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Identification of starch and determination of its botanical source in ancient manuscripts by MEKC–DAD and LDA

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ABSTRACT

A simple and rapid Micellar Electrokinetic Capillary Chromatography method with UV diode-array detection (MEKC–DAD) has been developed for the identification of the two starch polysaccharides, amylopectin and amylose, in ancient manuscripts. Moreover, a linear discriminant analysis (LDA) has been used in order to determine the botanical source of the starch (wheat, maize or rice). The reason to develop this method is that starch has been used throughout history in paper manufacture as glue and sizing agent.

The LDA was applied to the amylopectin/amylose ratio using the area and height data recorded.

The separation was performed in an extended path-length fused-silica capillary ('bubble capillary') of 36 cm in length and 50 μm i.d.. The running buffer was composed of 20 mM sodium acetate, 1.2 mM I_2, 7.2 mM KI, and 50 mM sodium dodecyl sulphate (SDS) at pH 6. The potential applied was 22 kV in positive polarity, the temperature was 25 °C, and the detection was performed at 560 nm. Injection of the samples was performed at 20 mbar for 2 s.

An artificial ageing test was carried out in the three types of starch in order to determine the effect of the temperature, relative humidity and irradiance on this compound. The procedure was performed in an ageing chamber according to the ISO 5630-3:1996 and 11341:2004 standards.

The methods were applied to samples from manuscripts preserved in the Historic Archive of the University of Granada and the Royal Chancellery Archive of Granada (Spain).

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1. Introduction

Knowledge on the materials used in the manufacture of paper in ancient manuscripts is extremely important and essential for archivists, curators, and restorers of documents. Furthermore, this knowledge makes possible to go in depth in our History, confirming the materials employed several centuries before, apart that is high valuable information to preserve our Documental Heritage. Among these materials, starch is probably, together with animal glues and natural gums, one of the most commonly used substances throughout the history of paper manufacture [1]. A series of consecutive treatments were necessary to obtain the final paper, including to provide the paper with a glossier appearance and to prepare it for the application of inks. This process is called *sizing*, and it was used in Arab paper from the 8th to the 18th century [2]. This treatment was used in Spain since the 11th century until the 18th century, when animal glue sized papers were predominant over starch sized papers [3]. The main types of starch used were wheat, maize and rice [4]. Nonetheless, scientific evidence of its presence is needed to confirm this information.

Starch is a biopolymer with a very complex structure, formed by glycosidic linkages between glucose units, and presents functional properties that make the polymer very helpful for the paper, textile and food industry. Starch consists of two macromolecules: amylose (Am) and amylopectin (Ap). Am is a linear or slightly branched polymer consisting of long chains of hundreds to thousands of glucose units connected with α-D-(1,4)-linkages, with molecular masses of up to 2×10^6. Ap, the major component of starch, is a much larger, highly branched polymer consisting of relatively short segments of D-glucopyranose residues linked by α-D-(1,4)-bonds, connected by α-D-(1,6)-glucosidic linkages, with molecular masses ranging from 10×10^6 to 500×10^6 [5]. The Ap/Am ratio is essential for the determination of the functional properties of different starches and this ratio varies depending on the botanical source of starch (wheat, maize and rice among others) [6].

No articles have been found in the literature on the identification of starch and its components in ancient paper using highly selective and sensitive techniques. They have been determined widely in food samples, but not in graphic documents. Indeed, different techniques have been used in food matrixes: colorimetric methods have been employed to determine Am and Ap in different starches [7–9], infrared (IR) techniques have been applied in samples of rice and starch [10,11], the precipitation of Ap with concanavalin A has been utilised in cereals [12] and even with thermal analysis starch has been determined in potato samples [13] and

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in plastics [14]. Starch in cereal samples has also been analysed by separation techniques; among these techniques, size-exclusion chromatography (SEC) [15–20], and high-pressure size-exclusion chromatography (HPSEC) [21–24] have been used to measure starch content. Capillary electrophoresis with diode-array detection has been employed due to the capacity of Am and Ap to form helical inclusion complexes with iodine [5,25]. In the separation of polymer mixtures, particularly in starch, capillary electrophoresis has demonstrated a high separation efficiency [5,25–27].

Micellar Electrokinetic Capillary Chromatography (MEKC) is a powerful separation technique that can provide high resolution efficiency. The main application of MEKC is in clinical, food and pharmaceutical samples. In the case of ancient manuscripts, the analytical methods for binder identification must be highly selective and sensitive due to the complexity and low purity of the matrixes, the presence of pigments, dyes and other substances, and the limited quantity of sample available from graphic documents and archive materials [28]. For these reasons, MEKC is the technique of choice for developing a new method to identify starch components in ancient graphic documents.

In the chemometric analysis, LDA is a well-known supervised classification technique based on linear discriminant functions, which maximizes the ratio of between-class variance and minimizes the ratio of within-class variance. LDA examines the set of variables associated with a given object and assigns the object to a group or class based on similarities and differences between variables. This chemometric technique has been widely used in classification of food samples [29,30] although can be found in other applications. In the case of graphic documents, LDA has been chosen to obtain classification models to assign a category of starch (wheat, maize or rice) to aged and non-aged unknown and historical samples of paper.

In addition, we performed an artificial ageing study of different starches selected. Over time, the climatic conditions may change or modify the composition of starch. For this reason it is necessary to understand the behaviour of this component under conditions of artificial ageing prior to the analysis of the historical samples.

There are previous artificial ageing experiments that focus on the determination of starch over paper and that take into account only the effects of temperature and humidity but not the effect of radiation (this factor has to be controlled in the case of graphics documents). The results showed that starch was degraded partially into D-glucose units [31]. For this purpose, we have carried out a complete ageing study in different media, using different techniques to determine the progression of the Ap/Am ratio over time and keeping the samples under different conditions to obtain as much information as possible.

The main objective of this work is to develop a simple and rapid methodology to extract and identify starch and to determine its botanical source using for the first time the combination of MEKC and LDA in ancient manuscripts. An issue worth mentioning is the complexity involved in the analysis of the unique samples obtained from an artwork and the extremely high limitations in the amount of sample. The application of this new methodology will provide information and/or confirmation about the use of starch as glue agent in the sizing process in ancient documents. Furthermore, the method will permit to know the type of starch employed in the manufacture and restoration of ancient paper samples with the subsequent utility of this information to restoration and maintenance of our Cultural Heritage.

The method was applied to the analysis of samples obtained from manuscripts preserved in Granada (Spain). From the Historic Archive of the University of Granada a minute book from 17th century, located in the Faculty of Political Science, restored and resized with wheat starch was analysed. This document contains valuable information about the first internal operations of the University of this city. From the Royal Chancellery Archive of Granada an ancient notarial register, called *Registro de Torres* (14–15th century, 1382–1400) was analysed. This document has a huge importance because of being the oldest notarial register preserved in Andalusian, and the second of the Castilla Crown (Spain).

2. Materials and methods

2.1. Instrumentation and software

All the MEKC measurements were carried out with a ^{3D}HP CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a DAD, a thermostated column cartridge, a high voltage built-in power supply and an autosampler. The HP ChemStation version A.0901 (Agilent) software package was used for retrieval and processing of electropherograms.

An extended path-length fused-silica capillary ('bubble factor': 3, Agilent Technologies) of 36.0 cm in length (28.0 cm effective length) and 50 μm i.d. was employed in the electrophoretic method.

The LDA was computed using StatGraphics Centurion XVI program.

The artificial ageing was carried out in an ageing chamber Solarbox 3000e RH (Italy) equipped with a xenon lamp and an indoor filter S208/S408. Xen43 software was used to record the information.

An UV–vis spectrophotometer 8453E (Agilent, Waldbronn, Germany) was used to record the absorbance spectra of the samples from the ageing chamber.

All pH measurements were made with a Crison (Crison Instruments SA, Barcelona, Spain) combined glass-saturated calomel electrode using a calibrated Crison 2000 digital pH-metre.

An ultrasonic bath from Selecta (Barcelona, Spain) was also used for starch extraction.

2.2. Chemicals and reagents

All reagents were of analytical reagent grade. Powdered and pure wheat starch was obtained from Lineco (USA). Maize starch was obtained from Maizena® (UK), and rice starch was provided by Kremer (Germany).

The analytes Am and Ap were obtained from Sigma-Aldrich Chemie (Steinheim, Germany).

The reagents sodium di-hydrogen citrate, sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, iodine (I_2) and hydrochloric acid (37%) were provided by Panreac (Barcelona, Spain). Sodium acetate was obtained from Fluka Chemie (Buchs, Switzerland). Sodium dodecyl sulphate (SDS), hexadecyltrimethylammonium bromide (CTAB), tetradecyltrimethylammonium bromide (TTAB) and potassium iodide (KI) were provided by Sigma-Aldrich Chemie (Steinheim, Germany). Linear alkyl sulphate (LAS) was provided by Cepsa Química (Cádiz, Spain).

The solvents methanol, acetonitrile, 2-propanol, acetone (HPLC-hypergradient grade) were obtained from Fluka Chemie (Buchs, Switzerland), ethanol from Alcoholes del Sur (Córdoba, Spain) and dymethylsulphoxide (DMSO) from Panreac (Barcelona, Spain).

In all the solutions, the water was ultrapure obtained from a Milli-Q Plus system, Millipore Advantage Q-10.

2.3. Extraction procedure

2.3.1. Reference substances

For the electrophoretic method, two types of reference samples were used: polysaccharide standards in DMSO: H_2O (80:20, 5 mg/mL) and samples of starch from wheat, maize and rice in DMSO: H_2O (80:20, 1 mg/mL). Starch samples only needed a heating process at 100 °C during 1 h for their dissolution.

2.3.2. Samples to validate the electrophoretic method

For the validation of the method, samples were made in the laboratory applying a layer of each starch (wheat, maize and rice) with a paintbrush (three spoonfuls of starch in one cupful of water [32]) to

the paper without colorants or glues. In the validation, a piece of paper of $20 \times 10\ mm^2$ was selected to work with.

The treatment of the samples was extremely simple: they were weighted, 1 mL of DMSO:H_2O (80:20) was added, heated at 100 °C during 1 h and then, ultrasonicated for 30 min. The solvents were removed by passing a nitrogen stream, and then 200 μL of DMSO:H_2O (80:20) was added to reconstitute the final residue that was reheated 1 h at 100 °C.

2.3.3. Samples for the artificial ageing study

The following samples were prepared: (i) samples with different starches over paper without colorants or glues, (ii) samples with different starches over glass (to compare the behaviour of starch on an inert support), and (iii) samples with starches in solution (to measure their absorption of light).

Paper samples consisted of small pieces of paper ($20 \times 10\ mm^2$) similar to what was described in Section 2.3.2. and with the same treatment.

As for glass, a layer of the starches (15 mg/mL) was applied over the surface. Then we scraped 1 mg of starch off, and added 500 μL of DMSO:H_20 (80:20) that was heated at 100 °C during 1 h.

The samples in solution consisted of hermetically sealed vials divided in two groups: one with a concentration of 5 mg/mL of the different starches, and the other containing Am and Ap in DMSO:H_2O (80:20) at a 5 mg/mL concentration.

The samples were divided into groups based on the conditions applied:

- samples in the ageing chamber exposed to light at 550 W/m^2, temperature of 80 °C and relative humidity (RH) of 65% were called *light* samples;
- samples in the ageing chamber exposed to 80 °C, 65% RH and covered with foil were called *darkness* samples;
- samples of reference kept in a compartment in the laboratory at 22 °C, 50% RH and no irradiance were called *reference* samples;
- samples exposed to open-air, with no UV filter, were called *open-air* samples.

Three experimental and instrumental replicas of the samples and a blank of each group were analysed.

2.3.4. Historical samples

From the minute book, three samples (pages No. 6, 20, 21) were analysed. From the ancient notarial register, three samples (pages No. 1, 13 and 28) were analysed too.

In both cases the samples, smaller than $2 \times 1\ mm^2$, were carefully removed with a scalpel and underwent the same treatment with the samples used to validate the electrophoretic method.

2.4. Electrophoretic analysis

MEKC separation was carried out in an extended path-length fused-silica capillary of 36.0 cm in length (28.0 cm effective length) and 50 μm i.d.. Every new capillary was preconditioned by rinsing with 1 M NaOH for 15 min, followed by a 5 min rinse with deionized water and 15 min with buffer. For subsequent analyses, the capillary was rinsed with 0.1 M NaOH for 10 min followed by a 5 min flush to ensure good repeatability. The capillary was equilibrated during 5 min with the running buffer consisting of 20 mM sodium acetate, 1.2 mM I_2, 7.2 mM KI and 50 mM SDS. The temperature was kept constant at 25 °C. The pH of the background electrolyte (BGE) was adjusted to 6 with hydrochloric acid 0.1% (v/v). The voltage was set at 22 kV and the resulting current was 60 μA. After each run, the capillary was flushed for 3 min with 0.1 M NaOH and finally during 2 min with water. Hydrodynamic injection of the samples was performed at 20 mbar during 2 s.

The running buffer was replaced after four runs. UV–vis detection was carried out at 560 nm with a bandwidth of 10 nm.

2.5. LDA methodology

Two LDA were made in order to classify non-aged and aged samples paper with starch. In the first case, the prediction sets were obtained using one sample of each type of starch mentioned in Section 2.3.2. In the second case, *light* paper samples resulting of the artificial ageing experiment were employed. To study non-aged starch, 30 injections of each sample were carried out in order to obtain a prediction set with 90 data. To study the aged starch, 33 injections of each aged sample were carried out so as to obtain a prediction set with 99 data. The final prediction set consisted, in this case, of 99 data. Both LDA had one type of categorical variable of classification ("type of starch") divided into three categories: wheat, maize and rice. Each category had two independent variables: the Ap/Am ratio in height and the Ap/Am ratio in area from the peaks obtained in the electropherograms.

2.6. Artificial ageing study

The experiment was developed following the requirements of the ISO 5630-3:1996 and 11341:2004 standards (80 °C, 65% RH and 550 W/m^2 irradiance). The total duration of the experiment was 144 h, with sampling at 0 h, 24 h, 48 h, 72 h and 144 h.

2.6.1. UV–vis spectrophotometry analysis

This technique was utilised to analyse the changes in light absorption of the different starches in solution. In this case the blank was the DMSO:H_20 (80:20) mixture. Spectral absorbance curves were recorded for all the solutions from 240 to 900 nm with a 5 nm sampling interval, using a 10 mm path length cell.

3. Results and discussion

3.1. Optimization of the MEKC method

Electrophoretic separation is based on previous studies on the determination of starch with CE-DAD using iodine complexes [5] but improving sensibility and using shorter times. For this reason, the optimum conditions for MEKC method, experimental and instrumental variables were investigated.

First, different surfactants (SDS, TTAB and LAS) were added in order to improve separation in a running buffer containing 20 mM sodium acetate, pH 5, 1.2 mM I_2, and 7.2 mM KI. In all the cases, the concentration of surfactants was 50 mM. As shown in Fig. 1, the best results were obtained with SDS, probably due to an interaction between the analytes and the functional group of this micellar agent, making its addition necessary to improve the separation between Am and Ap.

Concentration of SDS was studied between 25 and 100 mM. A concentration of 50 mM was selected because it offered optimum signals of analytes and an acceptable current (60 μA).

Different buffers (sodium acetate, sodium di-hydrogen phosphate and di-sodium hydrogen phosphate) at 20 mM which provided pH 5 were tested in order to improve the separation between the analytes. In all cases, the buffer contained I_2 (1.2 mM), KI (7.2 mM) and 50 mM of SDS. The best results were obtained with sodium acetate.

The effect of pH was tested at different values: 4, 5 and 6. The optimum signals were obtained at pH 6, especially in the Ap peak.

Different concentrations of sodium acetate were tested (10–40 mM), and 20 mM was found to be the optimum concentration, increasing the resolution of the analytes and allowing for the complete separation between Am and Ap.

The following step was testing different organic modifiers (ethanol, 2-propanol, acetonitrile and methanol) at 5% (v/v). No improvement was observed, so the use of organic modifiers was not included in the separation process.

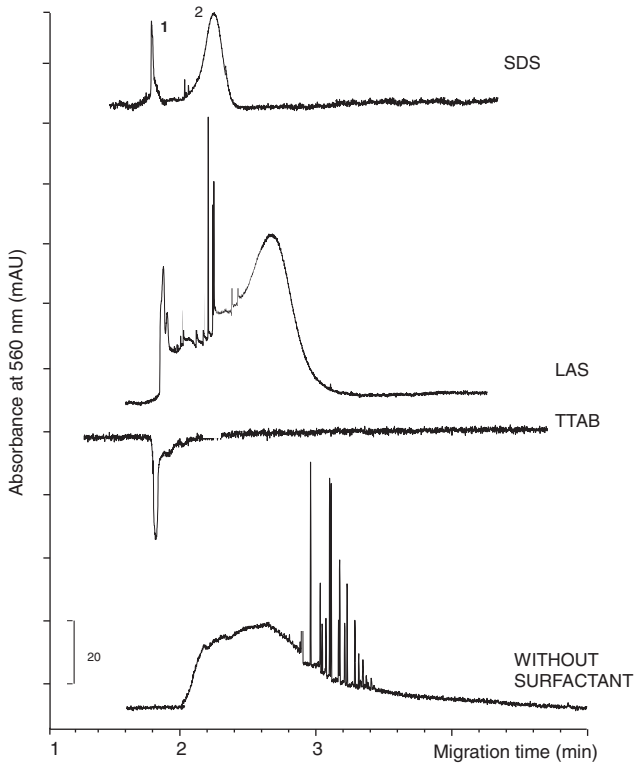

Fig. 1. Electropherograms obtained in surfactant selection. Conditions: pH 5; BGE: 20 mM sodium acetate, 1.2 mM I_2 and 7.2 mM KI; voltage 20 kV; temperature 25 °C; injection 34 mbar, 2 s (1: amylopectin; 2: amylose).

The temperature was investigated in the range of 20 to 30 °C. Increases in the temperature resulted in a reduction of resolution for the compounds studied. The selected temperature was 25 °C.

The voltage was studied at 15, 20 and 25 kV. A separation potential of 25 kV was optimal, but this value also resulted in high current values. Consequently, 22 and 24 kV were tested, with 22 kV selected as the most suitable value taking into account resolution and resultant current (60 μA).

To optimise the hydrodynamic injection, different injection pressures (50 to 10 mbar) and different injection times (1 to 4 s) were analysed. The optimum results were obtained at 20 mbar with 2 s, showing Ap a poor signal with larger injection pressures.

In Fig. 2 an electropherogram obtained with the optimal conditions where the concentration of sample is ten times lower than previous papers is shown [5] (1 mg/mL vs 10 mg/mL), and the intensity of peaks is in the same order, at least in Am, improving the sensibility of the method.

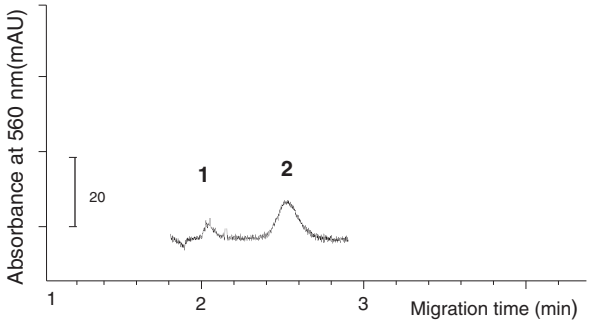

Fig. 2. Electropherogram obtained in optimal conditions. Conditions: pH 6; BGE: 20 mM sodium acetate, 50 mM SDS, 1.2 mM I_2 and 7.2 mM KI; voltage 22 kV; temperature 25 °C; injection 20 mbar, 2 s (1: amylopectin, 2: amylose).

3.2. LDA analysis

Two LDA were employed to determine the botanical sources of the starch. One for classifying non-aged starch and another for classifying aged starch. Both of the chemometric analyses arranged the data in three groups corresponding to wheat, maize and rice starch (Fig. 3).

In the non-aged starch, 100% of wheat and rice samples was correctly classified as wheat and rice respectively. In the case of maize, the samples were correctly classified in 58% of cases. As for the validation set, 100% of samples was correctly classified in wheat and maize, and 66.7% in rice samples (see Table 1).

In aged starch, 94% of wheat samples was correctly classified as wheat starch, whereas the percentage was 76% in maize and rice starch. As for the validation set, 100% of samples was correctly classified in all the categories.

3.3. Artificial ageing study

To evaluate the effect of the artificial ageing on the samples and make possible to determine starch in the historical samples, the following techniques were used: MEKC and UV–vis spectrophotometry.

3.3.1. Results and discussion using MEKC

This technique was used to study the ageing of starch on paper and glass in order to determine the potential changes in the Ap/Am ratio of the different starches.

In paper samples variations in the Ap/Am ratio were detected at the different sampling times in wheat, maize and rice starch, and in the *reference*, *light*, *darkness* and *open-air* groups. Fig. 4a shows the variation in Ap/Am ratio in *light* samples. The bell shape that appeared in the fluctuations of ratio Ap/Am until 72 h suggested that probably Am was the component affected initially, followed by Ap. This fact was corroborated by an artificial ageing experiment with Ap and Am (in DMSO:H_2O, 80:20) over paper in *light* samples. The results (Fig. 4b) showed that, effectively, Am was affected in the first place and later Ap began to disappear until both of them reach a stable state that is related to the stabilization of Ap/Am ratio.

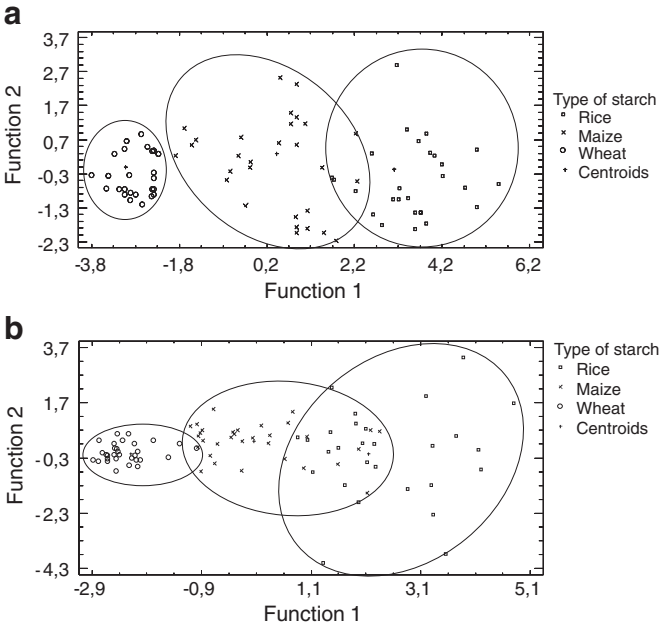

Fig. 3. Plot of the discriminant functions after the application of LDA in a) non-aged starch, and b) aged starch.

Table 1
Prediction and validation data in LDA.

Type of starch	Prediction data	Percentage correctly classified (%)	Validation data	Percentage correctly classified (%)
Non-aged starch				
Wheat	30	100	3	100
Maize	30	58	3	100
Rice	30	100	3	67
Aged starch				
Wheat	33	94	3	100
Maize	33	76	3	100
Rice	33	76	3	100

The total variation in Ap/Am ratio during the experiment was higher in rice starch, especially in the *light* and *open-air* samples (−69% and −77 respectively). Table 2 shows the variations in all the starches in the different samples.

No new compounds derived from the ageing process were detected in any of the samples.

As for glass samples, the Ap/Am ratio varied at the different sampling times in all the starches, with maize starch showing the highest values, especially the *open-air* samples (−66%) together with rice starch (−69%). In general, all the variations between the beginning and the end of the experiment were higher than paper samples.

When comparing the results between paper and glass samples, the greater variations in the Ap/Am ratio were seen in glass samples. This fact must be due to an interaction between paper fibres and the starch that cannot take place in the case of glass. This suggests that paper fibres provide some kind of protection to the starch applied over it, making it more resistant [33].

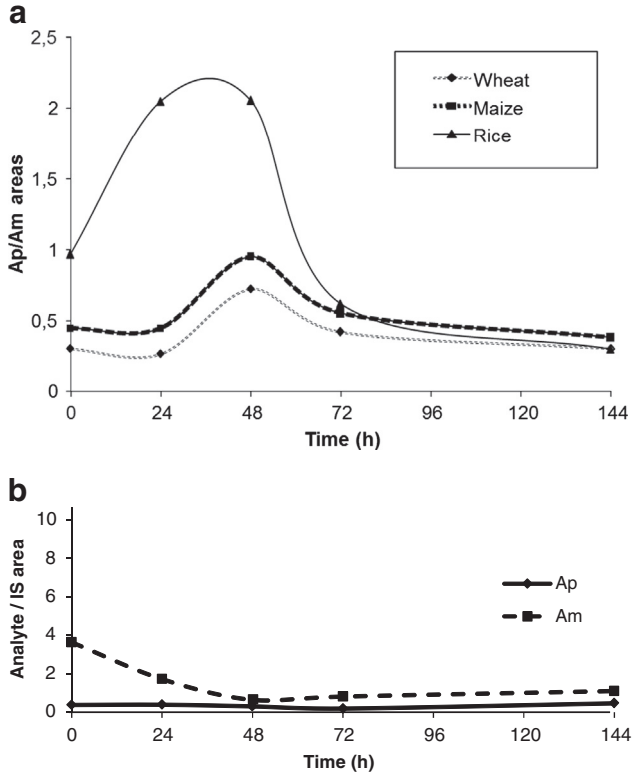

Fig. 4. a) Changes in the Ap/Am ratio in *light* paper; b) evolution of Ap and Am in the artificial ageing experiment in *light* paper samples.

3.3.2. Results and discussion using UV–vis spectrophotometry

The spectra of starch samples and the two polysaccharides in solution were obtained using UV–vis spectrophotometry. In all the cases (*reference*, *light*, *darkness* and *open-air*), the maximum absorption of starch samples at 285 nm was very low (<1 AU). The starch that showed the maximum absorption values was rice starch. Nevertheless, wheat samples exhibited a slight increase during the first 48 h of the ageing process, with a subsequent decrease. Absorption of maize and rice starch showed a tendency to decrease throughout the experiment. In general, no significant changes in absorption were observed for starches samples and standards during the UV study.

3.4. Validation of the method

The developed methodology was validated using papers with the three types of starch. The resultant electropherograms demonstrated a perfect extraction of the starch from the paper. It was possible to identify the starch in tiny papers through the presence of the two peaks corresponding to Ap and Am.

The validation of the method was performed in terms of precision. The intra-day and inter-day precisions of the ratio Ap/Am (as relative standard deviation, RSD) were determined in each kind of starch. The intra-day precision was obtained using three experimental and instrumental replicas in one day and inter-day precision in three consecutive days. The results are shown in Table 3.

Table 2
Variation in Ap/Am ratio between the beginning and the end of the artificial ageing experiment in all the samples.

Condition	Sample	Type of starch	0–144 h (%)
Reference	Paper	Wheat	12
		Maize	−6
		Rice	−52
	Glass	Wheat	44
		Maize	−49
		Rice	−27
Light	Paper	Wheat	1
		Maize	−14
		Rice	−69
	Glass	Wheat	6
		Maize	−43
		Rice	−20
Darkness	Paper	Wheat	−25
		Maize	8
		Rice	66
	Glass	Wheat	39
		Maize	−43
		Rice	−42
Open-air	Paper	Wheat	48
		Maize	19
		Rice	−77
	Glass	Wheat	31
		Maize	−66
		Rice	−69

Table 3
Precision of the method.

Starch	Intra-day precision		Inter-day precision	
	n	RSD	n	RSD
Wheat	9	16	27	19
Maize	9	14	27	15
Rice	9	8	27	15

3.5. Application to historical samples

The minute book was restored with wheat starch by our research team. The application of the methodology confirmed that starch was used as resized agent (Fig. 5a). The LDA of non-aged starch confirmed that the starch used was wheat starch (55.6% of samples were correctly classified as wheat starch). These results are in agreement with the restoration report.

In the case of the ancient notarial register, *Registro de Torres*, due to the date and the characteristics of the paper, the archivists supposed that the document contained starch. The tiny samples were subdivided to obtain three experimental replicas of each page. The resultant electropherograms showed the peaks of Ap and Am, confirming the presence of starch in the manufacture of this ancient document (Fig. 5b). The application of LDA of aged starch showed that the starch used in this case was maize (71% of the samples were classified as maize).

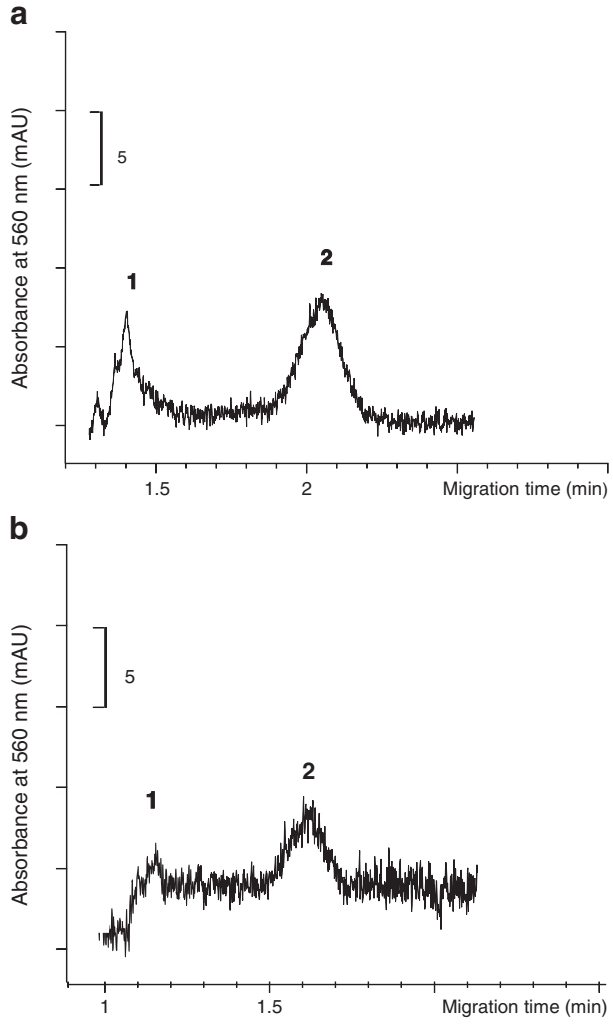

Fig. 5. Electropherograms obtained after application of the developed methodology to the historical samples from a) *Historic Archive of University of Granada*; b) *Registro de Torres*. Conditions: pH 6; BGE: 20 mM sodium acetate, 50 mM SDS, 1.2 mM I_2 and 7.2 mM KI; voltage 22 kV; temperature 25 °C; injection 20 mbar, 2 s (1: amylopectin; 2: amylose).

4. Conclusions

MEKC with diode-array UV–vis spectrophotometric detection has been proved to be an optimal technique for the identification and separation of the starch and its two components, amylose and amylopectin. We have demonstrated that it possible the determination of starch components after a process of artificial ageing and what component is affected initially. The application of LDA has made possible to determine the botanical source of non-aged and aged starch properly.

The developed methodology makes it possible to identify these compounds in ancient paper using a simple procedure of extraction and treatment of samples. The times of analysis have been reduced, and the sensibility of the method has been increased due to the addition of SDS, making working with concentrations of 1 mg/mL of the analytes possible. This improvement has permitted using minimal size of sample, one of the essential requirements in Cultural Heritage.

The information obtained with this methodology will help to determine the most suitable processes of restoration, preservation and cataloguing of the artworks studied.

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