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Pesquisa de adulteração e identificação da micoflora de amostras de mel comercializadas na região metropolitana de Belo Horizonte, Brasil

Assessment of adulteration and mycoflora identification of honey samples marketed in the metropolitan region of Belo Horizonte, Brazil

Investigación sobre adulteración e identificación de micoflora de muestras de miel comercializadas en la región metropolitana de Belo Horizonte, Brasil

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Resumo

O mel para consumo humano deve ser processado em condições satisfatórias de Boas Práticas de Fabricação e não deve conter matérias estranhas ou contaminação microbiológica. No entanto, vários estudos mostraram que uma alta porcentagem de amostras de mel de diferentes regiões do Brasil não está adequada em termos de segurança do alimento. Nesse sentido, este trabalho teve como objetivo avaliar a qualidade microbiológica, microscópica e físico-química de amostras de mel não inspecionadas na região metropolitana de Belo Horizonte, Brasil. Foram coletadas 30 amostras de mel (n = 30) e analisados coliformes totais, fungos totais, matérias estranhas e sujidades, teor de umidade, Aw, pH, acidez total titulável, teste de Lugol e 5-hidroximetilfurfural (5-HMF). Os fungos filamentosos também foram isolados e identificados ao nível de gênero. Pelo de roedor e formiga foram encontrados nas amostras, indicando risco à saúde dos consumidores. Os dados obtidos demonstraram que 56,7% das amostras estavam adulteradas. Todas as amostras positivas no teste de Lugol também apresentaram grânulos de amido na avaliação microscópica, sugerindo adulteração pela adição de cana de açúcar ou xarope de amido de milho. Nenhuma amostra mostrou a presença de coliformes e a contagem total de fungos foi considerada baixa. *Cladosporium* spp., *Penicillium* spp. e *Aspergillus* spp. foram os principais gêneros de fungos filamentosos isolados. Foram encontrados altos valores de 5-HMF, principalmente nas amostras adulteradas. Os dados obtidos serão comunicados às autoridades competentes, uma vez que tais produtos de composição desconhecida estão sendo comercializados, oferecendo riscos à saúde dos consumidores.

Palavras-chave: *Apis melífera*; 5-hidroximetilfurfural; Fungos filamentosos; Teste de Lugol; Matérias estranhas; Sujidades.

Abstract

Honey for human consumption must be processed under satisfactory conditions of Good Manufacturing Practices and not contain extraneous matters or microbiological contamination. However, several studies have shown that a high percentage of honey samples from different regions of Brazil are not adequate in terms of food safety. In this sense, this work aimed to evaluate the microbiological, microscopic, and physicochemical quality of uninspected honey samples not inspected in the metropolitan region of Belo Horizonte, Brazil. Thirty honey samples (n = 30) were collected, and total coliforms, total fungi, extraneous matter and filth, moisture content, water activity (Aw), pH, total titratable acidity, Lugol test, and 5-hydroxymethylfurfural (5-HMF) were analyzed. Filamentous fungi were also isolated and identified at the gender level. Rodent hair and ants were found in the samples, indicating health risk for consumers. The data obtained demonstrated that 56.7% of the samples were adulterated. All positive samples in the Lugol test also presented starch granules in the microscopic evaluation, suggesting adulteration by the addition of sugar cane or corn starch syrup. No sample showed the presence of coliforms, and the total fungal count can be considered low. *Cladosporium* spp., *Penicillium* spp., and *Aspergillus* spp. were the principal genera of filamentous fungi isolated. High 5-HMF values were found, mainly in the adulterated samples. The data obtained will be reported to the competent authorities, since such products of unknown composition are being commercialized, offering risks to consumers' health.

Keywords: *Apis mellifera*; 5-hydroxymethylfurfural; Filamentous fungi; Lugol test; Extraneous matters; Filth.

Resumen

La miel para consumo humano debe procesarse en condiciones satisfactorias de buenas prácticas de fabricación y no debe contener materias extrañas ni contaminación microbiológica. Sin embargo, varios estudios han demostrado que un alto porcentaje de muestras de miel de diferentes regiones de Brasil no es adecuado en términos de seguridad alimentaria. En este sentido, este trabajo tuvo como objetivo evaluar la calidad microbiológica, microscópica y fisicoquímica de muestras de miel no inspeccionadas vendidas en la región metropolitana de Belo Horizonte, Brasil. Se recogieron treinta muestras

de miel (n = 30) y se analizaron coliformes totales, hongos totales, materia extraña y suciedad, contenido de humedad, Aw, pH, acidez titulable total, prueba de Lugol y 5-hidroximetilfurfural (5-HMF). Los hongos filamentosos también se han aislado e identificado a nivel de género. Se encontraron pelos de roedores y hormigas en las muestras, lo que indica un riesgo para la salud de los consumidores. Los datos obtenidos mostraron que el 56,7% de las muestras estaban adulteradas. Todas las muestras positivas en la prueba de Lugol también mostraron gránulos de almidón en la evaluación microscópica, lo que sugiere la adulteración mediante la adición de caña de azúcar o jarabe de almidón de maíz. Ninguna muestra mostró la presencia de coliformes y el recuento total de hongos se consideró bajo. *Cladosporium* spp., *Penicillium* spp. Y *Aspergillus* spp. fueron los principales géneros de hongos filamentosos aislados. Se encontraron valores altos de 5-HMF, principalmente en las muestras adulteradas. Los datos obtenidos serán comunicados a las autoridades competentes, ya que dichos productos de composición desconocida se comercializan, lo que ofrece riesgos para la salud de los consumidores.

Palabras clave: *Apis melífera*; 5-hidroximetilfurfural; Hongos filamentosos; Prueba de Lugol; Materias extrañas; Suciedad.

1. Introduction

Brazil has a notable beekeeping potential with diversified melitophilous flora, favorable edaphic-climatic conditions, and the sizeable territorial extension (Caldas et al., 2019; Marques et al., 2011). Honey production contributes to income generation, rural work, and with the increase in the biological diversity of the ecosystem (Serafin, 2017).

Honey can be described as the food product produced by honey bees from the nectar of flowers, or the secretions of live plants, or plant-sucking insects. Bees collect, transform, combine with specific substances of their own, store, and let the honey mature in the combs of the hive (Brasil, 2000; CODEX, 2001).

This food has a variable chemical composition, which depends on several factors such as the composition of the nectar, climate conditions, soil type, bee species involved in the production, among others (Souza, 2017; Carvalho et al., 2005).

Honey for human consumption must not contain extraneous matter such as insects, larvae, and grains of sand. It must present only histological materials characteristic of the product, such as pollen grains (Brasil, 2014). However, several studies have shown that a high percentage of honey samples from different regions of Brazil, especially those from the street

market and not inspected, are not adequate in terms of the quality required by Brazilian legislation (Santos; Moura; Câmara, 2015; Mendonça et al., 2013).

In addition to the presence of extraneous matters, another worrying factor in the quality of honey is its adulteration through the addition of sugar cane syrup and sucrose. In this sense, it is of great importance to carry out microscopic analysis, together with microbiological evaluation, in order to obtain a broader set of information necessary to verify the safety of the honey (Gois et al., 2013).

The microbiological quality of honey is related to the hygienic-sanitary conditions along its production chain, from the management of hives in the field to the processing, and possible cross-contamination may occur. However, the Brazilian legislation (BRASIL, 2000) does not determine the microbiological parameters for honey, only establishing that good manufacturing practices must be applied. Also, The National Health Surveillance Agency (ANVISA) that regulates microbiological standards for processed food in Brazil does not include the honey product (Brasil, 2001).

Among the main microbiological contaminations of honey are fungi. In fresh honey processed under satisfactory conditions of Good Manufacturing Practices, the total fungal count is below 100 CFU/g. Higher values are associated with inadequate processing or storage conditions, enabling fermentation and development of the filamentous fungi (Silva et al., 2017).

The evaluation of the presence of filamentous fungi in honey is important because of some genera, such as *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. can produce toxic secondary metabolites called mycotoxins. These toxins are natural contaminants of low molecular weight and can cause mild symptoms, such as headache, to acute intoxications that can lead to death (Parisi et al., 2016). The principal genera that can be isolated from honey are *Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp. and *Botrytis* spp., varying according to the type of honey and the hygienic conditions of the processing (Seijo, Escuredo & Fernández-González, 2011).

It is important to note that honey has low water activity (Aw), preventing the development of filamentous fungi (Pitt & Hocking, 2009). However, the spores of these microorganisms can be transferred by honey to other foods, and, when they find favorable growth conditions, they can develop and produce mycotoxins.

The Brazilian legislation recommends some physicochemical analyzes to ensure the quality and safety of honey, such as the moisture content, 5-hydroxymethylfurfural (HMF), Lugol test, ash content, among others (Brasil, 2000). In this sense, this work aimed to

evaluate the microbiological, microscopic, and physicochemical quality of uninspected honey samples marketed in the metropolitan region of Belo Horizonte, Brazil.

2. Materials and Methods

The research aims to bring new knowledge to society, as stated by Pereira et al. (2018). The present study was carried out in the laboratory in a quantitative study with a small qualitative bias.

2.1 Sampling

Thirty honey samples (*n*= 30), stored in glass bottles, were acquired randomly from street markets in the cities of Sete Lagoas, Paraopeba, Contagem, Betim, and Belo Horizonte, in Minas Gerais, Brazil. The samples were transported to the Laboratory of Biochemical Engineering of the Federal University of São João del-Rei and then kept at room temperature until analysis. Microbiological analyzes were performed in duplicates, while physicochemical evaluations were performed in triplicates.

2.2 Microbiologic analysis

Microbiological analyzes were performed following the methodology described by Silva et al. (2017). For total coliforms, the multiple tube technique was applied with Lauryl Tryptose Broth (LTB). The number of coliforms, expressed as Most Probable Number (MPN/g), was obtained using the Hoskins table.

The enumeration of total fungi was performed in duplicates, using 25 g samples diluted in 225 mL of 0.1% peptone salt solution. Samples were homogenized for 60 s, corresponding to a dilution of 10^{-1}. From this, dilutions 10^{-2}, 10^{-3}, $10^{-4,}$ and 10^{-5} were made up using tubes containing 9 mL of 0.1% peptone salt solution. Surface plating was carried out in acidified potato dextrose agar (PDA), with 0.1 mL inoculum. Plates were incubated without reversing at 25 ºC for 5 days in a B.O.D. incubator. After this period, total colonies were counted and the results were expressed in CFU/g of honey.

Colonies of filamentous fungi were transferred to Malt Extract Agar (MEA) and Czapeck Yeast Extract Agar (CYA) and incubated in BOD at 25 ºC for 5 days. Afterward, the colonies were identified at the gender level, based on their morphological, macroscopic, and microscopic characteristics, according to Samson (2010). For that, a stereoscope microscope

(ZEISS Primo Star, Carl Zeiss, Germany) and an optical microscope (ZEISS Stemi 2000-C, Germany) were used, both coupled with a digital camera (ZEISS Axiocam ERc5s, Germany). In order to observe the precise arrangement of the conidiophores and accurately identify some genera were also prepared slide cultures using the Riddel's simple agar block method (Samson, 2010). After identification, isolates were seeded into tubes containing MEA and CYA agar in order to be stored at 5 ºC.

2.3 Microscopic evaluation

Extraneous matter and filth were evaluated according to the method 945.79 of the Association of Official Analytical Chemists (AOAC, 2016). For that, 200 g sample, previously homogenized, was dissolved in 200 mL of 2.5% HNO_3 solution, followed by filtration and microscopic examination of the material retained in the filter, using the stereoscope and the optical microscope, both coupled with a digital camera.

The presence of starch granules was investigated using aliquots of the sample diluted in distilled water (1: 1) and Lugol's solution. Starch granules were identified according to their histological characteristics, presented by Athié et al. (2006) and Oliveira et al. (2015). For the identification of rodent hair, the gallery of micrographs of Crutcher (2017) was also used.

2.4 Physicochemical and 5-HMF analysis

Physicochemical analyzes were carried out in triplicate and according to the methods of the Adolfo Lutz Institute (IAL, 2008), as presented: moisture content at 105 ºC (method 012/IV), pH (method 017/IV), and total titratable acidity (method 016 / IV). The Lugol test was applied to assess the presence of starch in the honey samples, according to method 0184/IV, in which 10 g of sample was diluted in 20 mL of distilled water and taken to boiling, followed by cooling and then added of 0.5 mL of Lugol's solution. The presence of starch was evidenced by a blue color in the samples.

The 5-hydroxymethylfurfural level was quantified by UV spectrophotometry, according to AOAC 980.23 method (AOAC, 2016), using a Shimadzu UV-1201 (Kyoto, Japan). The water activity (Aw) was evaluated using the Aqualab® 3TE (Rio de Janeiro, Brazil).

2.5 Statistical analysis

Data were evaluated by descriptive statistics, using Sisvar 5.0 software, and, to verify the existence of a correlation among the parameters evaluated, Pearson's Coefficient was calculated using the Microsoft Excel software, according to Devore (2006).

3. Results and Discussion

3.1 Microbiological analysis

There was no positive result for coliforms from the 30 samples analyzed, which can be justified by the low Aw of samples, that varied from 0.52 to 0.64. According to Villadiego et al. (2012), in food with low Aw (<0.6), there is no growth of microorganisms, although they can survive in samples as spores, such as fungal spores. In this research, only 5 samples showed Aw higher than 0.6, but with higher value corresponded to 0.64, which also can be classified as low Aw.

Regarding the total fungal count, 17 samples (56.7%) showed growth, with results ranging from undetected to 1.0 x 10^3 CFU/g. According to Silva et al. (2017), the total fungi count in fresh honey is below 100 CFU/g. In the present study, only 3 samples presented counts above 100 CFU/g, with values up to 1.03 x 10^3 CFU/g, indicating possible failures in the good processing practices. Also, fungal spores can contaminate honey in the management of hives. Considering the 39 colonies of filamentous fungi isolated, the predominant genera corresponded to *Cladosporium* (50%), *Penicillium* (34.62%), *Aspergillus* (11.54%) and *Engydontium* (3.85%). Similar results were described by Seijo et al. (2011), who identified the genera *Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp. and *Botrytis* spp., in honey samples from northwestern Spain. According to the authors, the contamination varies a lot according to the type of honey and the hygienic conditions of processing.

Nasser (2004) evaluated 45 samples of honey and found that 88.9% were contaminated with fungi. Nine genera were identified, and the most prevalent filamentous fungi isolated were *Aspergillus flavus, A. niger, A. fumigatus, A. versicolor*. It is expected that honey mycobiota may vary qualitatively and quantitatively, depending on geographic origin. Rodríguez-Andrade et al. (2019) evaluated the diversity of xerotolerant and xerophilic fungi in 84 samples of honey bee from Spain. Thirteen genera were identified, such as *Aspergillus, Bettsia, Candida, Eremascus, Monascus, Oidiodendron,* and *Penicillium*. Authors

demonstrated that these fungi could be considered as hot-resistant, which survives to the thermal treatment used to make honey non-crystallizable.

The data obtained in this work (qualitative and quantitative) point to the risk of the presence of filamentous fungi in honey that can produce mycotoxins.

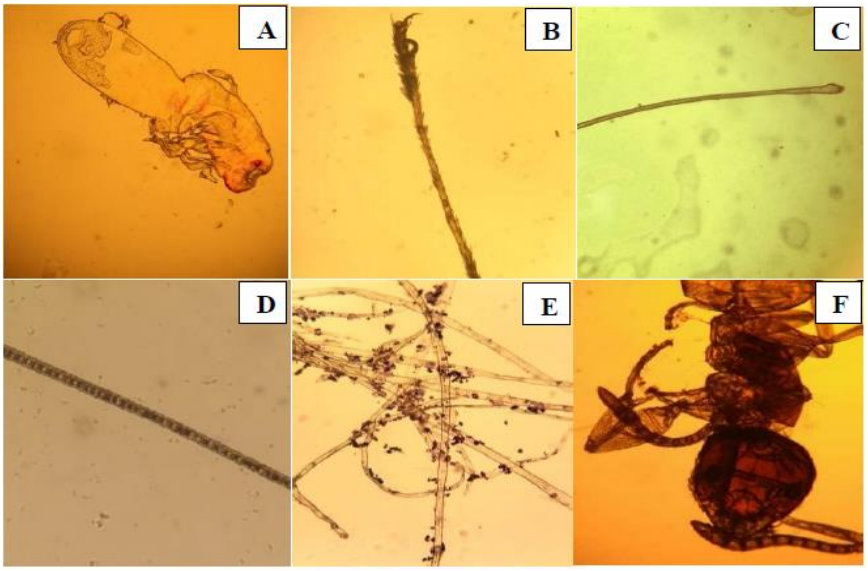
3.2 Microscopic evaluation and Lugol test

The presence of extraneous matter and filth was observed in 6 samples (20%), as shown in Figure 1. The Brazilian legislation classifies extraneous matter in two groups: i) indicative of risks to human health, such as the fragments of ants, cockroaches, rodents, pigeons and bats, which can transmit viruses and pathogenic bacteria and; ii) indications of failures in the good practices, including arthropods, human hair, filamentous fungi, sand, soil, among others (Brasil, 2014). In the present study, rodent hair and ants were found in the samples, both belonging to the first group, indicating health risk for consumers.

The presence of insects such as ants can give the false impression of not representing a danger. However, as presented by Fontana et al. (2010) and Sousa et al. (2016), such insects are vectors of several pathogenic bacteria, such as *Escherichia coli*, *Yersinia enterocolitica* and *Staphylococcus* spp. Also, the rodent hairs, identified by their typical shape, with a brown colored ribbon characteristic divided internally by partitions, are considered a health risk because they can carry pathogenic bacteria and viruses that cause human diseases (Silva; Martini, 2006; Athié, 2006).

In the present study, even though no coliforms were found, other undesirable microorganisms, including pathogenic ones, could be present in honey, since rodent hair was found.

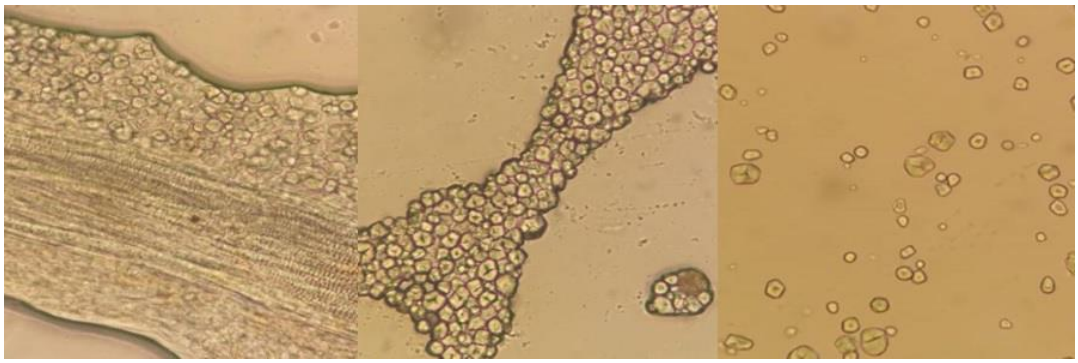
Figure 1 – Extraneous matter found in honey samples.



A: Mite (100x zoom); B: Bee fragment (100x zoom); C: Hair (100x zoom); D: Rodent hair (400x zoom); E: Vegetable fiber (100x zoom); F: Ant (100x zoom).
Source: Authors.

In the microscopic evaluation of starch, the granules were found in 17 samples (56.7%), as illustrated in Figure 2. Honey is considered a concentrated solution of sugars, water, enzymes, proteins, amino acids, organic acids, minerals, vitamins, aromatic substances, pigments, pollen grains, which may contain beeswax from the extraction process, however, it must not contain starch in its composition, which is indicative of fraud (Brasil, 2000).

Figure 2 – Starch granules and plant tissues found in the honey samples (400x zoom).



Source: Authors.

As demonstrated by Wang et al. (2015), using HPLC analysis, honey adulteration with starch syrup is one of the most common adulterations in this product. These syrups can be produced from high fructose corn, sugar cane, rice starch, cassava starch, and sucrose. The

conventional techniques, such as the optical microscope, can not identify the type of fraud, and for that, chromatographic and spectroscopic techniques have been used (SE et al., 2019).

3.3 5- 5-HMF and physicochemical analysis

The values obtained from the physicochemical analyzes are shown in Table 1. Regarding the moisture content, the national legislation determines the limit of 20% in honey. In two samples (6.66%), values higher than 20% were found, which can favor microbial growth and fermentation of the product. According to Marchini, Moreti and Otsuk (2005), one of the leading causes of the high humidity in honey is the early harvesting from immature honeycombs or inadequate storage conditions.

Table 1 – Results of physicochemical analysis of honey samples (*n*= 30).

Parameters	Range	Mean and variation among samples
Moisture contente (%)	13.77 – 20.64	82.42 ± 1.59
Aw	0.52 – 0.65	0.58 ± 0.03
pH	3.18 – 4.72	3.75 ± 0.47
Total titrable acidity (mEq/Kg)	18.93 – 59.46	34.7 ± 10.3
5-Hydroxymethylfurfural ($mg.kg^{-1)}$	0 – 772.5	227.1 ± 196.2

Source: Research data.

The pH range considered ideal for honey varies from 3.3 to 4.6 (BRASIL, 2000). The pH values in the samples ranged from 3.18 to 4.72, with 4 samples outside this range. Three samples were below pH 3.3, corresponding to 3.18 ± 0.2, 3.20 ± 0.1, and 3.26 ± 0.2, and all of these were positive in the Lugol test, indicating that they were adulterated. The low pH value may also explain the low microbiological contamination since it is known that low pH values inhibit microbial growth.

According to El Sohaimy, Masry and Shehata (2015), the pH can be related to the local conditions of honey production, mainly with the composition of the nectar collected by the bees and the low pH can be correlated with high acidity, indicating fermentation of sugars into organic acid. However, in the present research, there was no correlation between the parameters evaluated.

The total titrable acidity ranged from 18.93 to 59.46 mEq.kg^{-1}. Among the 30 samples analyzed, only 3 showed values higher than the maximum value established by legislation (50 mEq.kg-1) (Brasil, 2000). Two of them were also positive in the Lugol teste, indicating adulteration. The acidity of honey varies according to the chemical composition of the nectar and due to the action of enzymes and bacteria during the maturation and storage (Finco et al., 2010). Some bacteria and yeasts can metabolize the sugars present in honey and produce ethanol, which is later converted to acetic acid, altering the quality of the product (Brasil, 2000). As the samples analyzed were acquired from different locations and were processed under unknown conditions, since these are uninspected products, a considerable variation in this parameter was expected.

Regarding 5-HMF, values between 0 and 772.5 mg.kg^{-1} were found. The Brazilian law limits the maximum level of 5-HMF up to 60 mg.kg^{-1}. From the 30 samples analyzed, 20 showed much higher values. The 5-HMF is a toxic molecule formed in honey from reducing sugars through the Maillard reaction. When the honey is heated, processed or stored under inadequate conditions, 5-HMF can be formed, so it is a good indicator of honey quality (Shapla et al. 2018).

Grigoryan, K. (2016) described that 5-HMF is carcinogenic and cytotoxic, and the levels found in samples from different locations vary a lot, so it is one of the most critical parameters of honey quality to be evaluated. In the present research, the higher values of 5-HMF were obtained in samples that were positive in the Lugol Test, indicating that the levels of 5-HMF found may originate from vegetable syrups used to adulterate the products. Another point that should be highlighted is that the 5-HMF analyzes were performed in the present study by UV spectrophotometry, using a methodology intended for quantification in honey. In adulterated samples, overestimation of 5-HMF may occur, since the composition of the material under analysis is not known.

4. Final Considerations

The data obtained in the present research demonstrated that 56.7% of the honey samples uninspected obtained from the metropolitan region of Belo Horizonte were adulterated. All positive samples in the Lugol test also presented starch granules in the microscopic evaluation, suggesting adulteration by the addition of sugar cane or corn starch syrup. Due to the low Aw and pH, no sample showed the presence of coliforms, and the total

fungal count can be considered low. *Cladosporium* spp., *Penicillium* spp., and *Aspergillus* spp. were the principal genera of filamentous fungi isolated. High 5-HMF values were found, mainly in the adulterated samples and positive for starch granules. The data obtained will be reported to the competent authorities, since such products of unknown composition are being commercialized, offering risks to consumers' health.

As suggestions for future work, the evaluation of the physicochemical and microbiological properties of honey marketed in other regions should be evaluated in order to verify whether this is a recurring problem or not. The use of instrumental methods is recommended, especially those based on chromatography and spectroscopy techniques, to identify the types of adulterations.

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