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Qualitative tests for the determination of fraud in raw milk: evaluation of the influence of analytical parameters of the tests and the stability of the samples as a function of time and preservation form

Ensaios qualitativos de determinação de fraudes em leite cru: avaliação da influência de parâmetros analíticos dos ensaios e da estabilidade das amostras em função do tempo e forma de preservação

Ensayos cualitativos para la determinación de fraude en leche cruda: evaluación de la influencia de los parámetros analíticos de los ensayos y la estabilidad de las muestras en función del tiempo y forma de conservación

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Abstract
The present work aimed to evaluate the robustness of qualitative tests of frauds of addition of chlorine/hypochlorite, hydrogen peroxide and starch in raw milk, which parameters have the greatest influence on these analytical methods, and to evaluate the influence of time and mode of preservation of intentionally adulterated samples. The effects of the variation of some parameters of the analytical procedures were evaluated, making combinations between the different factors, determining the degree of influence of these variations on the tests. The stability time of the frauds in the samples was evaluated, keeping them under refrigeration (3±2 °C) and carrying out the tests on the same day, and under freezing (≤ -18 °C) for 5, 9 and 15 days, later analyzed, verifying if the frauds were detected. According to the results, it could be observed that the chlorine/hypochlorite detection method was adequate, detecting the addition of the adulterant in all combinations of variables and in all forms of conservation of the samples. The method for detecting the addition of hydrogen peroxide proved to be adequate for the samples analyzed immediately (< 24 h), detecting the presence of the substance in all combinations. However, the method did not detect the presence of peroxide in the combinations for the frozen samples, indicating that the method can generate false negatives in samples that are not fresh. The starch detection method did not detect the presence of this contaminant in all combinations of variables. The combinations that used only a drop of the Lugol's iodine solution showed false negatives, so the amount of added solution is a critical variable for the method. However, for the other combinations, the method proved to be adequate for both fresh and frozen samples, detecting the addition of starch, regardless of storage time.
Keywords: Adulteration; Hypochlorite; Hydrogen peroxide; Qualitative analysis; Starch.

Resumo
A adulteração do leite cru é um problema de ordem econômico-social, onde a adição de substâncias indevidas pode provocar efeitos deletérios à saúde humana ou mesmo inviabilizar o consumo/processamento do leite. A utilização de técnicas de detecção destas fraudes que sejam robustas e sensíveis, bem como o emprego de métodos adequados de armazenamento/estabilização das amostras, é fundamental para que fraudes e adulterações sejam detectadas e reprimidas. Com isto em vista, o presente trabalho teve como objetivo avaliar a robustez dos ensaios qualitativos de fraudes de adição de cloro/hipoclorito, peróxido de hidrogênio e amido em leite cru, quais os parâmetros de maior influência sobre estes métodos analíticos, e avaliar a influência do tempo e modo de preservação das amostras

adulteradas intencionalmente. Avaliou-se os efeitos da variação de alguns parâmetros da marcha analítica, realizando combinações entre os diversos fatores, determinando o grau de influência destas variações sobre os ensaios. Avaliou-se o tempo de estabilidade das fraudes nas amostras, mantendo-as sob refrigeração (3±2 °C), realizando os ensaios no mesmo dia, e sob congelamento (≤ -18 °C) por 5, 9 e 15 dias, sendo posteriormente analisadas, verificando se as fraudes eram detectadas. De acordo com os resultados, pode-se observar que o método de detecção de cloro/hipoclorito foi adequado, detectando a adição do adulterante em todas as combinações de variáveis e em todas as formas de conservação das amostras. O método para detecção da adição de peróxido de hidrogênio mostrou-se adequado para as amostras analisadas imediatamente (< 24 h), detectando a presença da substância em todas as combinações. No entanto, o método não detectou a presença de peróxido nas combinações para as amostras congeladas, indicando que o método pode gerar falsos-negativos em amostras que não sejam frescas. O método de detecção da adição de amido não detectou a presença deste contaminante em todas as combinações de variáveis. As combinações que utilizaram uma gota da solução de Lugol apresentaram falsos-negativos, de forma que a quantidade de solução adicionada é uma variável crítica para o método. No entanto, para as demais combinações, o método mostrou-se adequado tanto para as amostras frescas como as congeladas, detectando a adição de amido, independentemente do tempo de armazenamento.
Palavras-chave: Adulteração; Amido; Análise qualitativa; Hipoclorito; Peróxido de hidrogênio.

Resumen
La adulteración de la leche cruda es un problema económico-social, donde la adición de sustancias inapropiadas puede causar efectos nocivos para la salud humana o incluso hacer inviable el consumo/procesamiento de la leche. El uso de técnicas de detección de estos fraudes que sean robustas y sensibles, así como el uso de métodos adecuados de almacenamiento/estabilización de las muestras, es fundamental para que los fraudes y adulteraciones sean detectados y reprimidos. Teniendo esto en cuenta, el presente trabajo tuvo como objetivo evaluar la robustez de las pruebas cualitativas de fraudes de adición de cloro/hipoclorito, peróxido de hidrógeno y almidón en la leche cruda, qué parámetros tienen la mayor influencia en estos métodos analíticos, y evaluar la influencia el momento y el modo de conservación de las muestras adulteradas intencionadamente. Se evaluaron los efectos de la variación de algunos parámetros de la marcha analítica, realizando combinaciones entre los diferentes factores, determinando el grado de influencia de estas variaciones en las pruebas. Se evaluó el tiempo de estabilidad de los fraudes en las muestras, manteniéndolas en refrigeración (3±2 ° C), realizando las pruebas el mismo día, y bajo congelación (≤ -18 ° C) durante 5, 9 y 15 días, y posteriormente analizados, verificando si se detectaron los fraudes. De acuerdo con los resultados, se puede observar que el método de detección de cloro/hipoclorito fue adecuado, detectando la adición del adulterante en todas las combinaciones de variables y en todas las formas de conservación de las muestras. El método de detección de la adición de peróxido de hidrógeno resultó adecuado para las muestras analizadas inmediatamente (<24 h), detectando la presencia de la sustancia en todas las combinaciones. Sin embargo, el método no detectó la presencia de peróxido en las combinaciones para las muestras congeladas, lo que indica que el método puede generar falsos negativos en muestras que no son frescas. El método de detección de adición de almidón no detectó la presencia de este contaminante en todas las combinaciones de variables. Las combinaciones que usaron una gota de la solución de Lugol mostraron falsos negativos, por lo que la cantidad de solución agregada es una variable crítica para el método. Sin embargo, para las otras combinaciones, el método demostró ser adecuado tanto para muestras frescas como congeladas, detectando la adición de almidón, independientemente del tiempo de almacenamiento.
Palabras clave: Adulteración; Almidón; Análisis cualitativo; Hipoclorito; Peróxido de hidrogeno.

1. Introduction

Cow milk is one of the most consumed foods in natura, besides being an ingredient and feedstock in the production of several processed foods, dairy or not. World milk production in 2019 was 715.92 million tonnes, whereas Brazil produced 35.89 million tonnes (Food and Agriculture Organization of the United Nations, 2019). Rio Grande do Sul state, in turn, produced 4.27 million tonnes of milk in this same year (Instituto Brasileiro de Geografia e Estatística, 2019). Due to the *in natura* consumption and the wide variety of products that have milk as an ingredient, the origin and quality of raw milk are paramount regarding both an economic and public health standpoint (Callefe & Langoni, 2015).

Due to milk being a complex mixture in which diverse additives may be added without changing its physical appearance and organoleptic properties, it is of great importance to detect the occurrence of any fraud intentional or not. This aims to establish tight controls and care in milk handling and processing to preserve its physical-chemical and microbiologic quality (Gandhi et al., 2020).

Do exist reports on generalized cases of milk adulteration throughout Brazil, in general, carried out on a small scale.

In 2007, Operation *Ouro Branco* (Gold Milk, in Portuguese) was triggered in Minas Gerais state, which investigated the addition of several adulterants in milk produced in Uberaba region by a large productive chain; the investigation resulted in the conviction of the fraudsters (Polícia Federal do Brasil, 2012). In 2012, Operation Amalthea, carried out in Paraiba state, broke up a scheme that, among other felonies, added chemicals to milk unfit for consumption, aiming to resell it to the State (Tribunal de Contas da União, 2012).

Rio Grande do Sul state, one of the largest Brazilian producers, also had the credibility of its milk put in jeopardy after the discovery of adulterations. In 2012, a joint task force between the Federal Public Ministry (MPF) and the Ministry of Agriculture (MAPA) triggered Operation *Leite Adulterado* (Adulterated Milk, in Portuguese), which searched for milk adulteration schemes in North of Rio Grande do Sul state and West of Santa Catarina state (Santos & Vasconcellos, 2018). In May 2013, the State Public Ministry carried out several investigations that resulted in the discovery of a fraudulent scheme involving agents of milk productive chain. This operation was called *Leite Compen$ado* [sic] (Compensated Milk, in Portuguese), resulting in the conviction of the liable persons (Andreatta et al., 2019).

According to MAPA Normative n° 76 of November 26, 2018, refrigerated raw milk must not present foreign substances in its composition, such as biocide or biostatic agents, acidity neutralizers, or substances that alter milk density or its cryoscopic index. These substances are added intentionally to the milk and are regarded as adulterants, being classified as reconstituters, preservatives, or reducers, among others (Brasil, 2018b; Tronco, 2003).

Reconstituers are added to milk with the intention of reconstituting its density, which was changed with water addition. Substances that compose this group are sucrose, sodium chloride, soluble starch, urine, and whey. Preservatives are added to milk to maintain its microbiological quality, e.g., avoiding that microbial activity acidifies the milk, keeping it within standards (pH between 6.6 and 6.8 and titratable acidity between 0.14 and 0.18 g of lactic acid per 100 mL of milk). Hydrogen peroxide, potassium dichromate, chlorine, hypochlorites, and formalin are examples of substances that make up this classification. Reducers are employed aiming to neutralize milk acidity, such as sodium carbonate and bicarbonate, sodium hydroxide, and quicklime (Abrantes et al., 2014; Brasil, 2018b; Tronco, 2003).

Among the several frauds that exist, chlorine (Cl) addition as hypochlorite (ClO^-) is regarded as a fraud with the intention of preserving milk. The analytical procedure used in its detection is based on the formation of free iodine (I2) from potassium iodide (KI), either by the action of free chlorine or hypochlorite (which generates free chlorine in its decomposition) existent in the frauded sample. If free chlorine is present, a yellow color develops; if there is no color change, the procedure continues and, existing hypochlorite in the sample, there is the development of yellow color in later stages of the procedure (Brasil, 2005).

Hydrogen peroxide (H_2O_2) addition is also considered a milk preservation scam. This fraud may be detected using a colorimetric method that uses guaiacol. The reaction occurs by the action of peroxidase enzyme (which is naturally present in milk and active in raw milk); peroxidase hydrolyses hydrogen peroxide, releasing oxygen (O_2). The released oxygen reacts with guaiacol, changing it from a leuko to a colored form, giving the milk sample a light-pink color (Brasil, 2018a).

Starch addition scam is considered a reconstitution of milk, used to increase its density after water addition to increase the volume, which causes a decrease in overall fluid density. The analytical method for its detection uses iodine (Lugol's iodine) as the indicator. Starch reacts with iodine as triiodide (I_3^-), forming a complex that absorbs light in the yellow region of visible spectrum, giving the mixture a blue-violet color (Brasil, 2018a; Holló & Szejtli, 1957).

For an efficient identification of these frauds, it is important to verify the robustness of the analytical methods used in their detections. According to Leite (2006), robustness is defined as the method sensibility to small variations in the analytical procedure, where a method is considered robust if it has a low sensitivity or is not influenced by small changes in analysis conditions.

Several procedures may be proposed to verify method sensibility relative to an existing analytical method. For this, there are as examples changes of temperature, pH, and variations of the quantity of reagents and samples used in the methods, and comparing the obtained results with standard procedure, to evaluate a new method (Leite, 2006).

Many analytical conditions/factors may vary, this can occur by purpose or by motives beyond the control of the technician. Thus, the variables that are believed to have an influence on the result are evaluated to determine their real impact on the analytical results. Firstly, a problem must be defined, e.g., plan what must be evaluated, carry out the collection of the necessary data, eliminate possible errors and verify the data to observe the distribution of the results as a function of the evaluated variables (Mergulhão, 2009).

Besides the issues regarding the analytical procedures, sample stabilization before analysis is paramount, seen that samples improperly stored may have changes that render analysis inconclusive or generate false-positive or false-negative results (Albano & Raya-Rodriguez, 2009). The main methods employed in the stabilization of milk samples are refrigeration and freezing (Pinto Júnior et al., 2012; Porcionato et al., 2008).

Sample stabilizing procedures must not adversely affect the composition and physical-chemical properties of the samples once any change may cause sample mischaracterization or the induction of incorrect results. According to Porcionato et al. (2008), Firmino et al. (2010), and Pinto Júnior et al. (2012), both refrigeration and freezing do not have any deleterious effect on the composition and properties of raw milk, even for storage periods greater than 90 days. However, there is no consolidated data on the effect of refrigeration and freezing on the stability of adulterants added to milk.

Thus, the present work aimed to evaluate the influence of varying some analytical parameters of qualitative analysis of chlorine/hypochlorite, hydrogen peroxide, and starch addition frauds in raw milk and evaluate the influence of time and storage mode on the stability of intentionally adulterated milk samples.

2. Methodology

2.1 Obtainment of raw milk

The sampling of the raw milk used as the basis for the preparation of the adulterated samples was carried out in a rural property located in the countryside area of the city of Estrela, located in Taquari Valley, in Rio Grande do Sul state (geographical coordinates: 29°28'51"S; 51°54'34"W). The milk sample was collected once, homogenized, stored, and transported under refrigeration (4-7 °C) to avoid its degradation, which may cause an increase in milk acidity (Dias & Antes, 2014).

2.2 Sample preparation and performed analyzes

A 500 mL aliquot of raw milk sample was taken to carry out the analyses of physical-chemical properties and composition, which were the determination of protein, fat, lactose, and mineral content, as well as the total dry extract (TDE) and defatted dry extract (DDE), following the MAPA Normative n° 76 (Brasil, 2018b) and the procedures proposed by Tronco (2003).

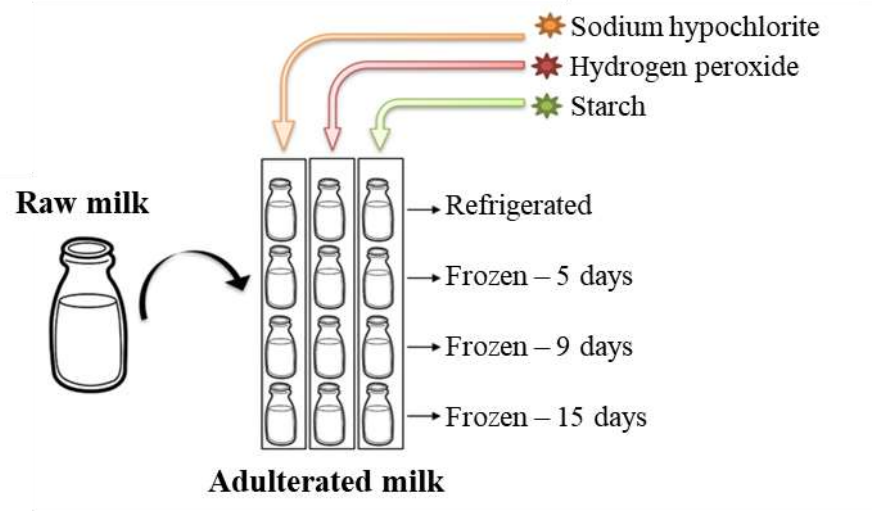
These analyses were carried out to verify whether the sample of raw milk collected was attending to the established quality parameters before adulteration and storage. This practice aimed to guarantee that the milk sample would not have any parameter out of specification, which may cause false results and increase the uncertainties of the analytical procedure.

The rest of the sample was split into twelve bottles of 300 mL, which were intentionally adulterated as follows: four bottles were adulterated with 2.4 mL of sodium hypochlorite 2.25% w/v for the analysis of chlorine/hypochlorite detection (final sample concentration of 0.018% w/v – 180.0 $mg \cdot L^{-1}$); four bottles were adulterated with 0.45 mL of hydrogen peroxide 3.00% v/v for the analysis of peroxide detection (final sample concentration of 0.0045% w/v – 45.0 $\mu L \cdot L^{-1}$); four bottles were

adulterated with 0.06 g of soluble starch for the analysis of starch detection (final sample concentration of 0.02% w/v – 200.0 mg·L^{-1}).

A simplified scheme showing the sample adulteration process and storage conditions of the treated samples is presented in Figure 1.

Figure 1 – Scheme of intentional adulteration and storage of raw milk samples.



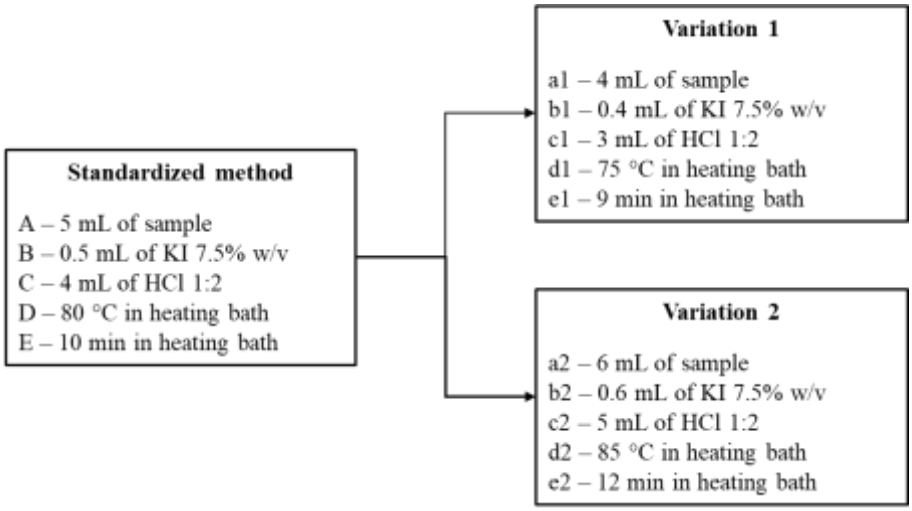
Source: Authors (2021).

After adulteration, the twelve bottles of frauded milk were stored in accordance with the specifications of each treatment. Three bottles were refrigerated (4±2 °C), being analyzed within 4 h after adulteration. Three bottles were frozen for 5 days, three bottles were frozen for 9 days, and three bottles were frozen for 15 days. All frozen samples were kept at temperatures below -18 °C. The frozen samples were defrosted using a heating bath at 25-30 °C.

The analytical procedures for the detection of starch and hydrogen peroxide were carried out following the Manual of Methods of MAPA (Brasil, 2019); the procedure for the detection of chlorine/hypochlorite followed the manual of Instituto Adolfo Lutz (Brasil, 2005). The limits of detection (LOD) of the laboratory were determined as 0.0056% w/v (55.8 mg·L^{-1}) for hypochlorite, 0.0015% v/v (15.0 μL·L^{-1}) for hydrogen peroxide, and 0.02% w/v (200.0 mg·L^{-1}) for starch, respectively.

In Figure 2 are presented the analytical parameters of the chlorine/hypochlorite test in raw milk samples, carrying out two variations (variations 1 and 2). These variations were defined by the authors, varying the parameters above and below the reference values specified in the standardized method, aiming to observe the impact of these parameters on test results. A codification of letters and numbers was used to identify the variations in each combination.

Figure 2 – Analytical parameters and their variations relative to the standardized values for the chlorine/hypochlorite detection test in raw milk samples.



Source: Authors (2021).

The combinations for the tests of hypochlorite detection based on the standardized method, which totaled 15 combinations of variables, are presented in Table 1.

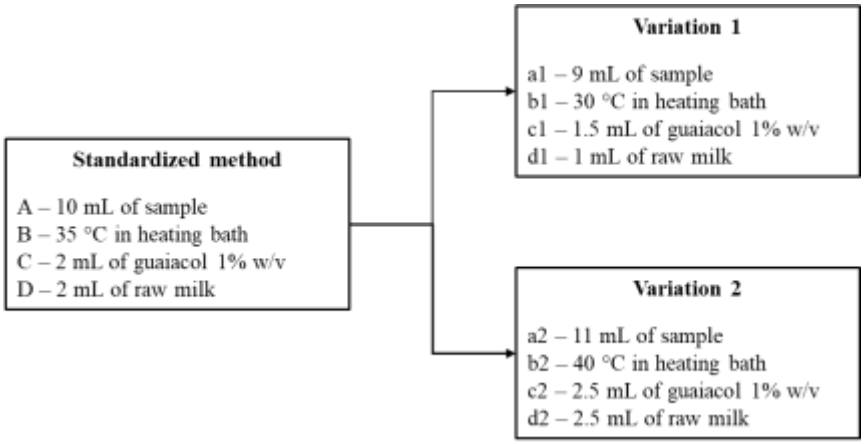
Table 1 – Combinations of varying parameters for the chlorine/hypochlorite detection test in samples of raw milk.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	A	A	A	a1	a1	a1	a1	A	A	A	a2	a2	a2	a2
B	b1	B	b1	B	B	b1	b1	b2	B	b2	B	B	b2	b2
C	C	c1	c1	C	c1	c1	C	C	c2	c2	C	c2	c2	C
D	D	d1	d1	D	d1	d1	D	D	d2	d2	D	d2	d2	D
E	e1	e1	e1	e1	E	e1	E	e2	E	e2	e2	E	e2	E

Source: Authors (2021).

In Figure 3 are presented the parameters and their variations for the hydrogen peroxide detection test, as well as the standardized values.

Figure 3 – Analytical parameters and their variations relative to the standardized values for the hydrogen peroxide detection test in raw milk samples.



Source: Authors (2021).

The fifteen combinations of variables evaluated for the hydrogen peroxide detection test are presented in Table 2.

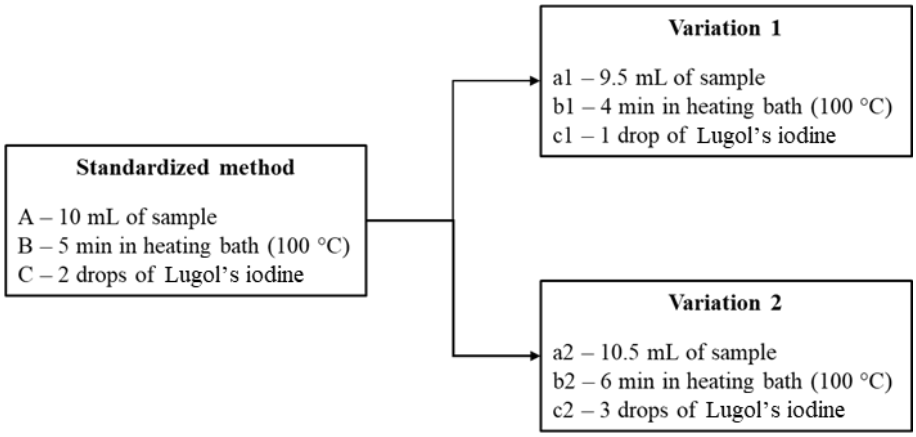
Table 2 – Combinations of varying parameters for the hydrogen peroxide detection test in samples of raw milk.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	A	A	A	a1	a1	a1	a1	A	A	A	a2	a2	a2	a2
B	b1	B	b1	B	B	b1	b1	b2	B	b2	B	B	b2	b2
C	C	c1	c1	C	c1	c1	C	C	c2	c2	C	c2	c2	C
D	D	d1	d1	D	d1	d1	D	D	d2	d2	D	d2	d2	D

Source: Authors (2021).

In Figure 4 are presented the parameters and their variations for the starch detection test in raw milk samples, as well as the standardized values.

Figure 4 – Analytical parameters and their variations relative to the standardized values for the starch detection test in raw milk samples.



Source: Authors (2021).

The fifteen combinations of variables evaluated for the starch detection test are presented in Table 3.

Table 3 – Combinations of varying parameters for the starch detection test in samples of raw milk.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	a1	A	A	a1	a1	a1	A	a2	A	A	a2	a2	a2	A
B	b1	b1	B	B	b1	B	b1	b2	b2	B	B	b2	B	b2
C	c1	C	c1	C	C	c1	c1	c2	C	c2	C	C	c2	c2

Source: Authors (2021).

To evaluate the effect of storage conditions and time on sample stability, all fifteen combinations proposed of the three adulterations were analyzed in the four storage conditions (refrigeration and freezing for 5, 9, and 15 days), in duplicates, totaling 150 assays for each adulteration kind (fifteen combinations of variables and four storage conditions, carried out in two replicates each).

3. Results and Discussion

3.1 Raw milk sample evaluation

Firstly, the physical-chemical parameters and the composition of the raw milk sample were verified; the obtained

results and the acceptance ranges established by law are presented in Table 4.

Table 4 – Physical-chemical parameters and composition of the raw milk used as basis for this study.

Parameter	Unit	Acceptance range	Result	Reference
Composition parameters				
Fat	wt. %	≥ 3.0	3.2	Brasil (2018b)
Defatted total solids (DTS)	wt. %	≥ 8.4	8.8	Brasil (2018b)
Total solids (TS)	wt. %	≥ 11.4	12.0	Brasil (2018b)
Protein	wt. %	≥ 2.9	3.0	Brasil (2018b)
Minerals	wt. %	0.6 to 0.8	0.76	Tronco (2003)
Lactose	wt. %	≥ 4.3	4.8	Brasil (2018b)
Quality parameters				
Density at 15 °C	$g \cdot cm^{-3}$	1.028 to 1.034	1.031	Brasil (2018b)
Titratable acidity*	% w/v	0.14 to 0.18	0.16	Brasil (2018b)
Cryoscopic index**	°H	-0.530 to -0.555	-0.544	Brasil (2018b)

* – titratable acidity informed as equivalent-gram of lactic acid; ** – Horvet degrees (ºH): cryoscopic index, °H = 1.03562 × cryoscopic index in Celsius degrees (Dias; Antes, 2014).
Source: Authors (2021).

According to the results compiled in Table 4, the analyzed milk was within the established standards, being regarded as adequate for consumption. Besides the composition of the milk, some quality parameters were also evaluated, such as titratable acidity, which indicates the preservation state of the sample, the density at 15 °C, which is the relation between the mass and volume of milk and is linked to the chemical composition, and the cryoscopic index, which defines the freezing point of the sample (Dias & Antes, 2014).

It was possible to observe that there was no microbial activity on the milk constituents, such as carbohydrates, proteins, and lipids. If microbial activity occurred, lactose (largest energy source for microorganisms in milk) would be transformed into lactic acid (lactic fermentation), which denatures casein, causing milk coagulation and increasing its acidity (Dias & Antes, 2014).

According to the density value observed, no fraud aiming to increase sample volume was carried out, such as water addition, which reduces milk density through a diluting effect. The cryoscopic index is also evaluated to identify milk adulteration by water addition. The analyzed sample had a result in accordance with the legal standards. Sample freezing temperature is related to the contents of the components that form the total dry extract (TDE). Water addition causes the cryoscopic index to approach the freezing temperature of pure water (Dias & Antes, 2014; Zenebon et al., 2008).

Only after verifying that the milk which would be used as the basis for the study was within the established quality standards the adulterations were carried out, followed by storage and analysis.

3.2 Chlorine/hypochlorite detection test

The results of chlorine/hypochlorite detection test for each storage kind and combination of parameters are presented in Table 5.

Table 5 – Results of chlorine/hypochlorite detection test for the fifteen combinations of variables tested and storage conditions.

Storage conditions	Combination of variables (analytical parameters)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Refrigerated	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Frozen - 5 days	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Frozen - 9 days	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Frozen - 15 days	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

The tests were carried out in two replicates for each sample; there was no difference between each replicate in any test. The qualitative results were presented as '+' when the fraud was detected and '-' when the adulteration was not detected.
Source: Authors (2021).

As can be seen in Table 5, all samples tested positive for the presence of chlorine/hypochlorite, regardless of the storage conditions and the combinations of analytical parameters evaluated.

Gondim et al. (2017), proposing a new spectrophotometric method for the determination of multiresidues of adulterants in milk, commented on the limitations of this strategy due to the low specificity (56.7% for hypochlorite adulterated samples at 200 $mg \cdot L^{-1}$). The same authors commented that, although traditional analytical methods (such as the one evaluated in this work) are costly and take a long time to be carried out, due to the high specificity and absence of interferences from other chemical species, they become preferable for legal oversight and quality control due to their reliability.

On the other hand, Scherer (2015), evaluating the applicability of this test for quantitative analysis, reported that the method has low robustness due to the difficulty in observing the resulting sample coloration. However, this may be mitigated by using specific equipment (colorimeter, spectrophotometer) or employing the method only for qualitative analysis (screening) and using other methods for quantitative analysis.

Silva et al. (2015), analyzing the presence of several adulterants added in amounts commonly found in samples of tampered milk, observed that, even after 48 h of refrigeration, hypochlorite was detected in concentrations in the range of 13.0 $\mu L \cdot L^{-1}$. Nevertheless, the addition of 750 ppm chlorine solution and chlorinated detergent (final concentration in the samples of 0.0013% v/v – 13.0 $\mu L \cdot L^{-1}$) were not detected by the method. The same authors commented that free chlorine is quickly inactivated by organic matter in a catalyzed reaction, which may explain the no detection of the addition of both chlorine solution and the chlorinated detergent.

Thus, regarding the results observed in the present study, it can be considered that the official method for the detection of chlorine/hypochlorite is robust since no result was affected by the different combinations of variables tested during the analytical procedures. It is also important to highlight that the results were not influenced by the storage conditions, being possible to store the samples (up to 15 days), provided that they are kept frozen (≤ -18 °C) until analysis.

3.3 Hydrogen peroxide detection test

The results of hydrogen peroxide detection test in relation to the storage conditions and the combination of the variables of the analytical method tested are presented in Table 6.

Table 6 – Results of hydrogen peroxide detection test for the fifteen combinations of variables tested and storage conditions.

Storage conditions	Combination of variables (analytical parameters)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Refrigerated	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Frozen - 5 days	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Frozen - 9 days	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Frozen - 15 days	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The tests were carried out in two replicates for each sample; there was no difference between each replicate in any test. The qualitative results were presented as '+' when the fraud was detected and '-' when the adulteration was not detected.
Source: Authors (2021).

As seen in Table 6, the presence of hydrogen peroxide was only detected in the refrigerated samples (adulterated and promptly tested). In the frozen samples (≤ -18 °C) that were stored for longer periods (5, 9, and 15 days), it was not possible to detect the presence of hydrogen peroxide.

It is believed that hydrogen peroxide decomposes gradually in the sample, releasing oxygen molecules and water. Thus, this fraud can be detected only when there is a short amount of time between adulteration and analysis, being a fraud whose detection is difficult (Olival & Spexoto, 2004). Scherer (2015) commented that analyte loss may occur more due to volatilization than decomposition, but the frozen samples were not stable, which means that other mechanisms beyond possible volatilization may play a role in not detecting hydrogen peroxide after a short amount of time.

Azad and Ahmed (2016), in a review study, pointed that, for qualitative methods of hydrogen peroxide detection in milk samples, the LOD customarily lies in the range of 0.004 – 0.025% v/v (40-250 $\mu L \cdot L^{-1}$); concentrations below this range may be more prone to generate false-negative results, especially with sample storage (analysis not carried out immediately).

In the study carried out by Silva et al. (2015), it was possible to detect the presence of hydrogen peroxide in samples that were analyzed immediately after adulteration, with a final concentration of peroxide of 0.003% v/v (30 $\mu L \cdot L^{-1}$). However, the same authors reported that it was not possible to detect the substance after 24 h of refrigeration, which indicates a quick decomposition of hydrogen peroxide, preventing the detection of this adulterant by industry or regulatory agencies.

Goulart et al. (2019), evaluating different qualitative methods for the detection of hydrogen peroxide in fresh milk samples, commented on the effect of storage time before analysis on the stability of raw milk samples refrigerated for 24 h. According to the authors, for samples that were adulterated and immediately analyzed, the standardized method detected hydrogen peroxide in concentrations in the range of 0.005% v/v (50 $\mu L \cdot L^{-1}$); for samples analyzed after 30 min after the addition of hydrogen peroxide, the lowest starting concentration detected was 0.010% v/v (100 $\mu L \cdot L^{-1}$); lastly, 24 h after the addition of hydrogen peroxide, only the samples with starting concentrations equal or above 0.5% v/v (5.0 $mL \cdot L^{-1}$) tested positive.

Thus, even freezing is not capable to avoid hydrogen peroxide decomposition, so that analysis must be carried out as soon as possible after sample receipt. It is also important to point out that samples that have small hydrogen peroxide levels (e.g., near the LOD) might, in a matter of hours, present levels of this adulterant that cannot be detected due to the instability of the analyte.

However, it is important to cite that the variation of analytical parameters had no effect on the results, being all adulterations detected in the samples that were adulterated and immediately tested. Thus, the method presented itself as robust, being necessary to have the care of analyzing the samples immediately to mitigate the loss of analyte with time.

3.4 Starch detection test

The results of the starch detection test as a function of the storage conditions and the combinations of variables tested in the analytical procedures are presented in Table 7.

Table 7 – Results of starch detection test for the fifteen combinations of variables tested and storage conditions.

Storage conditions	Combination of variables (analytical parameters)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Refrigerated	+	-	+	-	+	+	-	-	+	+	+	+	+	+	+
Frozen - 5 days	+	-	+	-	+	+	-	-	+	+	+	+	+	+	+
Frozen - 9 days	+	-	+	-	+	+	-	-	+	+	+	+	+	+	+
Frozen - 15 days	+	-	+	-	+	+	-	-	+	+	+	+	+	+	+

The tests were carried out in two replicates for each sample; there was no difference between each replicate in any test. The qualitative results were presented as '+' when the fraud was detected and '-' when the adulteration was not detected.
Source: Authors (2021).

According to the results presented in Table 7, it was possible to verify that starch addition was not detected in combinations 2, 4, 7, and 8. In these combinations, only one drop of Lugol's iodine solution (c1) was used. It is believed that only one drop of this reagent may be insufficient since the other combinations, in which the fraud was detected, two and three drops of Lugol's iodine were used. Probably, a smaller amount of Lugol's iodine prevented the detection of starch using the proposed method.

Holló and Szejtli (1957) and Figueira and Rocha (2013) commented that the reaction between triiodide ion (I_3^-) and the helicoidal chains of starch follows a stoichiometric pattern, where a minimum amount of triiodide must de adsorbed to starch chains for the iodine-starch complex (which has a characteristic purple color) to be formed; additional iodine is also adsorbed in starch chains up to saturation, not interfering with the already formed complexes. Thus, very probably, one drop of Lugol's iodine has not supplied enough triiodide ions to form the blue complex that results from the adsorption reaction of iodine with starch chains.

Regarding method sensibility, Azad and Ahmed (2016) and Pradeep et al. (2016) reported that starch detection methods based on the action of iodine as an indicator (Lugol's iodine and derivatives) have LOD values in the range of 0.02-0.04% m/v (200-400 $mg \cdot L^{-1}$). This reference range was evaluated in the present study, the tested samples had a final starch concentration of 200 $mg \cdot L^{-1}$ (very close to LOD), aiming to verify the robustness of the method in smaller degrees of adulteration.

Despite the influence of the amount of Lugol's iodine, on the other hand, the other analytical parameters had no effect on the results, indicating that the method is robust for other variations of the analytical procedure, being necessary to have additional care with the amount of Lugol's iodine used in the test.

Relative to storage conditions, it is possible to observe that they had no effect on the results, being the adulteration detected in all samples analyzed with two or three drops of Lugol's iodine solution, regardless of storage time. Gondim et al. (2016), evaluating different storage times (zero up to 48 h) and temperatures (4, 30, and 40 °C) of milk samples adulterated with starch, reported that storage time and conditions had no influence on starch stability, indicating that this analyte remains stable in the matrix (milk).

4. Conclusion

The chlorine/hypochlorite detection method was not affected by variations in the analytical procedure, neither the storage conditions, being the adulteration detected in all combinations of variables and all storage forms. This indicates that the method was adequate and may be used for samples stored under freezing. The hydrogen peroxide detection test only yielded correct results (adulterated samples) for the samples stored under refrigeration (and immediately analyzed), not being capable to detect the fraud in the frozen samples, regardless of the storage time. Thus, the test for the detection of hydrogen peroxide must be carried out immediately after the sample arrives, aiming to avoid false-negative results. The starch detection test was

the only one that was influenced by variations in the analytical procedure, where the combinations that used only one drop of Lugol's iodine yielded false-negative results. Storage conditions did not influence the results, being the adulteration detected in all samples that were analyzed using two or three drops of Lugol's iodine. Therefore, the amount of Lugol's iodine added to the sample is a critical factor for the starch detection test in raw milk, being necessary to have additional care with this analytical parameter. It is recommended to increase the volume of Lugol's iodine added to the sample, aiming to mitigate the possibility of false-negative results due to the addition of insufficient amounts of this reagent.

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