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ARTICLE

Development of a Green Methodology for the Determination of Artisanal Danbo Cheese Quality Parameters

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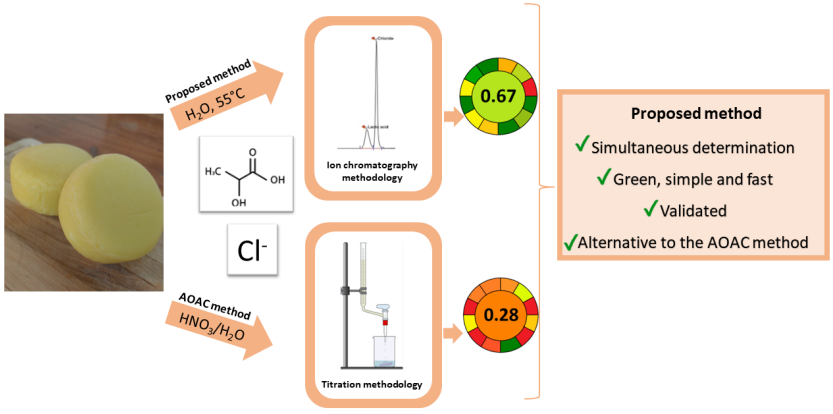

A simple and fast method for the simultaneous determination of chloride and lactic acid in artisanal Danbo cheese was developed and validated. The proposed methodology combined a sample treatment using only ultrapure water as an extracting agent and ion chromatography with suppressed conductivity for the detection of the analytes. One gram of sample exactly weighted is added to 40.0 mL of ultrapure water at 55 ºC. The suspension was stirred for 5 min and after centrifugation the supernatant obtained is ready to be used for the determination of both analytes. The performance of this method was evaluated by comparing the results obtained with those acquired after the analysis of the cheese samples using the AOAC reference methods using a *t*-student test of mean concentration values. At a 95% significance level, the means obtained by the different methods were comparable. Analytical precision expressed as relative standard deviation (RSD %) was less than 6. Artisanal cheese samples were analyzed (n=12) using the validated methodology, obtaining chloride levels between 0.6 and 1.1% w w^{-1} expressed in sodium chloride and levels of lactic acid between 1.1 and 2.1% w w^{-1}. Additionally, a greenness analysis was presented, using three different metric tools, concluding that the developed method is significantly in better agreement with the principles of Green Analytical Chemistry than the official methods.

Keywords: ion chromatography, chloride, lactic acid, cheese, green chemistry

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INTRODUCTION

Artisanal cheese can be defined as cheese made with raw, pasteurized, or thermalized milk, produced by a natural or legal person who makes artisanal cheese individually, family, or associative. The different artisanal cheeses can be characterized by their appearance in terms of composition and sensory features. Danbo or Dambo cheese is a semi-hard and matured firm cheese, which develops its characteristics of flavor and body during at least 2-3 weeks of maturation. This type of cheese is originally from Denmark, but its composition has been changed in Uruguay. Nowadays, Danbo cheese is one of the most exported cheeses elaborated in Uruguay.[1,2]

The determination of chloride (Cl^-) is relevant for quality controls in the production of artisanal cheeses. Chloride is added in the cheese preparation using different salts as calcium chloride, to incorporate calcium externally to achieve adequate curdling or as sodium chloride (NaCl) in the salting process. These aggregates, especially the NaCl solution in the salting process, promote the correct elaboration of the cheese, to achieve adequate curdling or the proper rind formation. Also, microbial growth, various enzyme activities, syneresis of the curd and reduction of moisture, and changes in texture and conformation are controlled by them.[3]

Lactic acid is normally present in cheese due to the fermentation activity of lactic acid bacteria.[4] The fermentation of lactose, through hydrolysis via different biochemical routes of sugar transport and utilization, produces lactic acid and acetic acid. Those acids play an important role in acidification during cheese making and during the maturation it could control the growth of microorganisms that can cause food poisoning.[5]

To determine the composition of cheese, official methods are listed under the guidelines of the AOAC (formerly Association of Official Analytical Chemists).[6] Chloride levels are determined by a potentiometric method and lactic acid by a titrimetric method as reference methodologies. However, different alternatives to determine chloride concentration were previously developed, for example ion electrode method,[7] high-performance liquid chromatography technique (HPLC),[8] conductometric technique,[9] and ion exchange chromatography.[10]

The reference methodology (AOAC) determines lactic acid by a titrimetric method as acidity in cheese.[6] However, recent works have proved the application of HPLC to determine lactic acid in cheese.[4,5,8]

Ion chromatography is an indispensable tool to separate complex mixtures of anions and cations and individual ions can be quantitatively measured in a short time.[11] Previous works showed the versatility of the technique for different analytical determinations in cheese and dairy products, referring to simultaneous analysis of various inorganic and organic analytes.[12-14]

Reference analytical methods are being questioned form the point of view of the environmental protection. Thus, are constantly in evaluation and new alternative methods are emerging. The knowledge of the consequences that some sample preparation practices can bring to the environment has led many analytical chemists to better review critical parameters in the development of new methods. Green Analytical Chemistry (GAC) is a statement that seeks to eliminate or minimize the environmental impact caused by the execution of analytical processes.[15] Thus, over the years, several metrics have been developed to evaluate this impact.[16-18] To compare the developed method with the official ones from the point of view of greenness using the GAC, Eco-sale, GAPI, and AGREE metrics.

Eco-scale

In the 1990s, Anastas, stated in the definition of green chemistry that the principal objectives of green chemistry are the reduction and elimination of the use and the generation of hazardous substances.[19] An approach to evaluating the greenness of analytical methods was proposed by Galuska (2012) comprehensive tool for semi-quantitative evaluation of analytical methodologies that will make possible comparisons and selection of the greenest alternative and will enable the greenness of new or modified methods to be tested. Although it is a semi-quantitative metric, it allows us to make a first observation of the greener of our method. Nowadays the Eco-scale is still used as a GAC metric.[19-21] It consists on

following the steps of the developed method and penalizing it with a certain number of points. Penalty points depend on various factors such as hazards, reagents, waste, and energy, among others, which are not following the GAC. The Eco-scale score is calculated as 100- penalty points. The Eco-scale ranking corresponds to >75 points, which represents an excellent green analysis; 50> represents an acceptable green analysis, and <50 represents an inadequate green analysis.[17]

Green Analytical Procedure Index (GAPI)

The GAPI index uses a specific symbol with five pentagons. It allows for evaluating and quantifying, from green to yellow and red, the environmental impact. Each pentagon represents a different aspect of the analytical procedure carried out. In this way, the environmental impact may be evaluated in each step of the methodology.[16] This is a qualitative method that allows evaluation of which aspect of the methodology is more committed to the environment. Nowadays, this is one of the metric tools often used for this purpose.[21-25]

Analytical GREEnness

Analytical GREEnnes is a metric tool that allows evaluating the greenness of analytical methods based on the SIGNIFICANCE principles, proposed by Galuzkca (2013) and considered the principles of the GAC.[26] The evaluation of the analytical procedure consists of the evaluation of the 12 principles individually; each of them has a way of being evaluated that varies according to the principle evaluated. The method uses a system of colors and numbers, where the color green and the number 1 represent a complete approximation of the GAC. AGREE is an easy metric to interpret since the visualization in colors allows us to evaluate in which steps our method must improve to align with the GAC approach. In addition, it allows the numerical evaluation of the different methods, which allows better differentiation between them.[18] This metric tool, developed in 2020 by Pena-Pereira et al, is currently widely used for the greenness assessment of analytical methodologies.[21,22,24]

Due to the necessity of the determination of quality parameters in cheese, such as chloride and lactic acid levels, is important to have adequate methodologies to determine them. Reference methods usually involve long-term classical methodologies which are difficult to automate or require toxic or corrosive reagents that need special disposal.

Herein a simple analytical method for the extraction of Cl^- and lactic acid from cheese samples for its simultaneous determination by ion chromatography is proposed. Furthermore, a critical greenness profile is discussed, comparing results using different tools.

MATERIALS AND METHODS

Reagents

For potentiometric titration, $AgNO_3$ (Merck, Darmstadt, Germany) was dissolved using ultrapure water. For acidity titration, NaOH 99% (Mallinckrodt, Xalostoc, Mexico) was diluted in ultrapure water and standardized using $H_2C_2O_4.2H_2O$ (Mallinckrodt, Xalostoc, Mexico). The standardization of $AgNO_3$ and the Cl^- solutions for the calibration curve were prepared using NaCl 99% (Merck, Darmstadt, Germany). A standard solution for the calibration curve of lactic acid was prepared using $C_3H_6O_3$ (Merck, Darmstadt, Germany). For acid extractions, diluted HNO_3 was prepared from the reagent 67% w w^{-1} (Merck, Darmstadt, Germany). All reagents used were of analytical grade and ultrapure water was used (18.2 MΩ cm ASTM type 1) obtained from a Millipore™ DirectQ3 UV water purification system (Bedford, Massachusetts, USA).

Samples

Twelve samples of semi-hard cheeses Danbo type were analyzed. Samples were elaborated artisanally using bovine pasteurized milk with washed dough. The cheesemaking procedure was carried out in a cheese experimental plant at the Veterinary Faculty, Universidad de la República, Uruguay. The samples were elaborated in two groups during 3 days for each one. Firstly, the milk was collected during the

morning milking (5 a.m.) and the cheeses were made on the same day. The whole procedure consisted in transferring 10 L of milk collected from individual cows to a small stainless steel cheese tub. The milk was filtered and pasteurized at low temperature long-time pasteurization (63 °C, 30 min). Then, the milk was cooled to 33 °C and the external ingredients were added as follows: 4 mL of $CaCl_2$ 35% w v^{-1}, 1 g of $NaNO_3$, and the mesophilic/thermophilic starter culture (Lyofast MOS 062D - Sacco, Italy). After 30 min of incubation, the rennet (100% chymosin) was added. Flocculation occurred in 30 min approximately and the curd was cut using a curd-cutter at a size of 10 mm. The whey was partially removed (30%) and 1.5 L of the hot water (68 °C) was added for scalding. The curd was cooked at 41-42 °C for 15min. After that, the whey was removed completely and the cheese blocks were formed (~250 g each) and pressed for 3 h. Finally, the cheeses were salted by immersion into 20% NaCl for 40 min, vacuum packed, and ripened for 90 days.

Sample preparation

The rind of the cheese was removed using a knife. Then, samples were grated individually, using a 5 mm hole diameter cheese grater. Finally, a blade mill was used to further reduce the particle size. Samples were stored at -18 ºC in polypropylene tubes until analysis.

Chloride potentiometric method

The extraction of Cl^- for its subsequent determination by potentiometry was carried out following the AOAC guidelines (AOAC 983.14). Briefly, 2 g of sample was weighed in a Bohemian glass and 40 mL of ultrapure water at 55 ºC was added. Then 2 mL of a mixture of HNO_3:H_2O (1:3) was added and stirred for 5 minutes using a magnetic bar.

Acidity titration

To determine the acidity, an extraction using ultrapure water was carried out according to the AOAC guidelines (AOAC 920.124). Two grams of cheese were weighed in Bohemian glass. Twenty millilitres of water were added and the suspension was stirred with a magnetic bar for 5 minutes. After that, the titration was executed.

Proposed method: ion chromatography

For the extraction, 1 g of the sample was exactly weighed, and 40 mL of ultrapure water was added at 55 ºC. Then the suspension was stirred for 5 minutes. After that, samples were centrifuged for 10 minutes with 1903 *g*. The supernatant was placed in 10 mL vials (Polyvials, ThermoScientific, Massachusetts, USA) in the auto-sampler, and measurement was carried out.

Analytical determinations

Potentiometry

The determination of Cl^- was carried out according to AOAC recommendations. The titrant $AgNO_3$ was previously standardized with NaCl. A working silver electrode (Ag wire) and a double junction Ag/AgCl reference electrode (ThermoScientific, Massachusetts, USA) were used for the determinations. Once the acid extraction was completed, the titration was carried out and the expenditure at the endpoint was carried out using the second derivative method. Finally, the result was expressed in Cl^- % (w w^{-1}) (AOAC 983.14).

Acidity titration

The titrant was NaOH 0.1 mol L^{-1}, prepared as mentioned above and titrated with $C_2H_2O_4.2H_2O$. Once the extraction was carried out, the titration was performed using phenolphthalein and the result is expressed as g lactic acid / 100 g.

Ion chromatography experimental conditions

Chloride and lactic acid were determined using a reagent-free system ion chromatograph (RFIC™) Dionex 5000+ (Thermo Scientific, Massachusetts, USA). The chromatographic separation was performed with an analytical anionic exchange column IonPac™ AS20 (250 mm × 2 mm, Thermo Scientific, Massachusetts, USA) and IonPac™ AG20 guard column (50 mm × 4 mm, Thermo Scientific, Massachusetts, USA) held at 35 ºC. The particle size of the stationary phase was 7.5 µm. The mobile phase consisted of KOH 20 mmol L^{-1} at 1 mL min^{-1} flow and isocratic elution was applied. The generator of the KOH was included in the equipment (reagent-free system). An anion electrolytically-regenerated suppressor (AERS 500, 4 mm) was used for conductivity detection at 35 ºC. The injection volume was 25 µL. The software for data analysis was Chromaleon Data System 7. Finally, the total time for the separation and quantification of both analytes was 10 minutes.

Validation

Once the chromatographic critical variables were established, the validation was performed according to the recommendations of the Eurachem Guide.[27] The figures of merit evaluated were: precision, trueness, linear range, limit of detection (LOD), and limit of quantification (LOQ). Results obtained with this method were compared with those obtained by applying the AOAC standards methods using a *t*-student test of mean values.[28]

RESULTS AND DISCUSSION

Optimization

In the ion chromatography technique, the conditions must be optimized to have an adequate eluent concentration to promote ion exchange with the column, but at the same time, the lowest possible concentration to reduce noise. In addition to optimize eluent concentration, column temperature must be controlled to control baseline noise.[11] The chromatographic conditions were selected based on the basic parameters that describe the chromatographic efficiency. In this sense, resolution, number of theoretical plates, and signal-to-noise ratio, must be evaluated to establish experimental conditions.

Herein, the optimized parameters were the mobile phase concentration and flow, to obtain the best resolution, number of theoretical plates, signal-to-noise ratio and, resolution between the analytes of interest with the shortest possible time. After these studies the optimal conditions were obtained in 10 minutes of runtime with 1 mL min^{-1} flow of 10 mmol L^{-1} eluent concentration (KOH). Resolution was higher than 2.20, number of theoretical plates were between 1165 and 9558 for lactic acid and chloride respectively, and finally a suitable signal-to-noise ratio was higher than the theoretical criteria (lactic acid S/N: 1165, chloride S/N: 28183). The determination of chloride and lactic acid using ion chromatography, presented retention times of 4.60 and 3.91 min respectively. Similar retention times were reported in previous works for dairy product matrices.[10] Figure 1 presents the obtained chromatogram. The short runtime for the separation of both analytes presented an advantage over the reference methods considering that those methods involve individual batch analysis. The proposed methodology allows the simultaneous determination of both analytes in the same runtime with minimal use of reagents.

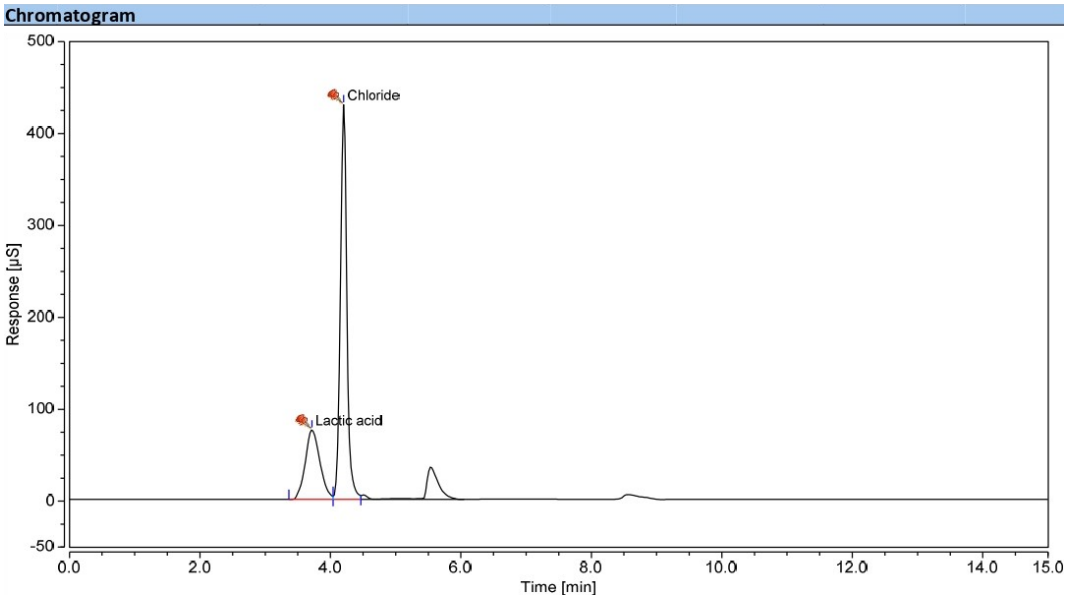


Figure 1. Ion chromatogram of the analyzed compounds: lactic acid (1.9% w w^{-1}) and chloride (0.8% w w^{-1}) in artisanal cheese sample using the developed method.

Validation

Linearity was evaluated for each analyte by constructing five-point calibration curves and the blank. Good results were obtained by evaluating visual inspection and determination coefficients (R^2) greater than 0.995. To characterize the method, limits of detection and quantification were calculated for each parameter as 3*s*/b and 10*s*/b respectively, where *s* was the standard deviation of 10 replicates of a low-concentration solution (1 mg L^{-1}) and b was the slope of the calibration curve. Precision was evaluated as repeatability expressed as relative standard deviation (RSD%) of the analysis of 6 replicates, was less than 6% for all the analytes and was considered suitable for the application. For trueness evaluation, three spiked samples were analyzed, and the percentage of recovery for both analytes was appropriate. Figures of merit are presented in Table I. These parameters were similar to those presented by Huang (2023). [29]

Table I. Figures of merit of the proposed method

Parameter	Chloride	Lactic acid
Linear range (mg L^{-1})	0.92 – 250	0.60 – 250
LOD (mg L^{-1} / % w/w)	0.28 / 0.0018	0.18 / 0.0004
LOQ (mg L^{-1} / % w/w)	0.92 / 0.0061	0.60 / 0.0012
Precision (RSD%, n=6)	5.2	5.9
Trueness (R%, n=3)	88 – 105	85 – 109

LOD / LOQ (mg L^{-1}) instrumental limits – LOD / LOQ (% w w^{-1}) method limits.
Spike concentration: Chloride 25 mg L^{-1}; Lactic acid 65 mg L^{-1}.

The performance of this method was also evaluated by comparing the obtained results with those acquired after the analysis of the cheese samples using the AOAC methods using a *t*-student test of mean values. The means obtained by the different methods were comparable since they did not differ significantly at a significance level of 95%. Results are presented in Table II.

Table II. Comparison between the developed method and the corresponding reference method, by Student's *t*-test

	Chloride (% w w^{-1})		Lactic acid (% w w^{-1})	
	Ion chromatography	Potentiometric method	Ion chromatography	acid-basic titration
Average	0.86	0.90	1.77	1.62
$t_{experimental}$	1.80		1.53	
$t_{(0.05, 5)}$	2.09		2.09	

Analysis of artisanal cheese samples

Figure 2 presents the levels of chloride and lactic acid in artisanal Danbo cheeses, applied to the analysis of 12 samples using the validated methodology.

Chloride levels were between 0.65 and 1.08% w w^{-1} expressed as sodium chloride. These results are in agreement with those presented before.[30,31] Chloride is incorporated into cheesemaking in the form of sodium chloride through immersion in a brine bath. In addition to the nutritional contribution, the addition of Cl^- modifies the hydration of proteins and participates in the development of the cortex. It also acts in the development of microorganisms and enzyme activity. Finally, it adds flavor to the cheese.[10,31] Furthermore, the Cl^- content coupled with moisture could alter the cheese's chemical composition.[32]

The amount of Cl^- is usually variable between the different types of cheese. But the role of Cl^- in cheese is important its determination, since composition, enzymology, water content, and lipolysis among other factors are regulated by the content of NaCl in the cheese-making process.[21]

On the other hand, the analysis of the artisanally made Danbo cheeses presented levels of lactic acid between 1.1 and 2.1% w w^{-1}. Lactic acid is normally present in cheese, due to the fermentation process. Its presence contributes to the pH of the cheese, which is important to the characteristics of the cheese, flavor, and microorganism control. The obtained results were in accordance with data presented before by Cuffia (2017) in cheeses with the same time of maturation (90 days), and also, presented similar levels with other types of cheese with a lower time of maturation process but similar production process.[4,5,34] The levels of lactic acid could be variable depending on the type of cheese, but the interest in the determination is relevant due to its role in the maturation process of the cheese.

To the best of our knowledge, there are no reported data about the Cl^- and lactic acid levels of artisanal Danbo cheese in Uruguay. This work contributes to establishing the levels of Cl^- and lactic acid for the evaluation of artisanal cheese parameters.

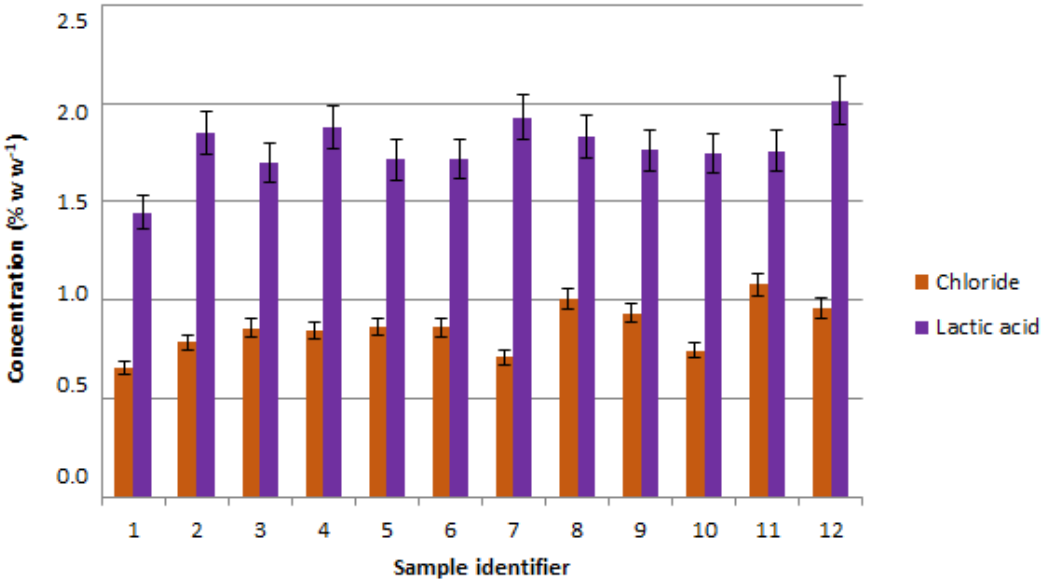


Figure 2. Levels of chloride and lactic acid in the analyzed artisanal cheese samples.

Greenness analysis

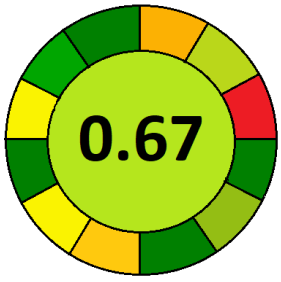
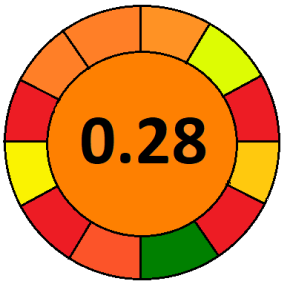
Ion chromatography has been reported and evaluated as a green analytical technique. Due to its efficiency, since the analysis times are short, the number of analytes that can be determined is high and simultaneous. In addition, the type of sample preparation methods, reagents used and waste generated tend to be more aligned with GACs, as well as energy costs.[35-37] Furthermore, the use of water as an extracting reagent enhances the greener aspect of the methodology.[38] Table III presents the greenness evaluation metrics resulting from the analysis of the proposed method and the reference methodologies.

Using the Eco-scale metric, the developed method and the officials are above 75, which represent excellent green analysis. Even though the chromatographic method has fewer penalty points, using this metric there is no difference between the methods. We can evidence that the Eco-scale is a useful but limited tool since it does not allow better differentiation between the different methods.

In GAPI, the first pentagon, the sample preparation, preservation, collection, and storage are the same in both cases. Then, the other two, corresponding to sample preparation and reagents and compounds are greener in the developed method. This is predictable since our method consists of hot water extraction only. However, we must be capable of making it greener. In the instrumental part, we can still work to make a greener method, for example, decrease water extraction. This tool is more efficient to evaluate the greenness of the different methods than Eco-scale. Also, it is useful to evaluate the most critical parts of the methodologies in terms of GAC.

Finally, AGREE metrics reinforce that the proposed method is the greenest. This methodology is easier to visualize, not only by the colors but also by the overall score. It provides more information than the other two metrics because it has more categories in each step of the metric system.[16-18] Using AGREE is possible to notice that the main problem of the developed method is that it is an offline method.[31] The use of AGREE as a green metric system provides more specific information and allows us to visualize the differences between methods, better than the Eco-scale and GAPI metrics.

Table III. Greenness evaluation metrics result from the analysis of the proposed method and the reference methodologies

Method	Eco-scale		GAPI	AGREE
Proposed method (ion chromatography)	**Reagents**	**PP**		
	KOH (10 mL)	2		
	Instruments			
	IC	0		
	Magnetic stirrer	0		
	Centrifugation	0		
	Occupational hazard	3		
	Waste (>10 mL)	5		
	Total PPs	10		
	Eco-scale	90		
Potentiometric method for chloride determination	**Reagents**	**PP**		
	HNO_3	4		
	$AgNO_3$	2		
	NaCl	0		
	Instruments			
	Magnetic stirrer	0		
	Voltimeter	0		
	Occupational hazard	3		
	Waste (>10 mL)	6		
	Total PPs	15		
	Eco-scale	85		
Titration method for lactic acid determination	**Reagents**	**PP**		
	NaOH (10 mL)	2		
	$C_2H_2O_4.2H_2O$	2		
	Phenolphthalein	1		
	Instruments			
	Energy	0		
	Occupational hazard	3		
	Waste (>10 mL)	6		
	Total PPs	14		
	Eco-scale	86		

PP: penalty points; IC: ion chromatography.

CONCLUSIONS

A simple analytical method for the simultaneous determination of chloride and lactic acid in cheese by means of ion chromatography was developed and validated. This method was adequate for the proposed purposes, being also very simple, fast, and with only water as an extracting agent.

Three different metric tools were applied for the evaluation of the methodologies, being the AGREE the most useful and complete to compare them since it provides more information about the strengths and weakness of the methodologies from the green point of view.

Finally, this can be postulated as an alternative protocol in better agreement with the principles of Green Analytical Chemistry, compared to the classical reference methods for food analysis.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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