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Research Article

Investigation of Variations of Invertase and Glucose Oxidase Degrees against Heating and Timing Options in Raw Honeys

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Invertase and glucose oxidase are secreted by the hypopharyngeal glands of honeybees for the hydrolysis of sucrose and the preservation from microbial effects, respectively. It is also prominent to understand how the levels of invertase and glucose oxidase of raw honey samples are being influenced by different conditions because the behavior of these specific enzymes in raw honeys could be a marker for the quality parameters. On the basis of this expectation, three raw honey samples tagged as blossom, pine, and oak were investigated. To reach the desired aim, extraction conditions were diversified by the range of different periods as 0, 1, 3, and 6 h and of different temperatures as 24, 45, and 65°C. The optimal heating condition and the period of extraction for invertase activity of all honey types were 24°C and 1 h because data of this condition were 147.960–178.266 enzyme unit per kilogram (U/kg) and 20.179–24.313 invertase number (IN). Although the variety of glucose oxidase activity was not evaluated as a worthwhile indicator of quality for raw honey due to its abnormal activity behavior, the change of invertase activity should be considered as a quality parameter due to showing the gradual decreasing level from initially a quite high one.

1. Introduction

Honeybees tirelessly collect nectar and pollen to produce the natural substance known as honey. The humankind who has noticed this natural product has used it as both food and medicine since ancient times [1]. If this natural product is not exposed to heat treatment and left in its natural state, it is called raw honey [1], but this easily transforms into a crystallized form. To prevent crystallization, honey must be well filtered and heated, but this time it is tagged as processed honey. All kinds of honeys, except adulterated honeys, are natural sources for enhancing health because these contain different antioxidant compounds and also host some biocompounds such as enzymes, amino acids, organic acids, carotenoids, vitamins, minerals, and aromatic substances [2–4]. But nowadays, consumers have started to prefer the less processed products as they believe that these are healthier. The fact that consumers demand more raw and more natural food does not mean that the quality parameters are ignored.

Although many quality parameters of processed honey were identified by standardization committees such as International Honey Commission (IHC), Codex Alimentarius Honey Standard, and the European Union Honey Directive, this situation is not the same for raw honey [5–7]. Furthermore, it seems that the quality parameters are not sufficient to distinguish between raw and processed honeys, and the optimal situations of raw honeys have not been adequately emphasized in the literature.

Honey enzymes, which are known as a well-defined parameter of these optimal situations, continue to be popular because these are accepted as an influential quality parameter in standardization. Generally, the current enzyme activity of honey shows a decreasing tendency with some conditions such as storage and heating of honey, and it can only be used as an indicator for the classification of the freshness of unifloral honeys. These enzymes are given specifically titled as invertase, α-and β-glucosidase, catalase, acid phosphatase, diastase, and glucose oxidase, and these

are classed under a small fraction of current protein. Nectars and the salivary fluids and pharyngeal gland secretions of honeybees can be cited as the source of these enzymes, but the degree of enzyme activities can show noticeable differences in honey types [8, 9]. Invertase and glucose oxidase mentioned in the current study received the attention of the scientific research studies thanks to their specific properties. While the invertase is used to invert the sucrose into fructose and glucose [10], the main role of glucose oxidase, which is a member of the flavin enzyme, is to catalyze the oxidation of β-D-glucose to gluconic acid. This reaction ends up with utilizing molecular oxygen as an electron acceptor and the flavin adenine dinucleotide (FAD) as a redox prosthetic group and simultaneous production of hydrogen peroxide (H_2O_2) [11]. Also, the H_2O_2 forming provides the possible antibacterial activity which results in the improvement of shelf-life of honeys [11].

In this context, the present study targeted to determine the differentiation of the invertase and glucose oxidase activities against the heating and timing options in some raw honeys. Also, the current article purports to find a marker in relation to the quality parameter of raw honey, too.

2. Materials and Methods

2.1. Chemicals. All chemicals used were of the analytical grade and supplied from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Samples and Their Extractions. Three raw honey samples were supplied from Turkish local beekeepers in the 2018 harvest period. The botanic origin was recorded according to the beekeepers' declaration, and the classification was performed as blossom, pine, and oak honey. In particular, blossom raw honey was the priceless sample owing to coming from Anzer Plateau (Rize, Turkey) which hosts many species of endemic plants. In addition to this specific sample, pine raw honey was obtained from Muğla, Turkey, and oak raw honey was also from Kırklareli, Turkey.

For invertase activity assay, the different combinations of aqueous extract of raw honey were prepared under three types of extraction temperatures (24°C, 45°C, and 65°C) and four types of extraction time (0 h, 1 h, 3 h, and 6 h). Also, for the glucose oxidase assay, all conditions were kept stable, but the extraction was privatized by preparing in 100 mM sodium phosphate buffer (pH 6.1). All concentrations were adjusted as 0.2 g/mL for each enzyme assay.

2.3. Determination of Invertase Activity. The invertase activity of the raw honey samples was determined by using *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) as a substrate in Bogdanov's method [12]. According to this method, *p*NPG is split into glucose and *p*-nitrophenol by α-glucosidase. By adjusting the pH value to 9.5, the enzymatic reaction is stopped, and at the same time, nitrophenol is transformed into nitrophenolate anion, which corresponds to the amount of converted substrate and is determined spectrophotometrically at 400 nm by using a spectrophotometer (Shanghai Mapada Instruments Co., China). The relevant results were expressed as the unit of enzyme per kg (U/kg) and Hadorn units (IN: invertase number). The calculation of IN was detailed by Duisberg and Hadorn [13].

2.4. Determination of Glucose Oxidase Activity. Glucose oxidase activity in honey was determined by the horseradish peroxidase/*o*-dianisidine method as previously described by Flanjak et al. [14]. Glucose oxidase catalyzes the oxidation of D-glucose to D-gluconolactone and subsequent transformation to gluconic acid and hydrogen peroxide (H_2O_2). H_2O_2 is reduced to water by peroxidase utilizing *o*-dianisidine known as a cosubstrate. The maximum absorption of the formed colored product was observed at 400 nm using a spectrophotometer (Shanghai Mapada Instruments Co., China). The quantification was performed using H_2O_2 as the standard (10–100 μmol/L) with peroxidase and *o*-dianisidine. The results were expressed as μg H_2O_2/h g honey.

2.5. Statistical Analysis. All analyses were expressed as means and standard deviation thanks to the triplicate administration. The interrelations between enzyme activity and extraction conditions of raw honeys were tested by the SPSS Statistic 11.5 (IBM SPSS Statistics, Armonk, New York, USA). The significance was determined as $p < 0.01$ under the guidance of Spearman correlation assumptions. In addition to the evaluation of correlation, analysis of variance was also used to calculate statistical significance of each group and the significance of difference among the means was determined using one-way ANOVA at 0.05 probability.

3. Results and Discussion

3.1. A General Overview of Some Parameters of Raw and Processed Honeys. There have been numerous honey studies in the literature; however, there are a limited number of studies on raw honey in journals which is defined as the scientific or unscientific. Moreover, to the best of our knowledge, it has not been reached a consensus about the quality standards of raw honey. If this situation goes on, the consumers could be aggrieved owing to the irregular production labeling. Hence, the importance of international standardization, which is related to physicochemical and biochemical properties, becomes prominent once again due to checking the quality of products and comparing them. It is expected that the current results could serve this reality, too. It must be known that the discussion of obtained results was done without an internationally accepted standard about the raw honey, and most of the actual references were compared with processed ones.

When the literature has been searched regardless of the type of honey, it has been seen that raw honey presents many advantages for some titles. Here, some study examples of them were given by taking into account the biochemical properties of raw honeys. The study of Aumeeruddy et al. [15] revealed the differentness between raw and commercial honey in terms of pharmacological activities, chemical profile, and physicochemical properties. Interestingly, the

antimicrobial activity of raw honey was found to be higher than that of the commercial one against most tested bacteria, but commercial honey showed some advantages about the total phenolic, flavonoid, and tannin contents. This statement was not to say that raw honey did not consist of antioxidant compounds because it was confirmed that the raw honey has much more potential in terms of flavonoids and other polyphenols which were known as antioxidant components [1]. Raw and commercial honeys were also subjected to another antibacterial study [16]. To detail the comparison data, the researchers tested some bacteria which were identified as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella* spp., and *Salmonella* spp. While both samples were noneffective against *Salmonella* spp., these were impressive against other bacteria. Moreover, it was purported that raw honey possessed more inhibitory activity against *S. aureus* and *Shigella* spp. than commercial honey [16].

3.2. Evaluation of Studied Enzymes Results

3.2.1. Invertase Values of Honey Samples. Honey filtration is an important process to remove unwanted dirt, bee parts, wax, and other impurities from honey, but it can result in some undesirable situation as elimination of some useful biocomponents, too. Honey enzymes are located among the mentioned biocomponents known as nutritionally important. For example, diastase is used for the hydrolysis of carbohydrates to be digested easily while invertase also known as β-fructofuranosidase is used for the hydrolysis of sucrose and maltose; hence, no loss of these enzyme activities can be wanted due to filtration operation [10, 17].

Taking into consideration the importance of invertase, this study was designed and the results of raw honey samples are summarized in Table 1 with two different expressions as the unit of enzyme per kg (U/kg) and Hadorn units (IN: invertase number). The values obtained ranged between 28.481 and 178.266 U/kg, and also invertase number scale was calculated as 3.884–24.313 IN. The invertase content of honey samples was given in respect to the U/kg and IN in the following order: pine honey > Anzer blossom honey > oak honey. While the optimal extraction time was defined to 1 h, the optimal temperature of extraction was found to be 24°C. Regarding these results, pure natural conditions mentioned above were the most suitable ones because it was found that the lower activation behavior of invertase was revealed when it was at high extraction time and at high temperature for all honey extracts. Moreover, long processing times and high temperatures (>6 h and >65°C) were required to start inactivation of the current enzyme (Figures 1(a) and 1(b)).

All these outputs were also statistically confirmed. When the extraction temperature was kept constant, the change of invertase activity (U/kg and IN) by the increase of the extraction time was investigated for three types of honey. As could be seen from Table 1, with the increase of the extraction period, the invertase activity significantly decreased in each honey sample ($p \leq 0.01$; $R^2 = -0.949$). Besides these findings, the statistical condition was changed by keeping the extraction time as a fixed value. The negative correlation coefficient showed again that invertase activity strongly decreased from the beginning of the heating for all samples ($p \leq 0.01$; $R^2 = -0.949$).

There have been plenty of reports on the analysis of invertase activity in honey, and many of them are about processed honeys. Actually, in the light of this information, it has actually arisen that the raw honey is superior to processed honey with respect to the invertase activity. Kamboj et al. [18] presented an investigation about invertase activity that targeted to optimize some conditions such as temperature, pH, and time without changing the quality parameters of honey by utilizing response surface methodology. They indicated fewer invertase degrees than that reported in our findings, varying from 10.84 to 16.96 IN [19]. Although there was a limited study of raw honey in the literature, fortunately a close study came from Dimins et al. [19]. They performed a study to reveal the change of the invertase activity in raw honey as affected by changing the heating duration and the heating temperature. They also confirmed that the invertase activity decreased with the increase of the heating temperature. And, they claimed that invertase activity started to decrease significantly at 50°C. In our opinion, the current temperature was seen as a high value for decreasing the activity; however, it should not be forgotten that 50°C was the initial condition in terms of temperature for the relevant study. If the temperature condition less than 50°C could have tried, the final opinion might have been different.

Briefly, the recommendations of invertase activity of the International Honey Commission for general types of honey are IN ≥ 10 and IU ≥ 73.45 [12] except for long-term extraction with high temperature; our findings about raw honeys indicated sufficiently high enzyme activity. Thus, the high values of invertase could be seen as a marker for the identification of raw honey.

3.2.2. Glucose Oxidase Values of Honey Samples. Glucose oxidase is another critical enzyme in honey that indirectly contributes to some bioactivity processes [17]. This enzyme was also studied, and the data are given in Table 1. For all honey samples, the activities of glucose oxidase were seen as the highest degree in the condition of 45°C. The values of Anzer blossom honey, pine honey, and oak honey were calculated as $33.269 \pm 0.204\ \mu g\ H_2O_2/h{\cdot}g$, $67.923 \pm 0.713\ \mu g\ H_2O_2/h{\cdot}g$, and $69.999 \pm 0.887\ \mu g\ H_2O_2/h{\cdot}g$, respectively.

Because of the presenting of complex enzyme behavior, the statistical evaluations of glucose oxidase were achieved separately for each honey type. According to the statistical data of pine honey, the increase in activity showed a positive correlation with the increase in the extraction time under each studied temperature ($p \leq 0.01$; $R^2 = 0.972$ for 24°C, $R^2 = 0.949$ for 45°C and 65°C). But the statistical situation of Anzer blossom and oak honey was little different because of its abundance of some exclusion criteria. For example, when the activity of 0 h of Anzer blossom honey was excluded, an increase in the extraction time caused the decrease in glucose

Table 1: Results of invertase and glucose oxidase of the studied raw honey extracts.

	Invertase (U/kg)*			Invertase number (IN)*			Glucose oxidase (μg H_2O_2/h·g)*		
	24°C	45°C	65°C	24°C	45°C	65°C	24°C	45°C	65°C
Extraction time									
Anzer blossom raw honey**									
0 h	165.096 ± 1.036^{a}			22.516 ± 0.141^{a}			1.077 ± 0.042^{c}		
1 h	$165.810 \pm 1.136^{a,1}$	$149.229 \pm 1.014^{a,2}$	$77.193 \pm 0.268^{a,3}$	$22.614 \pm 0.155^{a,1}$	$20.352 \pm 0.138^{a,2}$	$10.528 \pm 0.037^{a,3}$	$3.090 \pm 0.086^{a,3}$	$33.269 \pm 0.204^{a,1}$	$18.422 \pm 0.191^{a,2}$
3 h	$145.976 \pm 0.685^{b,1}$	$124.239 \pm 0.846^{b,2}$	$55.058 \pm 0.105^{b,3}$	$19.909 \pm 0.093^{b,1}$	$16.944 \pm 0.115^{b,2}$	$7.509 \pm 0.014^{b,3}$	$2.534 \pm 0.106^{b,2}$	$15.391 \pm 0.259^{b,1}$	$0.107 \pm 0.009^{b,3}$
6 h	$132.965 \pm 1.361^{c,1}$	$99.962 \pm 0.959^{c,2}$	$28.481 \pm 0.324^{c,3}$	$18.134 \pm 0.186^{c,1}$	$13.633 \pm 0.131^{c,2}$	$3.884 \pm 0.044^{c,3}$	$0.959 \pm 0.056^{c,1}$	$0.225 \pm 0.012^{c,2}$	$0.000 \pm 0.000^{b,3}$
Pine raw honey**									
0 h	167.000 ± 0.873^{b}			22.776 ± 0.119^{b}			4.184 ± 0.150^{d}		
1 h	$178.266 \pm 1.180^{a,1}$	$159.939 \pm 1.453^{a,2}$	$107.737 \pm 0.952^{a,3}$	$24.313 \pm 0.161^{a,1}$	$21.813 \pm 0.198^{a,2}$	$14.694 \pm 0.130^{a,3}$	$4.926 \pm 0.090^{c,3}$	$28.418 \pm 0.515^{c,1}$	$25.855 \pm 0.515^{c,2}$
3 h	$155.814 \pm 1.437^{c,1}$	$136.298 \pm 0.501^{b,2}$	$83.143 \pm 0.857^{b,3}$	$21.250 \pm 0.196^{c,1}$	$18.589 \pm 0.068^{b,2}$	$11.339 \pm 0.117^{b,3}$	$8.849 \pm 0.080^{b,3}$	$49.609 \pm 0.667^{b,1}$	$41.440 \pm 0.521^{b,2}$
6 h	$150.260 \pm 0.678^{d,1}$	$117.416 \pm 0.584^{c,2}$	$56.328 \pm 0.619^{c,3}$	$20.493 \pm 0.092^{d,1}$	$16.014 \pm 0.080^{c,2}$	$7.682 \pm 0.084^{c,3}$	$12.781 \pm 0.390^{a,3}$	$67.923 \pm 0.713^{a,1}$	$61.313 \pm 1.011^{a,2}$
Oak raw honey**									
0 h	135.742 ± 1.287^{b}			18.513 ± 0.176^{b}			1.733 ± 0.054^{c}		
1 h	$147.960 \pm 0.987^{a,1}$	$124.794 \pm 0.429^{a,2}$	$76.796 \pm 0.797^{a,3}$	$20.179 \pm 0.135^{a,1}$	$17.020 \pm 0.059^{a,2}$	$10.474 \pm 0.109^{a,2}$	$3.052 \pm 0.084^{a,3}$	$23.509 \pm 0.450^{c,1}$	$14.128 \pm 0.344^{c,2}$
3 h	$123.287 \pm 0.668^{c,1}$	$110.593 \pm 1.259^{b,2}$	$58.232 \pm 0.449^{b,3}$	$16.814 \pm 0.091^{c,1}$	$15.083 \pm 0.172^{b,2}$	$7.942 \pm 0.061^{b,2}$	$2.022 \pm 0.045^{b,3}$	$53.460 \pm 0.591^{b,1}$	$29.913 \pm 0.569^{b,2}$
6 h	$119.796 \pm 0.718^{d,1}$	$95.361 \pm 95.361^{c,2}$	$42.048 \pm 0.169^{c,3}$	$16.338 \pm 0.098^{d,1}$	$13.006 \pm 0.105^{c,2}$	$5.735 \pm 0.023^{c,2}$	$2.173 \pm 0.069^{b,3}$	$69.999 \pm 0.887^{a,1}$	$64.009 \pm 0.130^{a,2}$
Min-max	119.796–178.266	95.361–159.939	28.481–107.737	16.338–24.313	13.006–21.813	3.884–14.694	0.959–12.781	0.225–69.999	0.000–61.313

*In all the results given, the analyses were performed in triplicate. **Statistical evaluations were achieved for each honey sample separately. The same letters in each column for each honey sample were not significantly different at $p < 0.05$ (one-way ANOVA test). Also, the same numbers in each line for each honey sample were not significantly different at $p < 0.05$ (one-way ANOVA test).

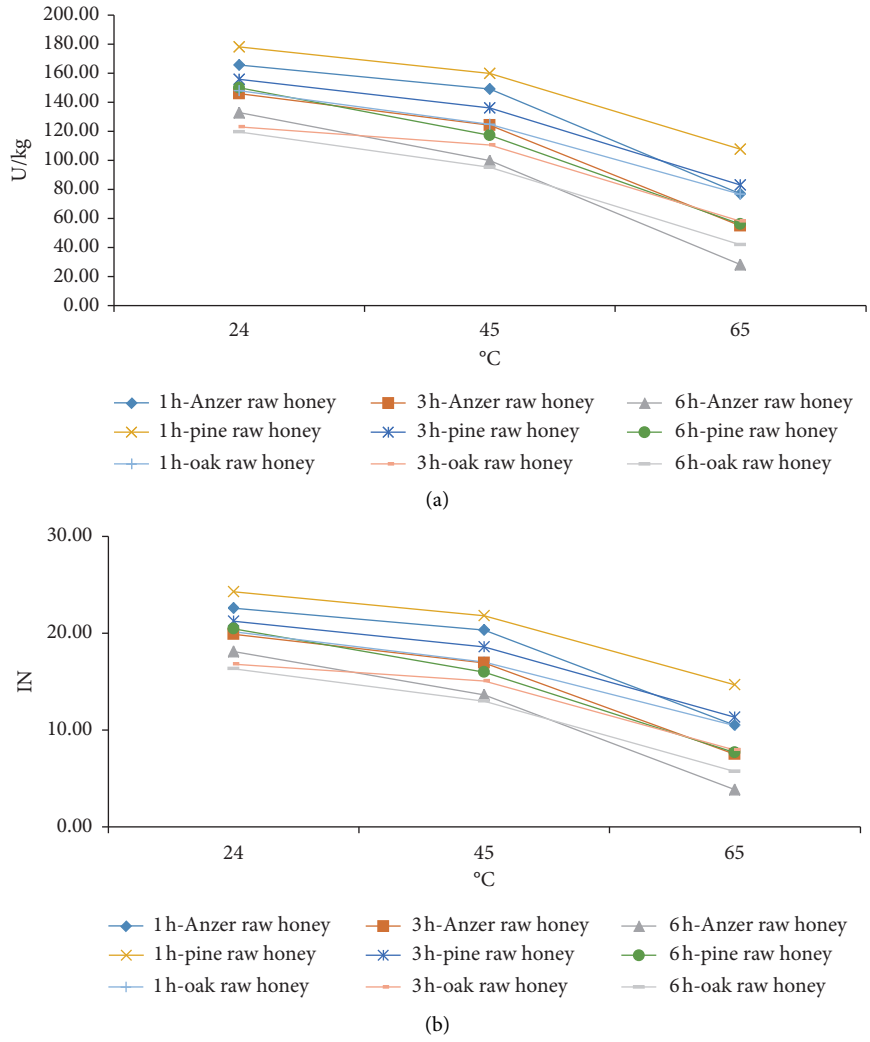

Figure 1: The invertase activity changing under the timing and heating conditions in terms of (a) U/kg and (b) IN.

oxidase activity under all heating temperatures ($p \leq 0.01$; $R^2 = -0.949$ for 24°C and 45°C, $R^2 = -0.965$ for 65°C). Namely, it presented the natural enzyme characterization because its activity was ended when it reached a certain temperature and extraction time and the activity rate of reaction drastically dropped such as 45°C and 65°C (Figure 2). Oak honey had much more exclusion criteria than others because the statistical evaluation on changing time periods was done by keeping constant the extraction temperature as only 45°C and 65°C. Under the current conditions, the Spearman correlation coefficient confirmed the activity as a positive correlation ($p \leq 0.01$; $R^2 = 0.949$). In our opinion, these complex behaviors were directly related to the honey types supplied from the different sources and their thermal stability of enzyme to produce hydrogen peroxide.

Although it seemed that the heating from 24°C to 45°C in some points of extraction periods resulted in a significant increase in glucose oxidase activity (5–35 times), the statistical response of utilizing different heating degrees under the constant extraction time remained unanswered due to the bell-shaped curve known as an unsuitable for statistical correlation ($p \geq 0.01$). As could be seen from Figure 2, glucose oxidase activity demonstrated the bell-shaped curve which was resulted in 45°C as the optimal degree.

Although it was achieved variable obtained results, the importance of glucose oxidase cannot be ignored. As a result of the antimicrobial effect, glucose oxidase can be seen as an indirect agent for food storage and packaging, also improving the shelf-life of foods because this enzyme shows impressive talent to inhibit the growth of microbes thanks to the self-produced hydrogen peroxide after glucose catalyzing. Moreover, some evidence from the scientific area has been revealed that also gluconic acid, a subreaction product, is responsible for the antimicrobial activity [11, 20]. Actually, it has known that the level of glucose oxidase in honey is not stipulated by any standard committee as a quality criterion. There have been some research studies about glucose oxidase activity in honey thanks to its potential bioproperties although raw honey studies also have been limited. Strelec et al. [21] used 20 honey samples which were not defined as raw honey and they reached the mean of glucose oxidase values ranging from 25.58 to 402.47 $\mu g\, H_2O_2/h{\cdot}g$. Also,

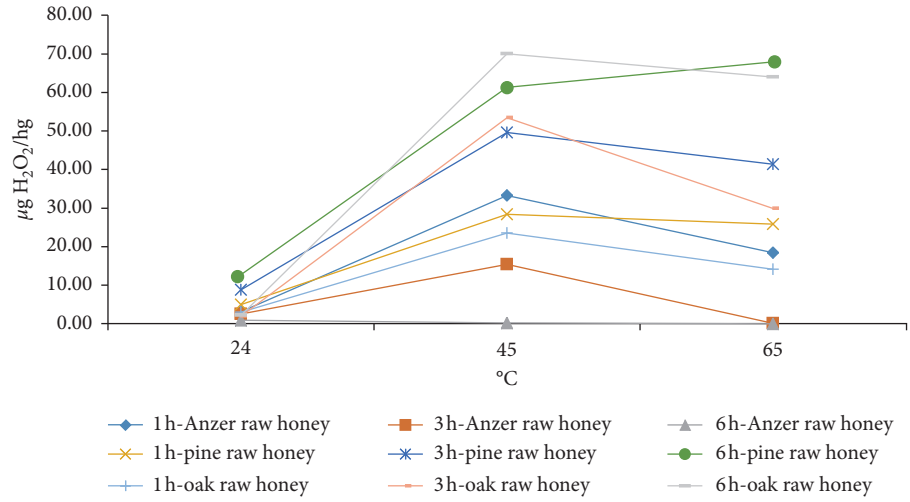

Figure 2: The glucose oxidase activity changing under the timing and heating conditions.

Flanjak et al. [14] observed the mean of glucose oxidase of 45 studied honey samples as 40.3–347.6 μg H_2O_2/h·g. According to another study, the average value of 57 blossom honey samples tagged as acacia ($n = 15$), wildflower ($n = 32$), and rapeseed ($n = 8$) were found as 29.7 μg/g [22]. When comparing relevant results, it was surprising that our initial values were quite low. This reason can be explained with a reality that enzyme constituents of honey vary according to the pollen types, floral characterization, and geographical location. Maybe, the essential reason is related to raw honey properties which do not involve any processing even though it has not been evidenced just like the current study.

4. Conclusion

Before this study, we noticed certain research gaps related to the identification of raw honey standards. For this reason, the study focused on investigating the degree of the invertase and glucose oxidase which was expected as the possible quality parameters of raw honey. Some extraction conditions as timing and heating on the invertase and glucose oxidase activities of blossom raw, pine raw, and oak raw honey were studied. Especially, the invertase results showed that the short period as 1 h and the low temperature as 24°C had major impacts on keeping this enzyme stable. And also, the current values were quite high when compared to previous studies. But the results of glucose oxidase showed the unexplainable behavior, unlike the general enzyme situation, so generalization was avoided as to whether there was a quality parameter or not for raw honey. As well, it was the reality that there were some limitations in our study. First, there was a deficiency in comparison and evaluation due to the fact that raw honey is not known clearly and quality parameters of it are not well defined in terms of the literature. The second one was the sample limitation. The use of much more types of samples could have been more beneficial to see the results clearly, and also investigated quality parameters of raw honey could have emphasized as assertively.

Briefly, one of the most practical applications based on the current results was the determination of the raw honey quality criteria because these results could offer an opportunity to easily distinguish the raw honey from others that are sold as processed. The enzymatic behaviors, summarized with a dramatic decrease in the invertase activity and supported with a partial increase following a partial decrease in glucose oxidase, showed that it should not be exceeded at temperatures above approximately 45°C for the long-term applications. Otherwise, the definition of raw honey should be replaced by pasteurized honey owing to the abnormal enzymatic behavior effected by the thermal situation. Taking into account the obtained information, our suggestion is that honey definitions of raw honey and pasteurized honey in the relevant codex rescript should be given definitively. It should not be forgotten that it is a legal right for consumers to know which product they purchased.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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