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Short communication

Optimisation of focused microwave digestion of proteinaceous binders prior to gas chromatography

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Abstract

The aim of the present study is to accelerate the acid-digestion step needed for the study of proteinaceous binding media by gas chromatography. The optimisation of the digestion step was carried out using full and half-fractioned factorial designs. The evaluation of the precision of the proposed digestion method and its comparison with the method considered as the reference showed the high irreproducibility of the derivatisation reaction needed for the analysis of the amino acids by gas chromatography. Although the optimal digestion time was found to be 90 min, the differences between different irradiation times were statistically non-significant, so an extremely high reduction on the analysis time is achieved.

Keywords: Binding materials; Amino acids; Rabbit-skin glue; Focused microwave; Digestion

1. Introduction

Binders are the media in which pigments have to be amalgamated so that they achieve the proper texture to be laid on the pictorial surface. Over the centuries, painting techniques have been distinguished according to the kind of binder used. Consequently, the paint medium determined the painting technique [1]. Artists have used a diverse variety of binding media, namely natural gums (carbohydrate compounds), oils, proteinaceous materials, milk and collagen glues made from animal skins and skeletons [2]. Artists have also used a wide variety of mixed binder recipes to improve and modify the painting properties having the binder features together. Consequently, it is of major interest for both conservators and curators to have information about the binder composition in order to plan the optimum conservation conditions and the best restoration actions.

The term ‘tempera’ originally meant the action of mixing the powdered pigments with a binder, in order to obtain a coloured mixture suitable to be applied in different layers on a support. Some time before the thirteenth century, with the extension of the use of the drying oils throughout Europe, the term ‘tempera’ began to refer only to the kind of binders used in aqueous dispersions [3], i.e. only to the media having a prevalently proteinaceous or polysaccharide constitution [1].

Collagen [4] is the predominant proteinaceous material in animal skeletons (both skin and bone) representing one-third of the total protein content in mammals. There are a number of different types of collagen but they all consist of molecules, which contain three polypeptide α-chains in a triple helix conformation. The types of collagen can be distinguished by slight differences in the sequence of their constituent amino acids.

Animal and fish collagen glues are widely used as strong adhesives for wood, binders in the preparation of grounds and size for canvas and pigment binders in decorative paintings [5].

Microwave-assisted sample preparation is being increasingly used in analytical laboratories. Microwave-assisted digestion and extraction constitute the two most relevant areas of analytical applications of microwaves. Since 1975, microwave digestion has gained widespread acceptance as an

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effective means of sample treatment because it dramatically shortens the digestion time [6].

Analytical procedures most commonly used to identify binding media comprise separation and identification steps, the chromatographic techniques being some of the most widely used [2,7–14].

The goal of the present study is to optimise a microwave-assisted method for the acceleration of amino acid hydrolysis, in order to improve a method already proposed for the identification of amino acids in binding media used in works of art [11,12].

2. Experimental

2.1. Instruments and apparatus

A Soxwave-100 focused microwave digestor (Prolabo, Fontenay-sous-Bois, France) with a maximum irradiation power of 300 W was used for accelerating sample digestion. This digester used a TX 32 device (Prolabo) for the control of the microwave unit. A Varian Star 3400 CX gas chromatograph equipped with a flame ionisation detection (FID) system (P.S. Analytical, UK) was used for the separation and identification of the resulting amino acids. Inlet and detector temperatures were 250 °C and 300 °C, respectively. The oven temperature was programmed from 100 °C to 275 °C with a ramp of 20 °C min^{-1}. The carrier gas used was He with an inlet pressure of 117 kPa in the splitless mode. The sample volume injected was 1 μl. To achieve a proper separation of the amino acids, a SPB-1701 fused silica capillary column (Supelco, Bellefonte, PA, USA) with 14% cyanopropyl phenyl methyl silicone (30 m × 250 μm i.d. × 0.25 μm film thickness) was used. A computer with a Varian Star Chromatographic Workstation software (version 5.51) was used for data collection and treatment.

In order to confirm the results obtained by GC-FID, a Varian Star 3800 gas chromatograph coupled to a Varian Saturn 2200 mass spectrometer was used with the same SPB-1701 capillary column for amino acids separation. The inlet temperature was 250 °C. The oven temperature was programmed from 100 ° to 275 °C at 30 °C min^{-1}. The ionisation technique used was electron-impact and the mass spectrometer conditions were as follows: source temperature 200 °C, electronic energy 70 eV, scan rate 0.5 s/scan over the range *m*/*z* 15–750. Helium was used as carrier gas with a head pressure of 47 kPa. Split injection at a 1:20 ratio was performed.

2.2. Reagents and solutions

The reagents used were of analytical grade: HCl 37%, chloroform and absolute ethanol for analysis (Panreac, Barcelona, Spain), and ethyl chloroformate (ECF, purity > 98% GC) and absolute pyridine (Fluka, Buchs, Switzerland).

The amino acid standard solution A9531 for collagen hydrolysates (Sigma, Saint Louis, MI, USA) contained L-alanine, L-arginine, L-aspartic acid, L-Cystine, L-glutamic acid, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine in 0.1N HCl at a concentration of 2.5 μmol ml^{-1}, except for L-cystine, hydroxy-L-proline and L-proline, whose concentration was 1.25, 12.5 and 12.5 μmol ml^{-1}, respectively. Norleucine (Sigma, Saint Louis, MI, USA) was used as internal standard.

2.3. Samples

The samples were prepared following a recipe found in a book on painting materials [15]. The dry rabbit-skin glue was purchased from a local drug store. Six grams of it was weighed and four times this amount water was added. After 24 h, once the rabbit-skin glue had swelled, it was softly heated in a water bath. The temperature was always below 60 °C to avoid protein degradation.

2.4. Experimental procedure

Ten milligrams of rabbit-skin glue was poured into a 5-ml glass tube. One millilitre of 10 M HCl was added. This tube was placed in the glass vessel of the microwave digester, which contained approximately 30 ml of water and it was irradiated under 240 W for different times. The glass tube had to be covered with a modified cork in order to purge the gases generated in the digestion process, so a hole was made on it and a PTFE tube of 0.8 mm i.d. was placed on the hole so that it was hermetically closed to avoid the entry of some of the water where the tube was immersed. During microwave irradiation, the solution evaporated so 1 ml of water was added for reconstitution. Then, 1 ml of chloroform was added, shaking vigorously to extract the hydrophobic species into the chloroform phase. Two hundred and fifty microlitres of the aqueous phase was placed into a 1-ml vial, and 250 μl of ethanol/pyridine (4:1) and 40 μl of ECF was added with vigorous agitation to facilitate the derivatisation reaction. The reaction mixture was then extracted with 250 μl of chloroform containing 1% ECF, and 250 μl of a saturated solution of $NaHCO_3$ was added to obtain a complete separation of the two phases (strong and careful agitation was also needed) [11,12].

The derivatisation of the amino acid standard solution began with the addition of the ethanol/pyridine solution and then, the same process as explained before was followed.

3. Results and discussion

3.1. Preliminary studies

Preliminary experiments were carried out in order to know the generic behaviour of some experimental variables, which could be significant for the digestion process.

Table 1
Tested ranges and optimal values for the focused microwave-assisted acid digestion variables

Variable	Tested range	Optimum value
Power (W)	60–300	240
HCl (M)	2–10	10
Irradiation time (min)	4–100	90
Delay time (min)	0–2	0
Number of cycles	2–8	8

Apart from the microwave-assisted digestion, two alternative ways for accelerating digestion were tried, namely ultrasound-assisted digestion and laser-induced digestion. None of these two sources of energy provided a good yield for the digestion, so the optimisation of the microwave-assisted digestion was carried out.

3.2. Optimisation of the acid digestion

The variables affecting digestion, namely microwave power, HCl concentration, number of cycles, irradiation time and delay time were optimised in order to obtain the best amino acid recoveries in the shortest time possible. The ranges over which the variables were studied and the optimum values found are listed in Table 1.

Five chromatographic peaks, which corresponded to L-alaline, glycine, L-proline, L-aspartic acid and L-phenylalanine were used as response variables.

A screening study of the behaviour of the main variables affecting the digestion efficiency was performed. A full two-level factorial design would involve an overall of $2^5 = 32$ experiments, in addition to the replicates for statistical evaluation of the coefficients for the fitted model and the degree of coincidence of the hyperplane obtained. The selection of a half-fraction 2^{5-1}, type-V resolution design allowing three degrees of freedom involved 16 randomised experiments plus three replicates of the centre point. Table 2a shows the three-level values (low, medium and high) given to each factor.

The conclusions obtained from the screening design were that the power and the irradiation time were the most influential factors on the digestion step and their increase had positive effects on the digestion yield; thus, higher values of these variables were tested. The concentration of hydrochloric acid was not significant for most of the analytes, except for L-proline that yielded slightly significant result with a positive effect, so the highest concentration value, i.e. 10 M, was chosen for further experiments. As the delay time always had a negative effect, its minimum value was chosen, i.e. 0 s therefore, irradiation was carried out in a continuous way instead of using cycles.

Table 2
Factor levels in the (a) first (half-fractioned) factorial design and (b) second factorial design

Factor	Units	Low level (−)	Medium level (0)	High level (+)
(a) First factorial design				
Power	W	60	150	240
Irradiation time	min	2	4	6
Delay time	min	0	1	2
Cycles		2	5	8
HCl concentration	M	2	6	10
(b) Second factorial design				
Power	W	240	270	300
Irradiation time	min	48	64	80

Taking into account these results, a full two-level factorial design was performed. This design involved an overall of $2^2 = 4$ experiments, in addition to three centre points. Table 2b summarizes the values used for the design.

The study showed that the power at these high values had a negative and non-significant effect, so the minimum of the present design, i.e. 240 W (80% of the nominal power) was chosen as the optimum for this variable and was the value used onwards. The irradiation time always had a positive effect and although it was non-significant for most analytes, for L-phenylalanine it was significant, and for glycine it was nearly significant; therefore, a kinetics study had to be done to check the effect on the digestion process.

3.3. Kinetics study

The kinetics of the digestion was studied preparing 10 identical experiments (10 mg of sample and 1 ml of 10 M HCl) and performing the digestion with different irradiation times, from 10 min to 100 min. Three replicates of each experiment were derivatised resulting that the optimal yield was obtained for 90 min of irradiation time, although the experiments with other irradiation times yielded high and well-defined chromatographic peaks as well. The analysis of variance of the results provided by the kinetics study (one-way ANOVA) for the 95% confidence level revealed that non-significant differences were found between them, so there is no need to lengthen the digestion step if the aim of the experiment is to find out the relative amounts of the different amino acids, in order to know which proteinaceous substance has been used as a binder for conservation and restoration purposes.

3.4. Evaluation of the precision of the method

To evaluate the precision of the method, within-laboratory reproducibility and repeatability were estimated in a single experimental set-up with duplicates [16]. The experiments were performed at the optimal values of each variable. Two experiments per day were carried out on seven days. For the sake of comparison, the same precision study was carried out with the method considered as reference in which, the digestion step was performed in an oven with 6 M HCl at 110 °C in Ar atmosphere for 24 h.

To determine the variance due to the between-day effect, Eqs. (1) and (2) were used:

$$s^2 = \frac{(\mathrm{MS}_{\mathrm{between}} - \mathrm{MS}_{\mathrm{within}})}{n_{\mathrm{j}}} \quad (1)$$

Table 3
Mean squares between and within days, and repeatability and within-laboratory reproducibility expressed as relative standard deviation for the method considered as the reference (O) and the proposed one (M) for A: L-alanine; G: glycine; P: L-proline; AA: L-aspartic acid; PA: L-phenylalanine

Analyte	$MS_{between}$ (10^{-6})		MS_{within} (10^{-6})		Repeatability		Reproducibility	
	M	O	M	O	M	O	M	O
A	9530	91900	40900	9650	60.21	27.14	47.28	62.24
G	78700	388000	341000	87300	60.27	24.24	47.29	70.53
P	30600	209000	67300	19500	67.30	27.21	57.41	65.89
AA	352	136	1490	7.47	59.09	37.77	46.47	117.11
PA	10600	9770	19500	908	63.24	28.11	55.54	68.18

where $MS_{between}$ and MS_{within} are the mean square between days and within days, respectively, and n_j the number of replicates per day. The within-laboratory reproducibility s^2_{WR} is equal to:

$$s^2_{WR} = s^2_r + s^2_{between} \quad (2)$$

where s^2_r represents the variance under repeatability conditions.

Table 3 shows the repeatability and within-laboratory reproducibility—expressed as relative standard deviation—for the five analytes chosen as response variables for the method considered as reference and the proposed one, with the mean squares values as well. As can be seen, the obtained values are extremely high but it is thought to be due to the irreproducibility of the derivatisation reaction, so a repeatability study of this step was done.

3.5. Repeatability study

In order to know if the large differences showed between replicates were due to the derivatisation reaction, 10 replicates of the standard amino acid solutions were first derivatised and then analysed. The repeatability values, expressed as relative standard deviation, obtained for the five analytes chosen, ranged from 54.16% to 66.42%, which emphasizes the fact that the high values found for the precision of the method were due to the derivatisation step.

4. Conclusions

The use of a focused microwave digester enables the rapid and complete digestion of proteinaceous samples used as binders in paintings. It dramatically reduces the digestion time (10 min are enough to achieve a very good yield versus 24 h in the method taken as a reference) and avoids the use of inert gases. Although the lack of reproducibility of the derivatisation step, the method can be used for the identification of proteinaceous binders because the ratio between amino acids is kept unaltered.

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