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ORIGINAL ARTICLE

The role of qualitative tests in detecting adulterants in stingless bee honey: A promising approach for honey producers and consumers

O papel dos testes qualitativos na detecção de adulterantes em mel de abelha sem ferrão: Uma promissora abordagem para produtores e consumidores de mel

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Abstract

Among the hive products of stingless bees (SB), honey is distinguished because it has different physicochemical properties than the honey produced by *Apis mellifera*. Its taste is more acidic and less sweet, and it naturally contains a higher percentage of water. Honey is one of the most frequently adulterated products marketed. Therefore, this work aimed to verify if the qualitative tests performed for *A. mellifera* honey are also valid for SB honey from *Tetragonisca angustula*, *Melipona quadrifasciata*, and *Tetragona elongata* and if they can detect the most common adulterations. Adulterations of SB honey with corn syrup, inverted sugar, and *A. mellifera* honey were simulated and tested with Lugol, Fiehe, and Lund reactions. For these three analyses, sample volume reduction was also tested. The Lund test did not work well with honey samples from SB because they have a higher water content, and reliable results could not be obtained. For the other two tests, the sample volume reduction used was efficient. The Fiehe test detected adulteration with corn syrup only. The Lugol test detected corn syrup and inverted sugar adulterations in all dilutions for all three SB species. No adulteration by added water or honey from *A. mellifera* was detected in any test. Therefore, using the qualitative Lugol's reaction test to evaluate SB honey quality is reasonably affordable. Since it is a rapid and inexpensive test, it allows the development of production chains for SB honey. Thus, detecting inauthentic honey can be done by combining qualitative tests as the first screening, followed by quantitative tests if necessary.

Keywords: Food control; Qualitative tests; Quality control; Food authenticity; Lugol; Lund; Fiehe.

Resumo

Entre os produtos da colmeia de abelhas sem ferrão, o mel se destaca por possuir propriedades físico-químicas diferentes do mel produzido pela abelha *Apis mellifera*. Seu sabor é mais ácido e menos doce, além de naturalmente conter maior porcentagem de água. O mel é um dos produtos mais frequentemente adulterados comercialmente. Dessa forma, este trabalho teve como objetivo verificar se os testes qualitativos realizados para o mel de *A. mellifera* também são válidos para o mel de abelhas sem ferrão das espécies *Tetragonisca angustula*, *Melipona quadrifasciata* e *Tetragona elongata*, e se é possível identificar suas adulterações mais comuns: adulterações do mel de abelhas sem ferrão com xarope de milho, açúcar invertido e mel de *A. mellifera* foram simuladas e testadas com as reações de Lugol, Fiehe e Lund. Para essas três análises, também foi testada a redução do volume da amostra. O teste de Lund não foi efetivo com as amostras de mel das abelhas sem ferrão, pois elas apresentam um maior teor de água, e resultados confiáveis não puderam ser obtidos. Para os outros dois testes, a redução do volume da amostra utilizada foi eficiente. O teste de Fiehe detectou adulteração apenas com xarope de milho. O teste de Lugol detectou adulterações com xarope de milho e açúcar invertido em todas as diluições utilizadas e para todas as três espécies de abelhas sem ferrão. As adulterações por adição de água ou mel de *A. mellifera* não foram detectadas em nenhum dos testes. Portanto, é possível utilizar o teste qualitativo da reação de Lugol para uma avaliação inicial da qualidade do mel. Como é um teste rápido e de baixo custo, ele permite o desenvolvimento de cadeias produtivas para o mel de abelhas sem ferrão. Assim, a detecção de mel inautêntico pode ser feita por uma abordagem combinada de testes qualitativos como triagem inicial, seguida de testes quantitativos, se necessário.

Palavras-chave: Controle alimentar; Testes qualitativos; Controle de qualidade; Autenticidade alimentar; Lugol; Lund; Fiehe.

Highlights

- The identification of inauthentic honey can be accomplished through Lugol's reaction
- Fiehe´s reaction detected adulteration with corn syrup but not with inverted sugar at any dilution
- The sample volume of stingless bee honey can be reduced in Lugol's and Fiehe's analyses

1 Introduction

Stingless bees (SB) (Hymenoptera: Apidae: Meliponini) are common in tropical and subtropical regions of the world (Michener, 2007). Their rational keeping, known as meliponiculture, can be considered a sustainable and profitable practice, with the marketing of hive products providing an important source of income for breeders (Barbiéri & Francoy, 2020).

Among the hive products of SB that can be commercially exploited, it is noted that wax, propolis, and honey stand out, as well as the multiplication and sale of the colonies themselves (Jaffé et al., 2015). The honey produced by these bees has different physicochemical characteristics than the honey produced by *Apis mellifera* bees (Faleiros-Quevedo & Francoy, 2022), with a more acidic and less sweet taste. It also has a higher water content in its composition (Nordin et al., 2018). This higher water content allows the development of beneficial microorganisms in honey, which modify some of its properties during maturation (Menezes et al., 2013).

Due to its differential organoleptic properties and high nutritional value, honey from SB has numerous benefits for preventive health care and treatment of diseases (Al-Hatamleh et al., 2020; Ávila et al., 2018; Biluca et al., 2016; Khongkwanmueang et al., 2020; Ooi et al., 2021; Samat et al., 2018). However, given its enormous diversity, it is a product that still needs proper legislation that reflects the territorial and ecosystem diversity of its distribution (Faleiros-Quevedo & Francoy, 2022; Koser et al., 2020), as well as tests that confirm the authenticity and purity of this product (Yong et al., 2022).

Commercial honey must not contain food ingredients or other additives (Food and Agriculture Organization, 2001). Determination of adulteration by adding sugars can be done by chromatography and stable carbon isotope analysis (Food and Agriculture Organization, 2001), however, these analyses require expensive and sophisticated equipment and the necessary infrastructure.

Some forms of honey adulteration for commercial purposes involve the addition of inverted sugar, corn syrup, chemicals, and water (Naila et al., 2018), as well as the addition of honey from *A. mellifera* bees to SB honey, to increase its volume. Therefore, it is essential for the production chain, like traders, buyers, and regulators, to identify honey without adulterants (Raypah et al., 2022).

Due to the complexity of methods for honey adulteration detection, more refined quantitative tests are constantly being developed to ensure its authenticity (Brar et al., 2023; Naila et al., 2018). However, as the commercialization of SB honey is still an emerging market, the laboratory tests and the sample size required for these tests further increase the value of the final product.

Regarding honey produced by *A. mellifera* bees, some rapid and inexpensive tests can be performed by beekeepers without the aid of well-equipped laboratories, such as the Lund, Fiehe, and Lugol tests (Instituto Adolfo Lutz, 2008). However, there is no comparative data in the literature on how these tests work with honey from SB.

The Lund test, which determines the purity of honey, measures the precipitation of albuminoids by tannic acid. Very low levels of albuminoids generally indicate honey adulteration. The Fiehe test is qualitative and is based on a colorimetric reaction in which a positive result shows a reddish or red color after reaction with a resorcinol-hydrochloric acid solution in the presence of sugar syrup or overheating. The Lugol's test is also qualitative and analyzes the presence of starch and dextrins in honey by a colorimetric reaction. After adding Lugol's solution, the mixture turns from reddish-brown to blue in the presence of commercial glucose or sugar syrups (Almeida-Muradian et al., 2013).

In particular, we aimed to test the reduction of traditional volume used to perform qualitative tests. For validation of tests, these tests were applied to SB honey aiming to detect the most common adulterations.

2 Materials and methods

2.1 Honey sampling

Honey samples of *Tetragonisca angustula* collected in January 2022 in the city of Jundiaí - São Paulo (23°11'11" South and 46°53'03" West), *Melipona quadrifasciata* collected in December 2021 in the city of Seara - Santa Catarina (27°08'58" South, 52º18'38" West), and *Tetragona elongata*, collected in December 2021 in the city of Atibaia - São Paulo (23°7'2" South, 46°33'1" West), were tested. Honey from orange flowers of *A. mellifera* collected in January 2022 in the city of Atibaia - São Paulo (23°7'2" South, 46°33'1" West) served as control.

2.2 Sample volume decrease test

Tests were performed to reduce the volume in the Lugol's reaction, where 10 g of honey is recommended (Instituto Adolfo Lutz, 2008), and 8, 5, and 2 g of honey were tested in triplicate. In the Fiehe reaction, the reduction of the sample volume from 5 grams (Instituto Adolfo Lutz, 2008) to 2 grams was tested in triplicate. In the Lund reaction, the sample volume was not reduced.

2.3 Qualitative tests

2.3.1 Lund reaction

Two grams of a sample were weighed and transferred to a 50-mL graduated cylinder, followed by the addition of 20 mL of water. After homogenization, 5 mL of a 0.5% tannic acid solution was added, and then water was added to bring the total volume to 40 mL. The mixture was stirred and allowed to settle for 24 hours to check whether a precipitate had formed, considering an interval of 0.6 to 3 mL of precipitate. In the presence of adulterated honey, either no precipitate is formed, or it does not reach the minimum volume.

2.3.2 Fiehe reaction

Five grams of the sample were transferred to a 50-mL graduated cylinder, and then 5 mL of water was added. After mixing, 5 mL of ethyl ether (PA grade) was added. After a 10-minute rest period, 2 mL of the ethereal layer

was transferred to a test tube, and then 0.5 mL of a 0.1% resorcinol solution prepared that day was added. After 6 minutes, the result can be observed, with a red color indicating adulteration (Instituto Adolfo Lutz, 2008).

2.3.3 Lugol reaction

Two grams of the sample were transferred to a 25-mL beaker, and then 4 mL of water was added. After thorough shaking, 0.1 mL of Lugol's solution was added, and a distinct color change from dark brown to reddish-brown to blue was expected.

2.4 Samples adulterations

To verify if the tests described above would be able to detect adulterations, we performed several different adulterations as described below. All the adulterations were tested with the three tests described above.

Commercial corn syrup and laboratory-produced inverted sugar were used for the adulteration simulations. In the preparation of inverted sugar, 350 g of sugar was weighed and heated with 150 ml of water and 15 ml of lemon juice. After the sugar had completely dissolved, the heat was turned off, and 3 g of sodium bicarbonate was added.

Ten different dilutions were performed for each sample of SB honey, as well as the control (Table 1). As a general control for adulteration of SB honey, nine different dilutions plus one control were performed for one sample of *A. mellifera* honey, using corn syrup, inverted sugar, and *A. mellifera* honey (see Table 2).

Table 1. Dilutions and adulterations were performed using honey from *Apis mellifera*, stingless bee (SB) honey from *T. angustula*, *M. quadrifaciata* and *T. elongata*, corn syrup, and inverted sugar.

	Dilutions	Percentage of stingless bees' honey
1	*A. mellifera* honey + Stingless bee honey	50%
2	*A. mellifera* honey + SB honey + water	33%
3	Corn syrup + SB honey	25%
4	Corn syrup + SB honey	50%
5	Corn syrup + SB honey	75%
6	Corn syrup + SB honey	90%
7	Corn syrup + SB honey	25%
8	Inverted sugar + SB honey	50%
9	Inverted sugar + SB honey	75%
10	Inverted sugar + SB honey	90%
11	Control - SB honey	100%

Table 2. Dilutions and adulterations were performed using honey from *Apis mellifera*, corn syrup, and inverted sugar.

	Dilutions	Percentage of stingless bees' honey
1	*A. mellifera* honey + water	50%
2	Corn syrup + *A. mellifera* honey	25%
3	Corn syrup + *A. mellifera* honey	50%
4	Corn syrup + *A. mellifera* honey	75%
5	Corn syrup + *A. mellifera* honey	90%
6	Inverted sugar + *A. mellifera* honey	25%
7	Inverted sugar + *A. mellifera* honey	50%
8	Inverted sugar + *A. mellifera* honey	75%
9	Inverted sugar + *A. mellifera* honey	90%
10	Control - *A. mellifera* honey	100%

3 Results

3.1 Sample volume decrease test

Examination of pure SB honey from three samples showed the formation of a small precipitate in the honey of all three SB species in the Lund reaction: *M. quadrifaciata* - 0.1 mL, *T. angustula* - 0.2 mL, and *T. elongata* - 0.2 mL. Honey from *A. mellifera* showed precipitation of 1.3 mL. For this reason, dilution tests were not performed, as the Lund reaction was not effective for use in SB honey.

In the Fiehe reaction, by obtaining an appreciable volume of the ether layer, it was not possible to continue the test with a sample weight of 2 grams. Therefore, as recommended, the tests were continued with a 5 g sample (Instituto Adolfo Lutz, 2008).

When the sample volume was reduced in Lugol's reaction, the use of 8, 5, and 2 grams presented the same positive result, thus indicating the use of 2 grams of honey sample.

3.2 Honey adulteration tests

The results of the dilution tests simulating adulteration showed that no tests detected the dilution to volume increase with water alone (Table 3). The Fiehe reaction was only effective in detecting adulteration with corn syrup (Figures 1.3, 1.4, 1.5, and 1.6). This test did not detect adulteration with inverted sugar at any dilution (Figures 1.7, 1.8, 1.9, and 1.10). In contrast, Lugol's test detected all adulterations with both corn syrup and inverted sugar (Figure 2). It is important to note that the test using 90% SB honey with inverted sugar (Figure 2.10) showed a color change compared to the control, even if it was not so evident.

A summary of all the results obtained by the tests can be found in Table 3, indicating the effectiveness of each test for each sample. It is important to note that regardless of the SB species that originated the honey, the results of the tests were always the same in all the triplicates, without any divergent outcome.

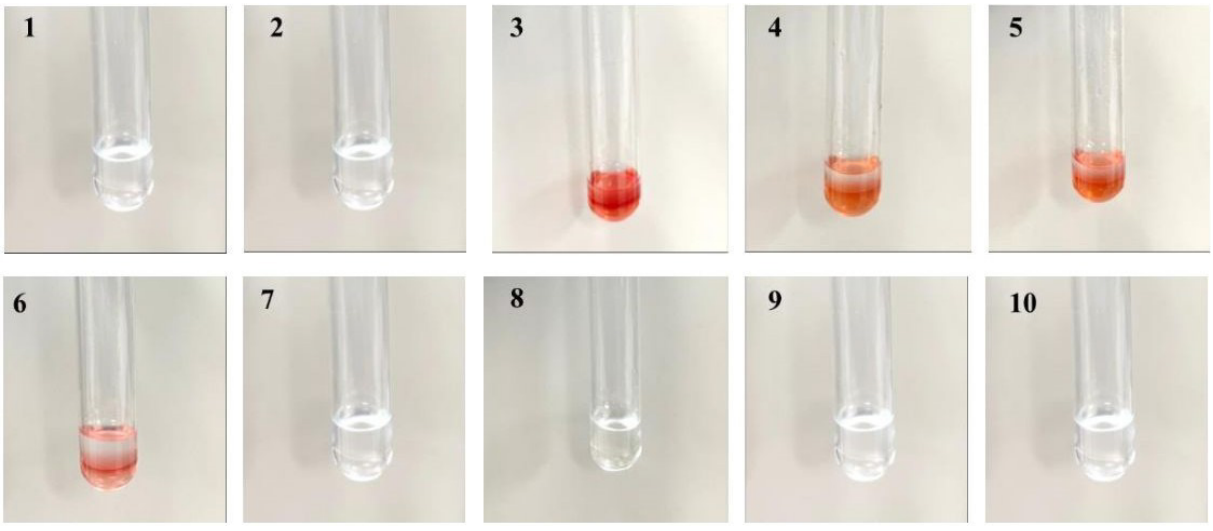

Figure 1. Results of dilutions and adulterations for the Fiehe reaction carried out with honey from *Apis mellifera*, stingless bee (SB) honey from *T. angustula*, corn syrup, and inverted sugar. 1 - *A. mellifera* honey + SB honey (50%); 2 - *A. mellifera* honey + SB honey + water (33%); 3 - Corn syrup + SB honey (25%); 4 - Corn syrup + SB honey (50%); 5 - Corn syrup + SB honey (75%); 6 - Corn syrup + SB honey (90%); 7 - Inverted sugar + SB honey (25%); 8 - Inverted sugar + SB honey (50%); 9 - Inverted sugar + SB honey (75%); 10 - Inverted sugar + SB honey (90%).

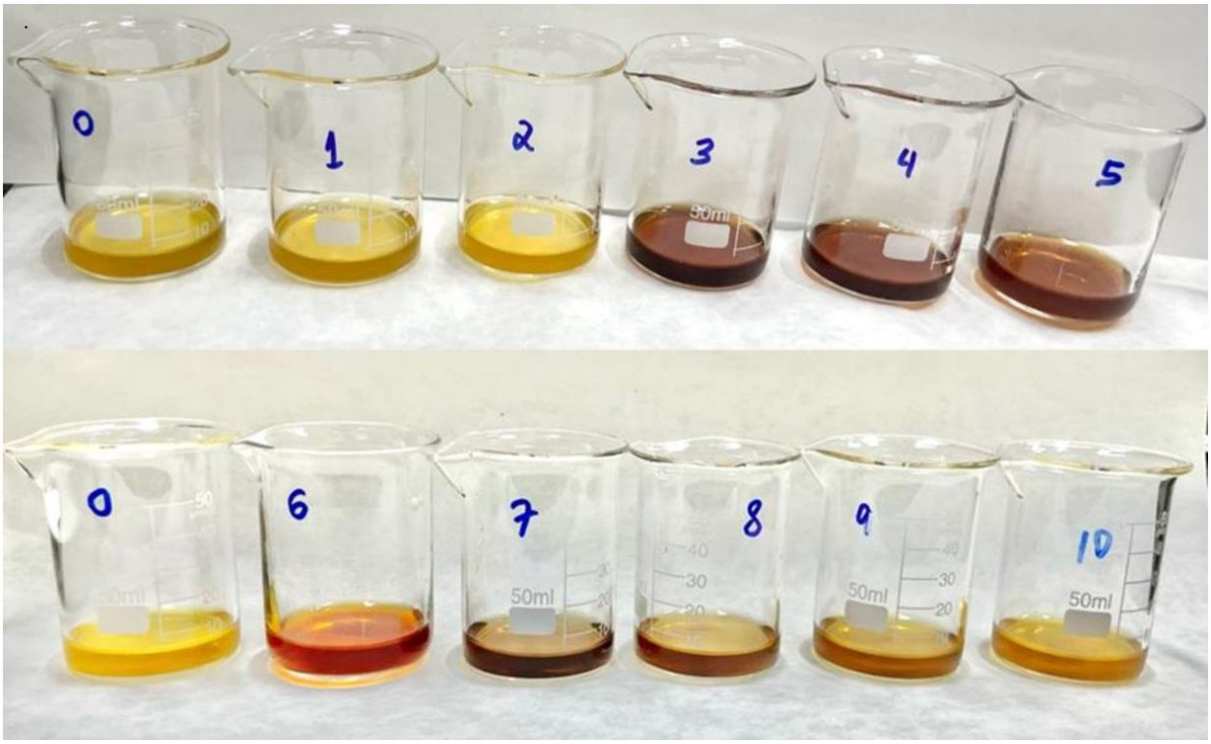

Figure 2. Results of dilutions and adulterations for the Lugol's reaction carried out with honey from *Apis mellifera*, stingless bee (SB) honey from *T. angustula*, corn syrup, and inverted sugar. 0 - Control; 1 - *A. mellifera* honey + SB honey (50%); 2 - *A. mellifera* honey + SB honey + water (33%); 3 - Corn syrup + SB honey (25%); 4 - Corn syrup + SB honey (50%); 5 - Corn syrup + SB honey (75%); 6 - Corn syrup + SB honey (90%); 7 - Inverted sugar + SB honey (25%); 8 - Inverted sugar + SB honey (50%); 9 - Inverted sugar + SB honey (75%); 10 - Inverted sugar + SB honey (90%).

Table 3. Results of Fiehe and Lugol tests on effectiveness and non-effectiveness for identifying adulteration.

	%	*T. angustula*		*M. quadrifasciata*		*T. elongata*		*A. mellifera*	
		Fiehe	**Lugol**	**Fiehe**	**Lugol**	**Fiehe**	**Lugol**	**Fiehe**	**Lugol**
1	50%	NC	non-effective	NC	non-effective	NC	non-effective	NC	non-effective
2	33%	NC	non-effective	NC	non-effective	NC	non-effective	X	X
3	25%	Effective	effective	effective	effective	effective	effective	effective	effective
4	50%	Effective	effective	effective	effective	effective	effective	effective	effective
5	75%	Effective	effective	effective	effective	effective	effective	effective	effective
6	90%	Effective	effective	effective	effective	effective	effective	effective	effective
7	25%	NC	effective	NC	effective	NC	effective	NC	effective
8	50%	NC	effective	NC	effective	NC	effective	NC	effective
9	75%	NC	effective	NC	effective	NC	effective	NC	effective
10	90%	NC	effective	NC	effective	NC	effective	NC	effective
11	Control	NC	non-effective	NC	non-effective	NC	non-effective	NC	non-effective

NC: no color; X: test not performed.

4 Discussion

Compared to the honey production of *A. mellifera* bees, which produce an average of 20 kg of honey per colony per year, the absolute production of SB is lower, ranging from 0.5 to 5 kg of honey per colony per

year, depending on the species (Chuttong et al., 2016). This difference in production, combined with regional factors and technical limitations in analysis (Faleiros-Quevedo & Francoy, 2022), raises the price of SB honey to two to five times the value of *A. mellifera* honey (Se et al., 2018; Zuccato et al., 2017). Thus, reducing sample volume for rapid tests and tests for physicochemical characterization of SB honey may facilitate analysis by small producers whose production is also small and who nevertheless need to separate larger quantities for quality testing (Faleiros-Quevedo & Francoy, 2022).

In the Lund reaction, all samples tested had a precipitate value that was below the limit recommended by the Adolf Lutz Institute (0.6 to 3 mL) (Instituto Adolfo Lutz, 2008), possibly because SB honey has a higher moisture content than *A. mellifera* honey, which dilutes more of the natural proteins in the honey, making their precipitation with tannic acid more difficult.

Almeida-Muradian et al. (2013) had already noted the ineffectiveness of the Lund test on honey samples from *Melipona subnitida*, and our data suggest that the honey from the species *T. angustula, M. quadrifasciata* and *T. elongata* does not respond effectively to the expected Lund reaction results. In addition, although this test is effective for *A. mellifera* honey, previous work shows it is unsuitable for SB honey because false positive results may occur (Almeida-Muradian et al., 2013). Ultimately, more significant amounts of honey must be used for this test to be effective. However, more extensive and in-depth studies must be conducted for this.

The Fiehe reaction detected effectively adulteration with corn syrup, by detecting a compound produced by high fructose content. This test did not detect dilution of adulterants with inverted sugar, possibly because it is composed of equal parts glucose and fructose, which makes it difficult to react with a resorcinol-hydrochloric acid solution.

On the other hand, Lugol's reaction detected all adulterations with corn syrup and inverted sugar, except for the adulteration with *A. mellifera* honey and water. It is important to note that the test with 90% SB honey and inverted sugar (Figure 2.10) displayed a color change compared to the control, although not as marked. This result underlines the urgent need to have a control sample to compare the reaction colors.

No dilution to increase the sample volume was detected in any test when only water and *A. mellifera* honey were used, indicating that it is difficult to detect this type of adulteration, which is very common because SB honey has a higher water content. It is necessary to perform more sensitive tests with other parameters to detect this type of adulteration.

When performed on adulterations of SB honey with *A. mellifera* honey, the tests did not show color changes as it is an adulteration of honey between different bee tribes (Apini and Meliponini) (Michener, 2007). This kind of adulteration is quite common to obtain a larger quantity of SB honey, which has a higher value. Thus, these tests are unsuitable for detecting adulteration with *A. mellifera* honey. For this type of adulteration, more in-depth studies would be needed to verify the exclusive differences between SB and *A. mellifera* honey.

SB honey is quite diverse in its characteristics (Biluca et al., 2016; Braghini et al., 2022; Faleiros-Quevedo & Francoy, 2022; Nordin et al., 2018), and since its characterization for commercialization is not yet fully regulated (Faleiros-Quevedo & Francoy, 2022; Koser et al., 2020), it becomes a target for various adulterants, mainly due to its high value compared to *A. mellifera* honey, as previously stated.

Hence, this highlights the need for methods to ensure the authenticity of SB honey, which is essential for the rapid development of the native honey production chain since the properties of these kinds of honey vary greatly between the different genera of Meliponini bees and within the same species, depending on the characteristics of the terroir. Several authors have striven to establish physicochemical parameters to ensure the authenticity of SB kinds of honey (Biluca et al., 2016; Braghini et al., 2022; Faleiros-Quevedo & Francoy, 2022; Nordin et al., 2018). However, the parameters identified so far do not correspond to the reality of the produced honey, making it difficult for both large and small producers to comply with the standards and obtain control or authenticity seals.

Future work should address current physicochemical standards and criteria to guide efforts and establish priorities for developing parameters for kinds of honey from different groups of SB in the medium and long term. However, to enable the marketing of SB honey in the short term, it is easiest to focus on rapid authenticity tests, such as Lugol's test, and hygiene criteria to protect consumer health. Our results suggest that Lugol's reaction can be used as an initial assessment of honey quality since honey production is an expanding activity in several countries.

However, the lack of comprehensive regulation of this activity hinders the emergence of several formal producers to meet consumption demand (Jaffé et al., 2015). In addition, informal producers complicate the traceability and reliability of the honey produced.

5 Conclusion

Apart from Lund's test, Lugol's and Fiehe's tests can be performed in reduced amounts of SB honey, reducing the losses that producers may encounter as they send samples for authenticity verification, especially if we take into consideration that some species produce only small amounts of honey per colony per year, allied to the high prices of that kind of honey.

As a rapid and inexpensive test, Lugol's reaction enables the development of the production chain for stingless bee honey. Developing a kit for SB honey authenticity testing that can be distributed to producers is also possible, helping to develop the production chain. More refined and accessible ways to detect adulteration with *A. mellifera* honey and water will still be tested in future works.

However, the results presented here are already a critical indication that detecting inauthentic honey can be accomplished by accessible tests, by combining qualitative testing as initial screening and, if necessary, quantitative testing.

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