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Polarized light microscopy guarantees the use of autochthonous wheat in the production of flour for the Protected Geographical Indication 'Galician Bread'

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A R T I C L E I N F O

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A B S T R A C T

'Galician Bread' is a traditional baked product and a national benchmark that has recently been granted the European mark of Protected Geographical Indication (PGI), which requires at least 25% of the flour to be produced from autochthonous varieties such as 'Caaveiro'. The objective of this work was to find a method that guarantees the presence and the percentage of 'Caaveiro' wheat in blended flours by using microscopy techniques.

Using optical microscopy, including bright-field and polarizing microscopy, autochthonous and foreign flours were analyzed and compared. 'Caaveiro' starch presented a different birefringence pattern (associated with a higher amount of amylose) with respect to other cultivars used to produce flours, a feature used to make a computation of the two starch granule types in the mixtures of 'Caaveiro' with foreign flours. Repetitions with different mixture percentages allowed us to develop a mathematical model to estimate the percentage of 'Caaveiro' flour present in the mixture. Firstly, the most effective method for preparing samples was determined by ensuring the homogeneity of the samples and, subsequently, a validation was carried out with blind samples.

Starch birefringence properties allowed the detection of 'Caaveiro' wheat flour in mixtures with foreign/Castilian wheat flours and to determine the percentages used in the flour mixtures applying a calibration line ($R^2 = 0.9577$). Deviations were due to the difficulty in obtaining precise mixtures of the blended flours, as happened with other Simple Sequence Repeat (SSR)-based methods used in the same samples. This is a novel method for detecting contraventions/infractions of the percentage of 'Caaveiro' used in wheat flours, which is simple, effective and inexpensive.

1. Introduction

'Galician Bread' is a traditional bread from Galicia (NW Spain) which has recently obtained the European mark of Protected Geographical Indication (PGI) (European Commission, 2019). This mark certifies that this traditional bread is made from common wheat flour (*Triticum aestivum* L.), and that at least 25% of this flour comes from Galician autochthonous varieties of wheat ('Caaveiro' and/or 'Callobre'). This wheat differs from other varieties because it has a high percentage of protein, but with a medium-low strength as well as a darker colour, resulting in a bread of greater intensity of aromas and flavour.

In addition to autochthonous flour, it is characterized by the use of sourdough to obtain very hydrated breads, and for its preparation, which requires long fermentations (minimum 3 h) and to be baked in stone ovens. The use of autochthonous varieties, as well as its elaboration process, make the 'Galician Bread' a reference of quality at national level (Câmara-Salim et al., 2020; Estévez-López et al., 2021; García-Gómez et al., 2022).

Currently, there are initiatives to recover autochthonous varieties, as is happening in Galicia with the varieties 'Caaveiro' and 'Callobre'

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(Royo et al., 2016). Promoting the recovery of these crops is of great importance since they are more resistant to the propagation of pathogens, they are better adapted to the environment and they require fewer inputs, also avoiding the loss of genetic diversity and recovering traditional agricultural practices (Varia et al., 2021; Zamaratskaia et al., 2021). Furthermore, consumers are increasingly interested in local products and are more concerned about sustainability throughout the entire production chain (Johansson et al., 2021). The production methods for these local products are often oriented to obtain products covered by different quality denominations that guarantee their exclusivity and differentiated quality (García-Gómez et al., 2022).

EU labelling rules protect consumers (Melini & Melini, 2019). In general terms, the application of the Protected Designation of Origin (PDO) and PGI logos on food packaging serves as a sign of quality and as a certification mark of the food product authenticity. This implies that: i) the product complies with the legislation (origin and production method), ii) it has the necessary composition for the legal name, and iii) no adulteration or substitution with cheaper, but similar ingredients occurs.

The objective of these EU marks is to protect the reputation of regional foods and promote good practices in rural and agricultural activity. These actions help growers and manufacturers to set better prices for authentic products and reduce unfair and misleading competition of non-original products, usually of lower quality and/or different flavour (European Parliament and European Council, 2012).

Since protected products (PDO, PGI) fetch higher prices than the corresponding standard products, there is a high risk of fraudulent labelling, unfair and misleading product competition and/or partial substitution of the ingredients of the protected product by other raw materials of inferior quality (Cervellieri et al., 2022; Melini & Melini, 2019). Hence, the demand for tools to authenticate and trace agri-food products has significantly increased in the last decades (Fanelli et al., 2021).

A wide variety of methods for food traceability and authentication have been developed and tested to date. Each method can provide specific information on the composition and characteristics of food, such as geographic origin, the presence of adulterants or the species or varieties used in the production process (Fanelli et al., 2021). To reveal the fraudulent use of food or species, several methods (including techniques and/or combinations of different techniques) are often employed, such as chromatography, spectrometry and spectroscopy, among others. These techniques are based on the chemical separation of similar compounds in complex food. Other methods, such as molecular biology, focus on DNA and proteins, allowing to distinguish the species and varieties used to produce a specific food (Ballin & Laursen, 2019; Dimitrakopoulou & Vantarakis, 2021). Even the use of sensory analysis has become important in many food sectors, since appearance, aroma, flavour and texture properties are important characteristics determining the quality-authenticity of food products (Danezis et al., 2016).

Another method indispensable in detecting foreign matter and adulterants is microscopic evaluation (Alamgir, 2017), including several levels of magnification, from optical to electron microscopy. Microscopy is very useful due to its capability to detect macromolecules (including fat, proteins, starch …) as well as structural features (such as different cell types and morphological characteristics) (Ballin & Laursen, 2019).

Nowadays, microscopy is routinely used in the evaluation and authentication of plant-based pharmaceutical drugs and to verify the presence/absence of adulterations (Kumar et al., 2011; Li et al., 2012; Li & Zhang, 2008; Wang et al., 2011), despite other sophisticated modern research tools. The microscopic method is still one of the simplest and most inexpensive methods to confirm the correct identity of the source materials (Kumar et al., 2011). For example, starch grains constitute an important diagnostic character in the examination of powdered vegetable as components of pharmaceutical drugs. Microscopic evaluation of botanical drugs may be used for both qualitative and quantitative determinations (for example stomatal number, stomatal index, palisade ratio, vein-islet number, vein termination number, among others, of drug components derived from leaves) (Alamgir, 2017).

In addition to its use in pharmacology, microscopy is also used in food analysis. Regarding foods of animal origin, histological methods allow the recognition of the components of meat products, so they also serve to detect fraud. Microscopy is used to detect adulterations in meat products (such as minced meat, kofta sausage, beef burger or meat sandwich products) with animal tissue including bone, heart, elastic, and degenerated skeletal muscle tissue and food additives, which are not allowed in these foods (Abd-Elhafeez et al., 2022). The use of different histological techniques is an efficient methodology for the qualitative evaluation of various meat products, and they could be used as an accurate technique for quality control (Abdel-Hafeez et al., 2016). Additionally, microscopic and morphometric techniques are suggested as effective methods for meat quantitative and qualitative estimation in meat products (Sadeghinezhad et al., 2015).

Light microscopy is the only official method for the detection and characterization of processed animal proteins (PAPs) in feed in the European Union (with the most severe restrictions), since the PAPs of mammalian and avian origin are prohibited in all animal feed, except for pets. The use of a microscope allows to detect the presence of constituents of animal origin in feed at the level of 1 g/kg with hardly any false negatives (Liu et al., 2011). Light microscopy also proved useful in detecting the presence of fishmeal in feed for ruminants, not being allowed, except for calves feed (Van Raamsdonk et al., 2017). In addition, it can distinguish between a single therapeutic dose of a tetracycline (permitted under the standards) and both multiple therapeutic dosing and prophylactic dosing (not permitted) in organic meat (Kelly et al., 2006).

Regarding products of vegetal origin, microscopy is currently the main technique used to control the quality of Brazilian coffee (ABIC, 2018). Fraud includes the trading of coffee with the addition of low-cost materials and/or the presence of defective beans, and even low-quality or beans from different geographical regions to that reported in the product label (Martins et al., 2018). These methods are based on the use of optical microscopy and scanning electron microscopy (SEM) that provides visual information of the surface. This analysis is based on three stages: sample degreasing, filtration to remove fine particles, and image processing (Ferreira et al., 2021). Horn and Häser (2016) were able to recognize adulteration of Bamboo tea by developing an anatomic diagnostic key for the differentiation of bamboo, lemongrass and carnation, using available markers after a simple procedure.

Regarding cereals, microscopic techniques are efficient in identifying species in archaeological studies based on the morphological characteristics of starch granules (Aceituno-Bocanegra & López-Sáez, 2012; Piperno & Dillehay, 2008). However, to date, there is no evidence that microscopic techniques have been applied to traceability studies. Therefore, the aim of this work was to develop a microscopic technique to verify the presence of ‘Caaveiro’ flour in a mixture and to quantify its proportion, guaranteeing 25% of ‘Caaveiro’, the minimum quantity required in ‘Galician Bread’.

2. Materials and methods

2.1. Flour samples

Two types of flour were used, ‘Caaveiro’ flour and a commercial wheat flour obtained from mixtures of various wheat cultivars used in Galicia (Castilla flour). The autochthonous flour was ground in a stone mill while the foreign flour was ground in an electric mill. The samples were provided by Da Cunha Group.

2.2. Sample preparation

On a microscope slide, a sample of flour was sprinkled with a spatula according to Tapia et al. (2012). To ensure adherence, a drop of a diluted gelatin solution was added with a pipette and allowed to dry at room temperature. However, accumulations of starch granules were formed, so it was necessary to improve homogenization.

Different methods of sample preparation were devised, and 18 dispersion methods were evaluated and compared to selected the best one: a) flour mixed with glycerol at different concentrations (100% glycerol and 50% glycerol) according to Cai and Wei (2013) and Wang et al. (2018); b) flour mixed with 50% ethanol solution simplifying the processing of samples used by Yang et al. (2017) and Zhang et al. (2012); c) flour mixed with surfactant (dishwashing detergent) at different concentrations (0.1%, 0.5%, 1%, 10% and 20%) simplifying the method used by Wang and Wang (2004), in which they combined surfactants and high-intensity ultrasound for the isolation of rice starch; d) 10 mg of flour were weighed, introduced into an eppendorf and 1 mL of gelatin solution was added and mixed homogeneously on a vortex mixer at different speeds (15 Hz and 25 Hz) and time lapses (3 min, 5 min, 10 min, 18 min and 28 min) based on Patel and Seetharaman's (2006) method. Then, 200 μL of the suspension was distributed onto a microscope slide and allowed to dry at room temperature.

Light microscopy was used to observe the appearance, shape and size of starch granules and to find morphological differences between the pure samples using a Nikon Type 104 microscope. In addition, these samples were also characterized under polarized light by adding polarizing filters (Chakraborty et al., 2020).

Next, flour samples were photographed in bright field and under polarized light conditions in a Zeiss Axiophot microscope equipped with a digital camera (Zeiss AXIOCAM 208 color). From each slide, 15–20 photographs were taken until reaching between 400 and 450 starch granules per sample for counting. These photographed starch granules underwent morphometric analysis with the image processing program Fiji ImageJ (an open source software, https://imagej.net/Fiji).

2.2.1. *Pure flours*

The granule size distribution of autochthonous starch granules was determined by measuring the diameter of each starch granule. Since morphological differences were detected under polarized light, a manual classification was performed based on the birefringence observed in the granules into three types: Type 0, Type 1 and Type 2 (Fig. 1).

2.2.2. *Flour mixtures to obtain a calibration line*

To make an assessment of the variable percentages present in the flour mixtures of 'Caaveiro'/Castilla, and mixtures with a decreasing percentage of 'Caaveiro' were made according to these ratios: 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100. Additionally, a ratio of 25:75 was included, as 25% of 'Caaveiro' is the minimum required to produce 'Galician bread' under the auspicious of the PGI. Then, each sample was processed following the protocol indicated for pure flours, that is measuring and accounting for the three types of starch granules.

2.2.3. *Calibration curve calculation*

A calibration curve was made with the samples and procedures described in the previous point (2.2.2). Initially, the flour mixtures were sprinkled onto the microscope slide. However, after processing the results and evaluating how the starch aggregates affected them, and in order to optimize starch dispersion, the vortex was used for 10 min at 15 Hz. This procedure was done in duplicate. Once the different mixture data were obtained, a statistical analysis was carried out to complete a calibration curve.

2.2.4. *Calibration curve verification using blind samples*

To check the calibration line, and in order to investigate the reliability of the microscopic method for the detection of adulterations of 'Caaveiro' flours by intentional admixture of less expensive wheat flours, 'Caaveiro' wheat and commercial flours were blended in different ratios by someone external to this experiment. All the evaluations were performed blindly by one of the authors (NF–C). Different samples were included (for specifications, see Table 1):

1) 'Caaveiro' pure flour consisting of a sample harvested in a different year to check if it followed the same pattern (blind1, b1) and a sample of wholemeal flour (b2).
2) Foreign pure flours: different flours commonly used in bakeries in Galicia (b3-b5).
3) Eight flour mixtures with different proportions of 'Caaveiro' and foreign flours (b6-b13).

2.2.5. *Data analysis*

The count of the number of starches of each type was made with IBM SPSS Statistics 25. The results of starch analyses were treated statistically by regression analysis using Excel.

3. Results and discussion

3.1. *Morphological characterization of pure flour*

Using bright-field microscopy, no differences were observed in the morphology of the starch granules from 'Caaveiro' and Castilla flours. In both cases the starch granules had rounded, oval or elliptical shape, although 'Caaveiro' presented a greater number of starches with irregular shapes. In the case of 'Caaveiro', the starch grains showed a trimodal size distribution: small size (<12.5 μm), medium size (12.5–22.5 μm) and large size (>22.5 μm). Katyal et al. (2019) and Singh et al. (2010) also reported a trimodal classification in durum and soft wheat, where A-type was large size; B-type, medium size; and C-type, small size. On the other hand, the grains of Castilla followed a bimodal distribution: small size (<11.25 μm) and large (>11.25 μm). Kim and Huber (2010)

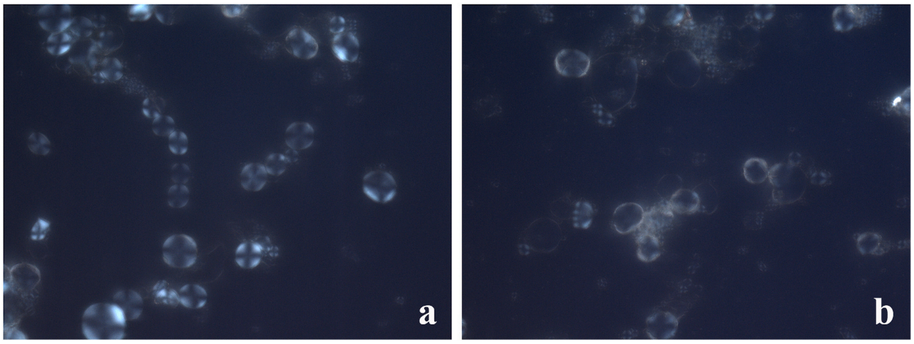

Fig. 1. Castilla flour (a) and 'Caaveiro' flour (b) observed under polarized light in optical microscopy. Maltese crosses predominate in flour from Castilla (Type 0) while most 'Caaveiro' starches did not present the Maltese cross (Type 1) (Magnification x400).

Table 1
The flours used as blind samples with their characteristics. The different letters indicate a different type of flour sample (D and G are the same as those used in the calibration line).

Code	% of 'Caaveiro' flour mixed with commercial flour	'Caaveiro' type				Commercial flour type	
		Sample	Year	Cultivation system	Wholemeal flour	Sample	Year
b1	100	A	2020	conventional	no		
b2	100	B	2020	conventional	yes		
b3	0					E	2020
b4	0					F	2020
b5	0					G	2019
b6	50	A	2020	conventional	no	G	2019
b7	50	C	2020	organic	no	G	2019
b8	50	D	2019	conventional	no	G	2019
b9	25	A	2020	conventional	no	G	2019
b10	25	C	2020	organic	no	G	2019
b11	25	D	2019	conventional	no	H	2020
b12	20	D	2019	conventional	no	G	2019
b13	20	D	2019	conventional	no	I	2020

and Salman et al. (2009) also used a bimodal classification in their studies on starch wheat.

In contrast, under polarized illumination, there were differences in the morphology of the Maltese cross of the starches from Castilla and 'Caaveiro' (Fig. 1). In the flour from 'Castilla', the Maltese cross was perfectly delimited, while in the autochthonous flour this characteristic was not present in most cases, however, the outline of the 'Caaveiro' starch was fully delimited, displaying a characteristic peripheral halo. Therefore, differences were found in the birefringence emission pattern between both types of flour. Taking these differences into account, a qualitative and quantitative starch count was made classifying them into three types:

- Type 0: those where the Maltese cross was perfectly displayed in a central position. The cross was dark, delimiting four bright quarters.
- Type 1: including those starch granules with the Maltese cross much less marked and barely outlined, although they displayed a birefringence halo on the periphery.
- Type 2: those that did not follow any of the previous patterns and, therefore, could not be classified in any of the previous types.

This difference between Type 0 and Type 1 is due to the proportion of amylose and amylopectin that have an effect on the morphology of the starch. Starch consists of two classes of glucose polymers that vary in their proportions: amylose, the minor component (~20%–25%), and amylopectin, the major component (~70%–75%) (Regina et al., 2015). In autochthonous starch granules, the main component of the amorphous region is made up of amylose and the branching points of amylopectin, while the linear branches of amylopectin and some amylose association in crystalline double helixes arranged in parallel fashion form the crystalline structure. Due to the orderly arrangement of the crystalline areas, these starch granules show birefringence, with an interference pattern seen as a Maltese cross under polarized light (Chakraborty et al., 2020). Increasing the average chain length of amylopectin, reducing the frequency of branching and increasing the amylose to amylopectin ratio are all factors that disrupt the ordered architecture of starch (Regina et al., 2015). Therefore, 'Caaveiro' shows a less birefringent pattern associated with high amylose starches. These starches with high amylose content have been positively correlated with lower glycaemic response as well as reduced insulin resistance (Chakraborty et al., 2020; H. Li et al., 2019).

We used this difference in the birefringence emission pattern of the starch granules to determine the proportion of 'Caaveiro' contained in a mixture of both flours, since the greater proportion of one type of flour implies the predominant starch would vary. For example, the greater amount of 'Caaveiro' present in a mixture, the higher is the percentage of granules Type 1 (Fig. 2).

Therefore, the birefringence pattern of the starches in each mixture was analyzed based on the type of starch. Starch morphology offers opportunities for an experienced microscopist to identify species origin (Wrigley, 2017), and further variety based on starch examination. After calculating a regression line and plotting the proportion of Type 1 and the corresponding amount of 'Caaveiro', it was possible to observe a trend (Fig. 3). With this sample processing technique (flour sprinkled on the microscope slide), a clear trend in the data has been seen, so that the greater the amount of 'Caaveiro', the greater the proportion of Type 1 starches in the mixture. However, there is a large dispersion in the data, such as the 100% Castilla sample, which deviated considerably from the calibration curve. This could be influenced by two factors: on the one hand, the samples on the slide were not completely homogenized and aggregates of granules were formed, which could not be classified, and, on the other hand, despite having analyzed more than 5000 starches in mixtures, it might be necessary to carry out a larger and, therefore, more representative sample. Consequently, we tried to optimize the sample homogenization to improve the pattern.

3.2. Homogenization of the sample/Selection of optimal treatment/comparative treatment

There are different procedures to isolate starches (Cai & Wei, 2013; García-Armenta et al., 2021; Kim & Huber, 2010), but we have focused on looking for simple, fast and cheap systems to make them accessible to

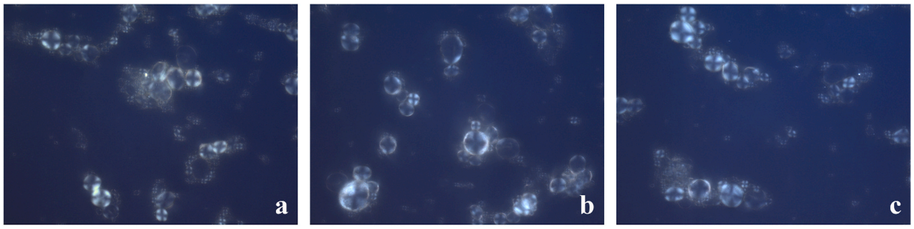

Fig. 2. Different mixtures of 'Caaveiro' and Castilla. (a) 75% 'Caaveiro'/25% Castilla, (b) 50% 'Caaveiro'/50% Castilla and (c) 25% 'Caaveiro'/75% Castilla. The lower the amount of 'Caaveiro' in a mixture, the lower the number of Type 1 granules it presented (Magnification x400).

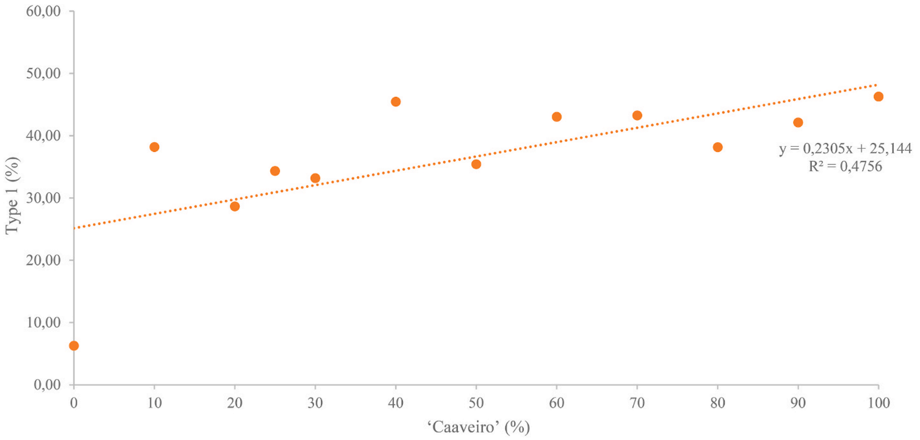

Fig. 3. Initial calibration curve, where a trend in the data was observed ($R^2 = 0.4756$), since the greater the amount of 'Caaveiro', the greater the number of Type 1 granule.

any laboratory. Therefore, different ways of preparing the samples were tested. Glycerol and ethanol solutions were discarded since they did not improve sample dispersion. The use of surfactants was ruled out because, at higher concentrations, the accumulations decreased, but there were marks/residues that we attributed to the high concentration of surfactant. However, at low concentrations, samples were not homogeneous. Finally, mechanical dispersion techniques were tested, combining different times and speeds of agitation in the vortex. Using shorter times (3 and 5 min), agglomerations of granules still formed. In contrast, at 10, 18 and 28 min, it was observed that the number of aggregates decreased considerably. No differences were observed either in the three times or in the speed of agitation, therefore, the shortest time combined with the lowest speed was chosen (10 min and 15 Hz).

3.3. *Calibration curve*

With the previously selected conditions, and after evaluating the 12 mixtures of 'Caaveiro' and Castilla flour, the starches of each sample were assessed according to the type of granule. In addition, in an attempt to develop a more robust model, all samples were done in duplicate (analyses 2 and analyses 3). As can be seen in Fig. 4, with this sample processing method, a better sample homogenization and better line fit were achieved ($R^2 = 0.9484$ and $R^2 = 0.8859$, respectively), a fact that corroborates that this sample preparation method is valid. The deviations found between the lines may be due to the heterogeneity in sample processing or errors in the weighing of the samples and their correct homogenization as reported by Morcia et al. (2020) in developing a method for quantifying common wheat throughout the pasta production chain using digital chip PCR (cdPCR). Data fit better if the sample is enlarged ($R^2 = 0.9577$), that is, analyzing the replicates together and assessing approximately 850 starches per mixture. In addition, the samples used in this work were also analyzed by simple sequence repeats (SSR) (Ramos-Cabrer et al., 2022), including those that showed specific alleles for 'Caaveiro' that were useful for traceability purposes and even to quantify the proportion of 'Caaveiro' wheat in the mixture, by using polymerase chain reaction (PCR) and droplet digital polymerase chain reaction (ddPCR). Moreover, when using ddPCR, the best line fit achieved presented R2 values of the same order as with microscopy (0.9516–0.9872), showing similar deviations due to the difficulty in obtaining the precise percentage of 'Caaveiro' in mixed flours.

In addition, we tried to examine whether the diameter influenced the mathematical model (data not shown), but it was found that it was not a significant variable for this model, hence, we have focused on the starch type analysis.

3.4. *Calibration curve validation*

Thirteen blind flour samples were analyzed (Table 1 and Step 2.2.4 of Materials and Methods), which included pure flours and mixtures made with 'Caaveiro' and foreign flours usually used in baking in Galicia, to check the reliability of the method (Fig. 5). All were correctly classified ($R^2 = 0.9574$), with a maximum difference of 7% with respect to the percentage of the real mixture. The average deviation was 5.17%,

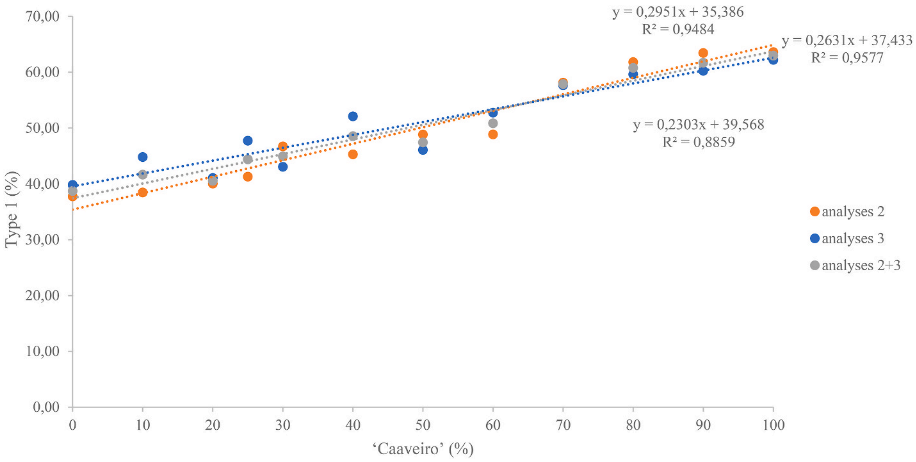

Fig. 4. Prediction calibration curve of 'Caaveiro' flour. Statistical errors are minimized when analyzed in duplicate (analyses 2 + 3).

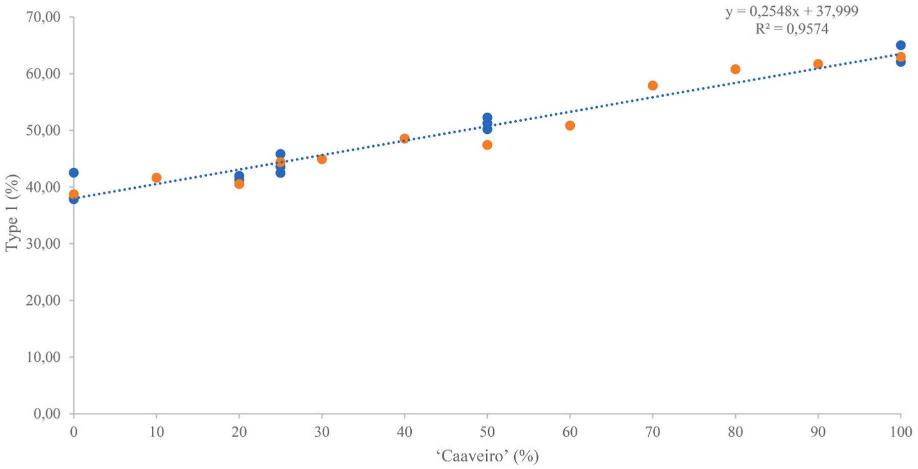

Fig. 5. Prediction calibration curve of 'Caaveiro' flour with 13 blind samples (blue points).

which could decrease, if a method is applied to improve homogenization.

Therefore, with this method it is possible to accurately detect the presence or absence of the 'Caaveiro' variety and quantify its proportion in a mixture. Both the classification of pure flours as well as the detection of adulterations through mixing are of high economic interest (Ziegler et al., 2016). Our method is a novelty, since there is no previous evidence that birefringence has been used as a resource for discriminating wheat varieties or for assessing the proportion of each flour in a mixture.

The microscopic method of standardization is one of the simplest and most inexpensive methods to start with, establishing the correct identification of the source material (Alamgir, 2017).

Different methods have been used to guarantee the control of authenticity in cereals and derivatives (González-Martín et al., 2014). For example, Morcia et al. (2020) applied cdPCR to detect adulterations with *T. aestivum* in pasta manufacturing, reaching a detection level of 3% (the limit to be considered contamination in the legislation). Pasqualone et al. (2007) showed that the use of DNA microsatellite for the detection of common wheat in 'Altamura Bread', a bread with a PDO mark, could detect soft wheat with a threshold of 2.5% by real-time PCR. Another example of the efficacy of using molecular techniques to preserve the authenticity of a protected product was the implementation of Denaturing High Performance Liquid Chromatography (DHPLC) technique in the bread 'Pane Nero di Castelvetrano' where the detection level was 2.5% (Giancaspro et al., 2016). Another method, such as Digital droplet PCR (ddPCR), was also used to detect the proportion of flour mixtures from different wheat varietal blends, reaching variation coefficients generally below 5% (Perry & Lee, 2017). These methods provide high sensitivity and satisfactory accuracy, however specialized personnel, quite long analysis times, and very expensive devices are required, involving high costs (Cocchi et al., 2006).

Other techniques, like isotope ratios were also applicable for authentication of cereals. Knödler et al. (2010) detected and estimated common wheat adulteration in durum wheat based on the analysis of the C17/C21 alkylresorcinol ratios with a sensitivity of 5%.

The use of spectrophotometry has also proved useful for these purposes. For example, near-infrared spectroscopy (NIR) was used by Cocchi et al. (2006) for detection of the adulteration of durum wheat flour with common wheat with a sensitivity of 0.5%. In the same way, Ziegler et al. (2016) also used NIR for identifying five wheat species (bread wheat, spelt, durum, emmer and einkorn), and detecting the adulteration of spelt flour with bread wheat. It was also possible to create a model to identify and quantify taro flour (fetching high prices in the market) in mixtures with sago and wheat, 5% being the smallest proportion quantified by this method (Rachmawati et al., 2017). Another method, the SW-NIR hyperspectral image technique, was used as well to differentiate wheat flour from cheaper grains, such as sorghum, oats and corn, achieving detection sensitivity of up to 2.5% (Verdú et al., 2016).

In general, the detection levels achieved with these techniques mentioned above are similar to those obtained in this investigation. However, our detection level is well below the minimum level of 25%, thus it can be used as a preliminary quality control required to consider the flour mixture suitable for producing Galician bread. Therefore, we corroborate that microscopic and morphological studies will provide reliable information for detecting adulteration, as stated by Alamgir (2017). In addition, it can be used as an alternative method for food authentication in all the categories of origin, substitution and extension (Ballin & Laursen, 2019), although not fully implemented yet. Authentication by microscopy has advantages, since it requires a small amount sample, very low cost, simple processing, and is completely reliable (Li et al., 2012; Wang et al., 2011). The disadvantages are that some training is needed, and it depends, to some degree, on the analyst's objectivity. Fortunately, software programs are increasingly addressing this issue nowadays.

4. Conclusion

The present study proposes a new way of guaranteeing traceability in wheat flour using polarized light microscopy. Our method is based on the different birefringence patterns shown by 'Caaveiro' and Castilla/foreign flours, due to the fact that the autochthonous flour has a larger amount of amylose. Thanks to this difference, a simple mathematical model has been developed that enables the quantification of the 'Caaveiro' proportion in a mixture up to a detection level of 5.17% in a simple and very low-cost way. To optimize its use, the next step will be to apply automatic image analysis software to increase the speed of sample processing.

CRediT authorship contribution statement

Nerea Fernández-Canto: Conceptualization, Formal analysis, Methodology, Writing – original draft, Investigation, Validation, Visualization. **María Ángeles Romero-Rodríguez:** Funding acquisition, Resources, Writing – review & editing. **Ana María Ramos-Cabrer:** Methodology, Validation. **Santiago Pereira-Lorenzo:** Data curation, Formal analysis, Writing – review & editing. **Matilde Lombardero-Fernández:** Conceptualization, Project administration, Writing – original draft, Writing – review & editing, Investigation, Methodology, Visualization.

Declaration of competing interest

The authors declared that they had no conflict of interests with respect to their authorship or the publication of this article.

Data availability

Data will be made available on request.

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