decomposed to oxygen and water. Adding a chromogen, in the presence of peroxidase, oxygen oxidizes this chromogen and changes it from colorless to colored state. In the present study, accumulation of H_2O_2 (as an activity of glucose oxidase) in honey was monitored in the presence of leuco-crystal violet. Three common Egyptian honeys from different floral sources; citrus, clover, and cotton produced during three successive seasons were tested. The obtained results showed that fresh honeys exhibited higher enzyme activity than those stored for two or one year, respectively. Cotton honey had the higher enzyme and H_2O_2 activities followed by clover honey, while citrus honey was the least active one. It was observed that dilution of honey increased enzyme activity gradually with increase in dilution rate, simultaneously the time needed for reaching a maximum concentration of H_2O_2 was extended. From these results it could be concluded that this method could be used to evaluate honey quality and distinguish those stored or adulterated honeys.

Key words: Bee honey, Glucose oxidase, Hydrogen peroxide, Quality
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Introduction

Many factors affect honey quality such as beekeeping practices, packing treatments, honey components and storage conditions. Enzymes are among the most interesting and the most important components of honey, not only because

they have nutritional significance in human diet, but also because they play a vital part in the production of honey from its ultimate raw plant material (Codex Alimentarius, 1998; Oddo *et al.* 1999, and Vorlovà & Celechovskà, 2002). Of these enzymes, glucose oxidase (GO) which oxidizes honey glucose and produces gluconic acid, the principal acid found in honey, and H_2O_2 which is an important microbicidal agent. GO is most active in diluted or unripe honey, and is most active when the sugar concentration is 25-30% (Crane, 1990). H_2O_2 quickly breaks down into water and oxygen, and its production and decomposition are continuous while the nectar is being converted into honey.

Prolonged storage of honey influences the peroxide accumulation (Dustmann, 1972; Wakhle & Desai, 1991).

The present study describes a quick and a simple enzymatic method for evaluating honey quality by determining H_2O_2 in fresh and stored main types of Egyptian honeys using leuco crystal violet (LCV) stain according to the following equations:

1. β-D- glucose + water + oxygen + GO → gluconic acid + H_2O_2
2. H_2O_2 + LCV (reduced form or colorless) → H_2O + LCV (oxidized form or colored). The oxidizes form LCV was colorimetrically measured and the quantity of H_2O_2 was determined using standards.

Materials and Methods

1. Locations

Citrus honeys were obtained from an apiary situated in Benha, Qalubia Gov., while clover and cotton ones were obtained from the apiary of the Faculty of Agriculture, Fayoum University, Fayoum Gov., Egypt. Bee colonies in these apiaries were situated in Langstroth's standard hives and headed with local hybrid Carniolan, *Apis mellifera carnica*, queens.

2. Samples

Nine honey samples from three unifloral sources; citrus (*Citrus* spp.), Egyptian clover (*Trifolium alexandrinum*) and cotton (*Gossypium vitifolium*)

were obtained by ordinary beekeeping practices. Floral sources of the tested honeys were ascertained by categorizing their pollen contents according to the mellisopalynological examination given by Louveaux *et al.* (1978) in addition to microscopical identification mentioned by Owayss *et al.* (2004) for Egyptian honeys.

Stored honey samples were packed in opaque glass bottles and kept in dark at normal room conditions (28 ± 10 ^{o}C). Timing of stored and non-stored samples and assay is shown in the following table:

Table (1) Timing of harvested honey samples and their assay.

Type of Honey		Date of Harvest	Date of Assay
Citrus	1	April 15, 2003	April 25, 2005
	2	April 17, 2004	April 25, 2005
	3	April 19, 2005	April 25, 2005
Clover	1	June 20, 2003	June 30, 2005
	2	June 22, 2004	June 30, 2005
	3	June 24, 2005	June 30, 2005
Cotton	1	July 26, 2003	August 06, 2005
	2	July 28, 2004	August 06, 2005
	3	July 30, 2005	August 06, 2005

3. Reagents

1.Horse radish peroxidase (Sigma Chemical Co.) solution was prepared on a daily basis (17.25 I.U. / ml phosphate buffer pH 6.8). 2.Leuco-crystal violet (LCV; Aldrich Chemical Co.) in 0.5% HCl. 3.Phosphate buffered solutions (0.15 M, pH 6.8, a mixture of KH_2PO_4 and Na_2 HPO_4) were prepared. All reagents were stored at 0-2 ^{o}C and kept protected from light.

3. Dilutions

Honey samples (H), 5g each, were diluted (D1, D2, D3& D4) with dist. water (W) as follows:

a) D1 (1 H : 1 W), b) D2 (1 H : 2 W), c) D3 (1 H : 3 W), and d) D4 (1 H : 4 W). Diluted solutions were magnetically stirred for one min., then filtered through Whatman No. 1 paper.

4. Assay

The proposed enzymic method was based upon the research works of Lerke *et al*. (1983) modified by López-Sabater *et al.* (1993). The method was based on the action at pH 6.8 of the diamine oxidase enzyme over the histamine present in the sample to be analyzed (fish extract or standard). This enzyme catalyses the breakdown of histamine. As products of enzymic activity, imidazole acetaldehyde, ammonia, and hydrogen peroxide were formed. A second enzyme (peroxidase) in the presence of a chromogen (LCV) in reduced form (colorless), caused its oxidation into crystal violet (colored form) measured colorimetrically.

Filtrate honey solution (500 µl) was pipetted into a cuvette and the following reagents were added: (a)1ml of phosphate buffer, (b) 500µl of peroxidase solution and (c) 100 µl of LCV. The mixture was blended by a vortex device, then the development in absorbance, as a result of GOA, was recorded for about 4 hrs. using a Spectronic 2000 (Bauch & Lomb) at 580 nm. The absorbance readings were converted into concentrations as ppm H_2O_2 by means of a standard curve prepared using different concentrations of H_2O_2 30% (Merck Chemical Co.).

5. Statistical analysis

Obtained data were statistically analyzed according to the methods given by Snedecor and Cochran (1967) and computerized by Minitab® for Windows® system.

Results

It is worth noting that in preliminary experiments of this study, sugar-adulterated honey or artificially-made honey (fructose 40%, glucose 30%, sucrose 3% and water 20%) had zero absorbance, *vice versa* natural honeys.

The effect of type of honey, season and dilution on H_2O_2 accumulation during about four hours as a result of GOA are illustrated in figures 1, 2 & 3. Also, standard deviation and standard error values were shown in tables 2 & 3.

From data in figure 1 (a, b & c) it was noticed that H_2O_2 formation, as a result of GOA, in citrus honey increased more slowly and the rate of formation was affected by season and dilution, however, citrus honey of season 2005 showed a slight increase in H_2O_2 as compared to seasons 2003 or 2004.

Data (figure 2) showed that GOA in clover honey was also affected by season and dilution. Clover honey of season 2005 showed the highest concentrations of H_2O_2. Also the same trend was noticed with the increase in dilution indicating H_2O_2 increase.

The data in figure 3 indicated that H_2O_2 in cotton honey (season 2005) increased gradually and reached maximum concentrations (1.15, 2.13, 3.05 & 3.98 ppm) after 15, 25, 35 and 45 min. in D1, 2, 3 & 4, respectively, but decreased gradually to minimum concentrations (0.42, 0.88, 0.79 & 1.08 ppm) after 150, 105, 140 and 150 min., respectively, then become stable till the end of experiment time. The same trend was noticed for season 2004, but cotton honey of season 2003 showed slow increase, reaching maximum amounts (0.92 & 1.94 ppm) after 120 and 140 min. at D1 and 2, respectively, then was stable thereafter.

Discussion

The low formation of H_2O_2 in citrus honey may be attributed to supplementary feeding (by beekeepers) of bee colonies on sucrose syrup in this time of season coinciding with short nectar flow. It was also observed that H_2O_2 increased (in all tested samples) with increased honey dilution. This relation was also mentioned by Crane (1990).

The decrease in H_2O_2 formation may be attributed to the reduction of chromogen LCV by some components in honey, and may also be related to color dilution of LCV. In this regard, White (1975) showed that decrease in

H_2O_2 is attributed to oxidation of some components in honey such as ascorbic acid by oxygen produced by breakdown of H_2O_2 into oxygen and water.

From the aforementioned results it could be observed that the glucose oxidase activity (GOA) was affected by both of season and rate of dilution, since, the activity decreased with the increase in age of honey sample, *vice versa,* increased with the increase of dilution rate.

Tested cotton honey showed the highest GOA followed by clover honey and that may be attributed, partially to the high temperature during production period. Contrary, citrus honey showed the lowest activity. Also, H_2O_2 decreased with the prolonged storage, *i.e.* fresh samples (season 2005) showed the highest activity compared to stored samples (season 2003). In this respect, some authors mentioned that prolonged storage of honey influences the peroxide accumulation (Dustmann, 1972; Wakhle & Desai,1991).

More diluted honey samples showed the highest H_2O_2 formation as compared to less diluted ones and that may be attributed to GOA which is reduced to the sugar concentration increases (Postmes, 1995). The glucose oxidase enzyme activated by dilutions of honey generates H_2O_2 which, generally, is the major antibacterial factor in honey. This enzyme is inactivated by heating honey, and by exposure to light in some honeys which contain a sensitizing factor. Some honeys also contain substances which destroy the H_2O_2 generated by the enzyme (Molan, 1992a&b).

Another explanation for glucose oxidase being inactive until the honey is diluted is that, its activity is suppressed by the unfavorable pH in ripened honey and the enzyme has an optimum pH of 6.1 with a good activity from pH 5.5 to pH 8, but the activity drops off sharply below pH 5.5 to near zero at pH 4. These findings could easily be explained by the acidity of some honeys keeping the pH too low for the enzyme unless diluted (White *et al.* 1963; White & Subers, 1964 and Schepartz & Subers, 1964).

Consideration needs also to be given to the effect of dilution on the concentration of substrate with the enzyme requiring such a high level of glucose for maximum activity. The rate of production of H_2O_2 decreases acutely when the level of glucose is lowered, as would happen when honey is diluted a lot (Molan, 1992a).

The maximum levels of accumulated H_2O_2 occurred in honey solutions diluted to concentrations between 30% and 50% (v/v) with at least 50% of the maximum levels occurring at 15-67% (v/v) (Bang *et al.* 2003).

On the other hand, some factors oppose H_2O_2 formation *e.g.* catalase or ascorbic acid (vit. C). Catalase, when present in relatively large amounts in honey, may destroy H_2O_2 to produce oxygen and water. Vitamin C, naturally present in a few types of honey, is oxidized readily, and therefore, reduces H_2O_2 (White, 1975 and Kerkvliet 1996). Removal of H_2O_2 produced by the addition of ascorbic acid, gave five-fold increase in the rate of reaction (Schepartz, 1966). According to Postmes (1995), a high iron content of the honey also results in a reduction in H_2O_2.

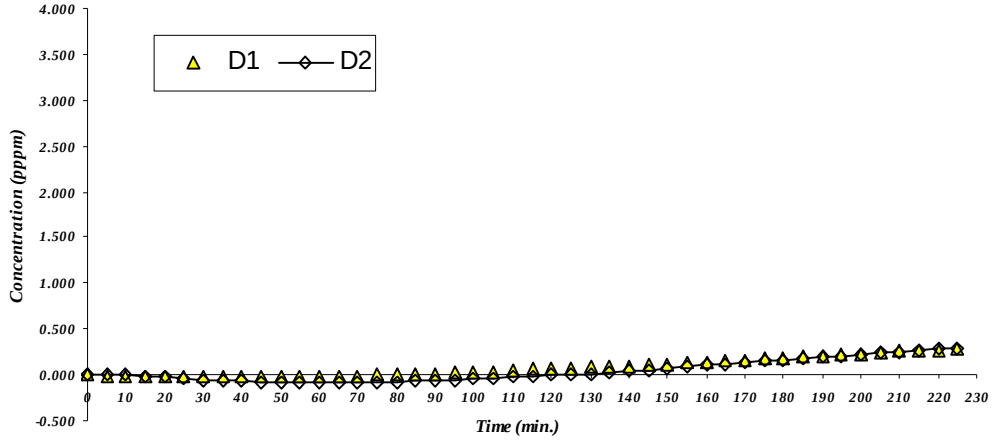
In a conclusion this present technique could serve as a simple and a quick method as a quality criterion for honey (freshness, storage and adulteration).

Table (2) Std. Dev. of hydrogen peroxide in tested honeys after dilutions (D1 to D4).

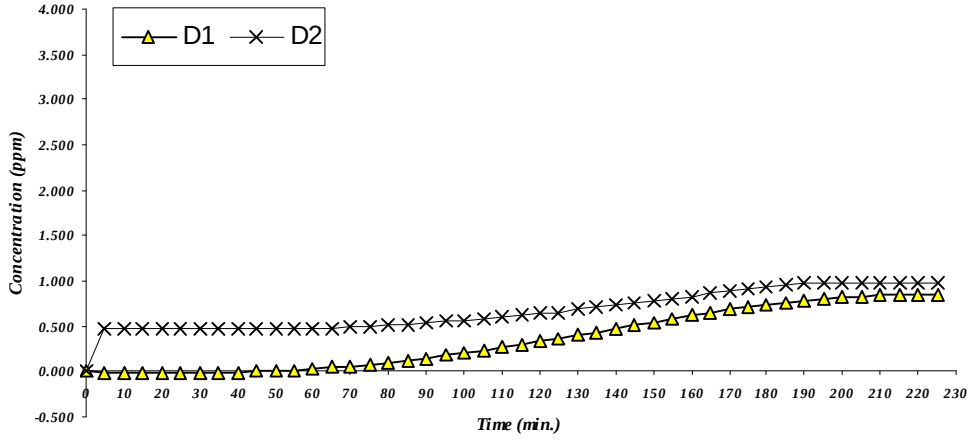
Type of	**Season 2003**				**Season 2004**				**Season 2005**			
Honey	D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4
Citrus	0.101	0.119	-	-	0.326	0.224	-	-	0.286	0.456	-	-
Clover	0.337	0.542	-	-	0.429	1.010	1.381	-	0.429	1.014	1.295	-
Cotton	0.400	0.846	-	-	0.333	0.435	0.811	-	0.293	0.491	0.861	1.168

Table (3) Std. Error ± mean of hydrogen peroxide in tested honeys after dilutions (D1 to D4).

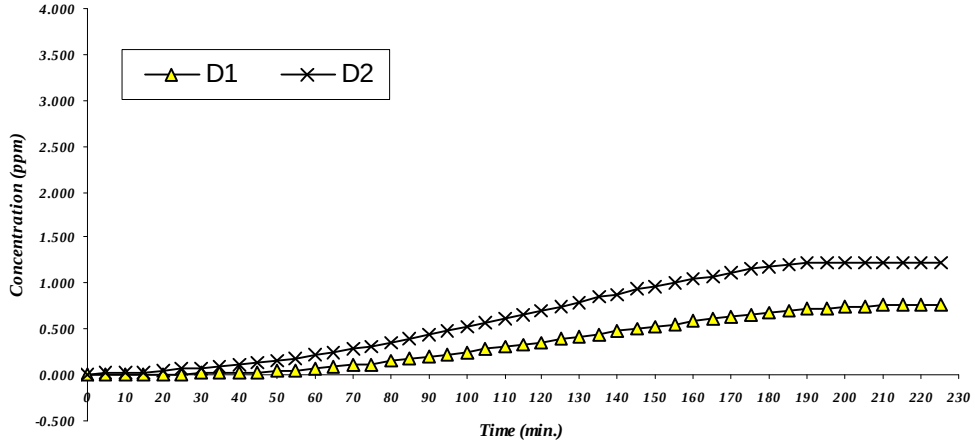
Type of	**Season 2003**				**Season 2004**				**Season 2005**			
Honey	D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4
Citrus	0.015	0.018	-	-	0.048	0.033	-	-	0.042	0.067	-	-
Clover	0.050	0.080	-	-	0.063	0.149	0.204	-	0.632	0.149	0.191	-
Cotton	0.059	0.125	-	-	0.049	0.064	0.120	-	0.043	0.072	0.127	0.172



(a) Season 2003

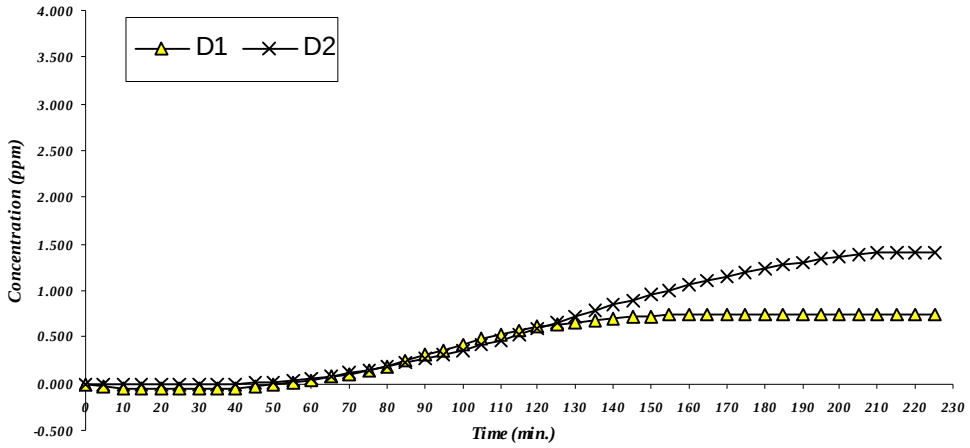


(b) Season 2004

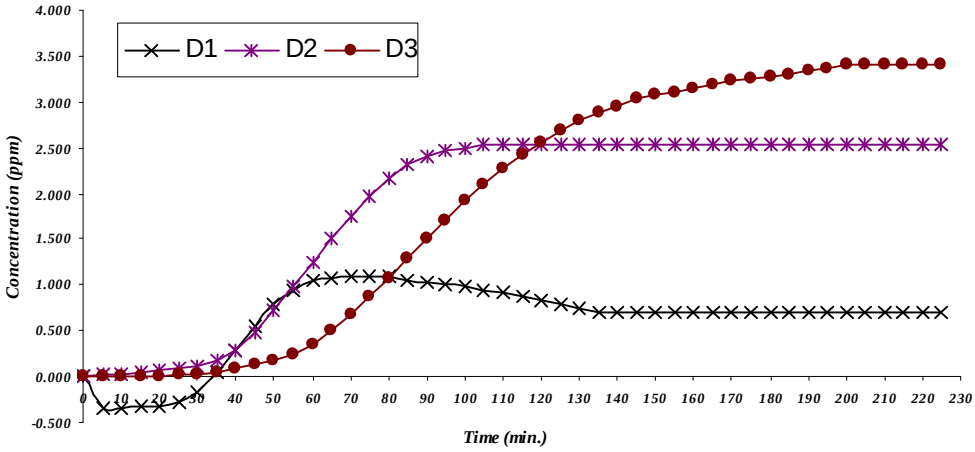


(c) Season 2005

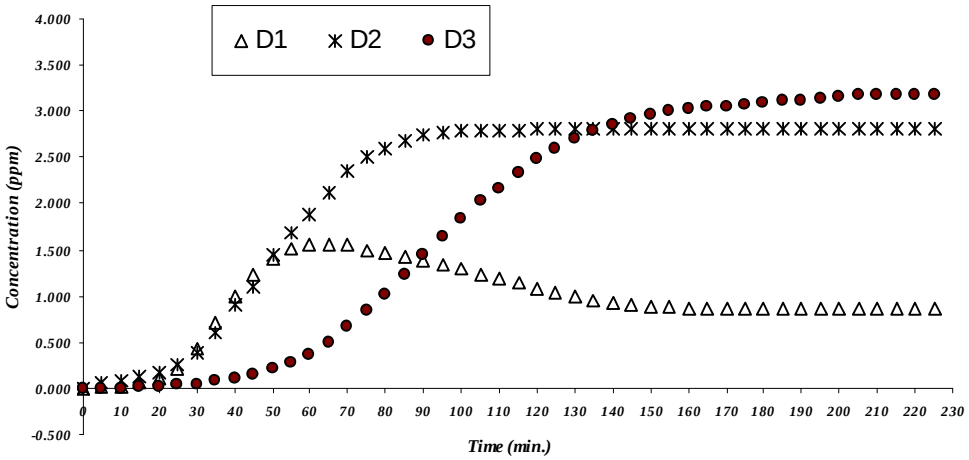
Fig. (1). Peroxide accumulation (ppm) in stored (a &b) and fresh (c) citrus honey. D1, …etc = dilutions



(a) Season 2003

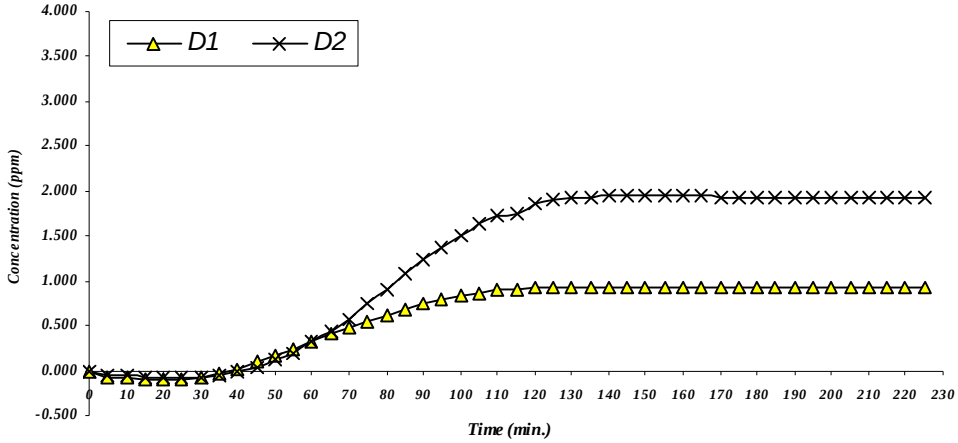


(b) Season 2004

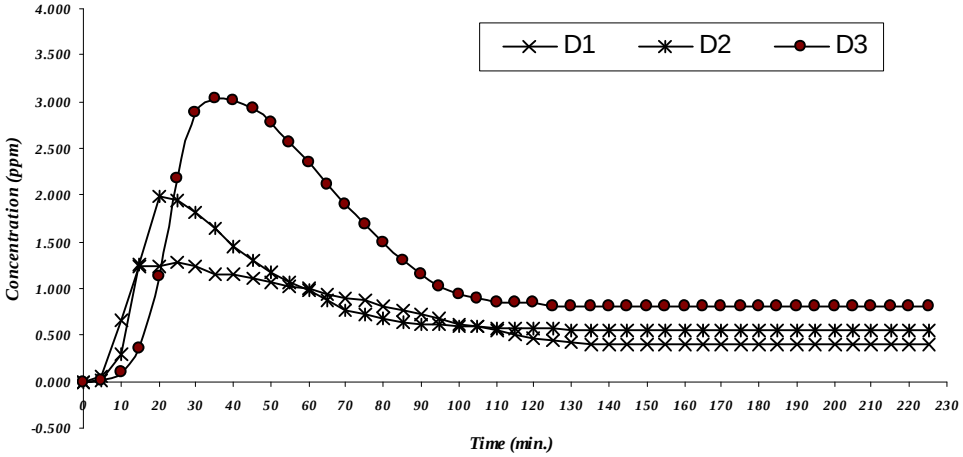


(c) Season 2005

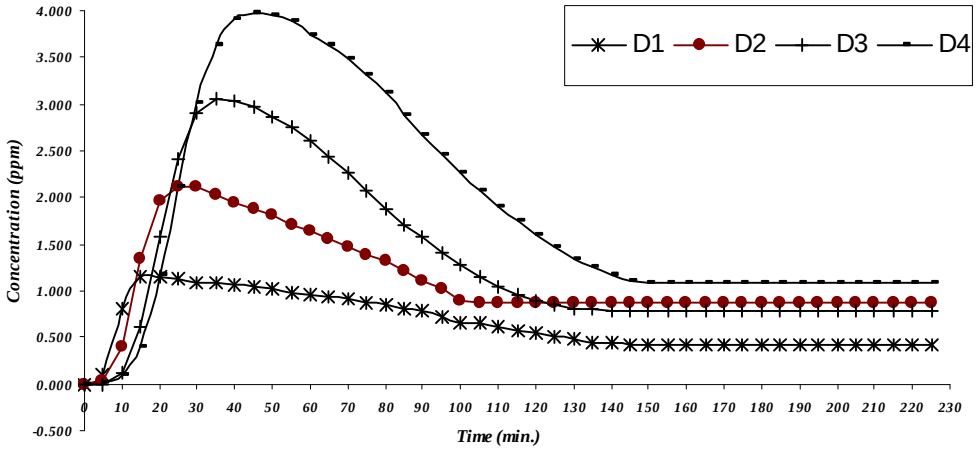
Fig. (2). Peroxide accumulation (ppm) in stored (a &b) and fresh (c) clover honey.



(a) Season 2003



(b) Season 2004



(c) Season 2005

Fig. (3). Peroxide accumulation (ppm) in stored (a &b) and fresh (c) cotton honey.

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طريقة معدلة لتقدير نشاط فوق أكسيد الهيدروجين كمؤشر على جودة عسل النحل

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يحتوى عسل النحل الطبيعى ضمن مكوناته على بعض الإنزيمات والتـى مـن بينهـا إنـزيم الجلوكوز أوكسيديز والذى ينشط فى حالة العسل غير الناضج أو عند تخفيف العسـل بالمـاء ويقـوم بتحويل سكر الجلوكوز فى العسل إلى حامض جلوكونيك وفوق أكسيد الهيدروجين وهو غير ثابـت لا يلبث أن يتكسر إلى ماء وأكسجين. وفى حالة وجود بعض الصبغات يعمل هذا الأكسجين على أكسـدة الصبغة وتحويلها من الصورة عديمة اللون إلى الصورة الملونة وذلك فى وجود أنزيم البيرأوكسيديز.

وقد أجريت هذه الدراسة بغرض تتبع نشاط فـوق أكسـيد الهيـدروجين، باسـتخدام صـبغة لوكوكريستال بنفسجى كمؤشر على نشاط إنزيم الجلوكوز أوكسيديز، فى ثلاثة أنواع رئيسة من عسـل النحل (الموالح – البرسيم – القطن) منتجة فى ثلاثـــة مواسم متتالية: 2003 ،2004 ، 2005. تم تخزين أعسال مواسم 2003 ، 2004 فى عبوات زجاجية فى الظلام تحت درجات حرارة الغرفـة.

وقد أوضحت النتائج ما يلـى:

أظهر عسل القطن نشاطا عاليا لإنزيم الجلوكوز أوكسيديز وأعلى كميـة مـن فـوق أكسـيد الهيدروجين يليه عسل البرسيم بينما كان عسل الموالح أقلها نشاطا.

تأثر النشاط الإنزيمى للعسل نتيجة التخزين حيث انخفضت كمية فوق أكسيد الهيدروجين فـى العينات المخزنة لمدة عامين عن تلك المخزنة لمدة عام بينما كانت العينات الطازجـة أعلاهـا نشـاطا وذلك فى الأنواع الثلاثة المختبرة من العسل. ومن خلال دراسة تأثير معدل تخفيـف عينـات العسـل المختبرة بالماء على النشاط الإنزيمى لوحظت زيادة فى النشاط الإنزيمى مع زيادة معدل التخفيف وفى نفس الوقت ازداد الوقت اللازم للوصول إلى أعلى تركيز من فوق أكسيد الهيدروجين.

ومما سبق يمكن استخدام هذه الطريقة كوسيلة بسيطة وسهلة وسريعة فى التعرف على جـودة عسل النحل والتمييز بين الأعسال الطبيعية والمخزنة والمغشوشة.