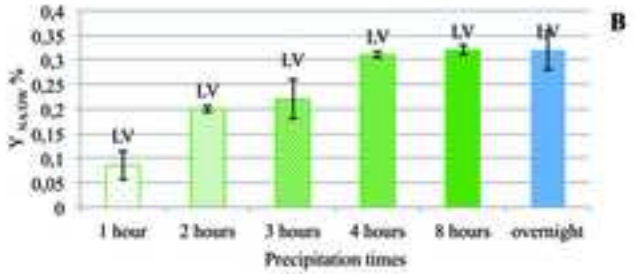
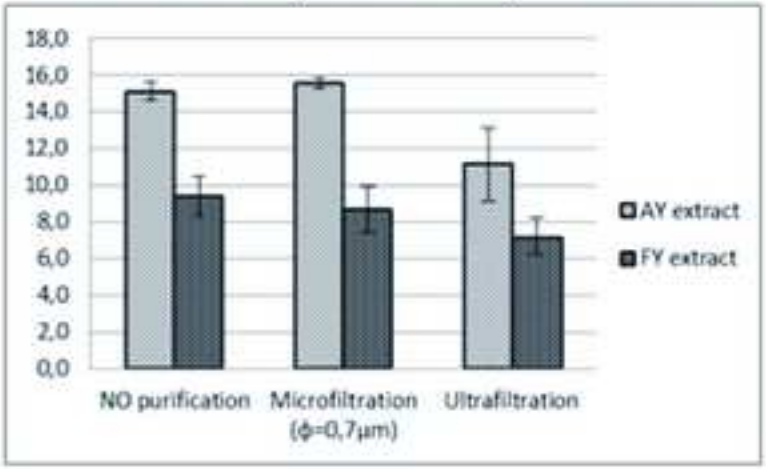
Nucleic acids from agro-industrial wastes: a green recovery method for fire retardant applications

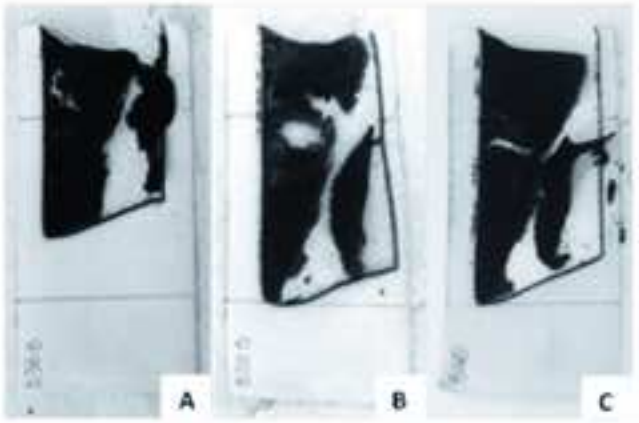
Graphical Abstract

Extraction yields of vegetable NAs



Extraction yields of yeast NAs





Residues after flammability tests

- Spent brewer's yeast and vegetable scraps were used as inexpensive sources of NAs
- A simple, cheap and green extraction method was set for each matrix
- The extraction procedures showed a high level of reliability for NAs recovery
- The extracted nucleic acids were suitable for fire retardant applications
- NAs from spent brewer's yeast cells were able to provide self-extinction to cotton

Nucleic acids from agro-industrial wastes: a green recovery method for fire retardant applications

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ABSTRACT

Two different agro-industrial biological wastes, namely spent brewer's yeast and vegetable scraps have been considered as inexpensive sources of nucleic acids (NAs) to be employed for conferring flame retardant features to cotton fabrics. A simple, cheap and green extraction method has been set-up for each matrix. The yields, purity grades and molecular sizes have been assessed and compared with two different commercially available purified DNA. The developed extraction procedures have shown a high level of reliability for the recovery of nucleic acids

from both agro-industrial wastes. The results of the flammability tests with the extracted NAs clearly indicated that, the add-on value on the fabrics being equal, the NAs extracted from spent brewer's yeast cells are able to provide self-extinction to cotton and reproduce the fire behaviour obtained with highly expensive commercially purified DNAs. Thus, the proposed procedures may be applied for large-scale NA recovery from inexpensive agro-food wastes to obtain partially purified NA suitable for fire retardant applications. Moreover, the developed extraction procedures can be considered an addition to the growing body of more rational waste valorisation techniques within the bio-refinery approach and circular economy concept.

Keywords: nucleic acids; wastes; spent brewer's yeast; vegetable scraps; cotton fabrics; flame retardancy.

1. Introduction

At present, agri-food waste and by-product valorization processes are focused on the recovery of low-value compounds such as fertilizers and building blocks, which can be used directly or as carbon sources in fermentation processes and for energy production (i.e. biogas). Some of these applications have been optimized over the last few decades, while other applications are still at their early stages of development. As far as the biomacromolecules from wastes are concerned, although the recovery of polysaccharides, proteins and lipids is a well-known process (Liguori and Faraco, 2016; Mohan et al., 2016), the nucleic acids (NAs) fraction has not been considered within a bio-refinery approach.

In the last five years, the scientific literature on textile flame retardants (FRs) has clearly indicated that new radical solutions are necessary, and efficient and environmentally friendly

products have to be found in order to replace the “traditional” FRs that, despite their good efficiency, may suffer from some environmental drawbacks. One of the latest approaches, which is worth of further investigation, is that of the use of biomacromolecules, such as proteins and DNA, as FRs, thanks to their chemical structures and composition. In fact, some of them have already been proven to be very effective at a lab-scale, exploiting green finishing processes (i.e. by applying aqueous solutions/dispersions directly to different textiles, using traditional processes, such as impregnation/exhaustion and spraying). Recently, low molecular weight DNA was employed as an effective intumescent flame retardant coating for cotton fabrics, since it has demonstrated to provide self-extinction in horizontal flame spread tests and a substantial reduction in the heat release rate in cone calorimetry tests (Alongi et al., 2013; Bosco et al., 2015). The DNA’s fire retardant activity has been ascribed to the simultaneous presence of phosphate groups, nitrogen bases and deoxyribose sugars in the same molecule, which makes the biomacromolecule to be considered as an intumescent-like product. On the other hand, proteins derived from milk, such as caseins and whey proteins, show a fire retardant effect when deposited on cotton fabrics. Although they cannot provide self-extinction of the flame, they are able to significantly reduce the burning rate and increase the final residue (Alongi et al., 2014; Bosco et al., 2013).

On the basis of these findings, it is possible to consider that DNA could be a valid alternative to traditional halogenated FRs, and could offer the opportunity of opening a new research scenario related to this unconventional application. However, the use of commercially available products might not be suitable because these chemicals mainly used in molecular biology for analytical purposes (Dellaporta et al., 1983), are available in very small quantities and are extremely expensive.

For this reason, valid DNA sources have to be found, and environmentally friendly and cost-effective recovery techniques have to be developed. Bearing in mind that the amount of extracted biomacromolecules has to be sufficient to treat large fabric surfaces, it seems logical that the available DNA sources that are inexpensive and accessible in large quantities should be considered.

In this context, wastes from agro-food industries that process biomass or food may represent good sources of nucleic acids that meet all of the above-mentioned requirements.

The traditional techniques that are usually applied in molecular biology and medical research fields cannot be exploited for the extraction of NAs from these bio-wastes. In fact, conventional techniques are suitable for the treatment of very small starting samples in the order of mg, and consequently the obtainable quantity of nucleic acids is very limited, i.e. of the order of μg. In addition, these techniques utilize a sequence of numerous separation and purification steps, which makes them time consuming. Finally, they can be expensive because the high grade of DNA or RNA purification requires highly pure, expensive and, sometimes, even toxic reagents, like phenol and chloroform (Dellaporta et al., 1983; Guillemaut and Marechal-Drouard, 1992; Lõoke et al., 2011).

In the present work, two different food industry wastes have been investigated as potential nucleic acids sources, namely spent brewer's yeasts and mixed vegetable scraps from a processing plant that handles vegetables and fruit to obtain IV range products. Brewer's yeast traditionally belongs to *Saccharomyces* spp; spent yeast is one of the main by-products of beer production, and it is mainly exploited in animal feed. Other applications, such as a substrate in microbial cultures or for the extraction of cell components, (e.g. β-glucans, proteins, B vitamins

and minerals), can also be mentioned (Suphantharika et al., 2003; Podpora et al., 2015). Vegetable wastes from food industry plants are discharged in remarkable quantities. The quantities and compositions of these wastes depend, to a great extent, upon the seasonal periodicity of the agricultural productions. They are mainly utilized for the production of compost, livestock feeding and fermentation for biogas or ethanol production (Di Donato et al., 2014; Nagarajan et al., 2014; Bouallagui et al., 2005).

An attempt has been made in the present work to develop and optimize simple, sustainable and cost-effective methods in order to extract NAs with high yields, using considerable quantities of spent brewer's yeasts or mixed vegetable wastes. Because of the different biological origins of the two wastes (vegetal tissue or microorganism), two different specific recovery methods were developed. The obtained low purified NA extracts have been applied to cotton fabrics and tested to assess their flame retardant properties in terms of horizontal flame spread propagation.

2. Materials and methods

2.1 Materials

The mixed vegetable wastes were supplied by a food processing plant (Stroppiana Ortofrutticola S.p.A. Chieri, Turin, Italy) and were derived from a IV range vegetable production line. Because of the seasonal variability of the agricultural cultivations, the batches, which arrived at the laboratory at different times, were heterogeneous in composition. Almost all of the batches consisted of a portion of leafy vegetables (lettuce, chard, celery, cabbage leaves, red chicory, rocket salad leaves) and another made up of small pieces of turnip, onions, peppers,

courgettes, potatoes, carrots and pumpkin. The vegetable matrices considered have been indicated as follows: mixed leafy vegetables (MLV) and leafy vegetables (LV). MLV mainly comprised leafy vegetables and a small portion of vegetable pieces (about 10%), while LV matrix was only composed of leafy vegetables. The matrices were separated into small portions (500 g) and stored at +4°C (for a maximum of 48 hours) or at -20°C before use.

Spent brewer's yeast was kindly supplied by Heineken Italia S.p.A. (Aosta, Italy) and Birrificio Della Granda (Cuneo, Italy) and stored at +4°C until it was used.

Cotton fabrics (COT, 200 g/m^2) were purchased from Fratelli Ballesio S.r.l. (Torino, Italy) and cut into 100x100 mm^2 square specimens.

A liquid commercially available detergent (containing 5-15% anionic surfactants) was used. Commercial DNA from herring testes and herring sperm, NaCl, ethanol (at 99.8% purity), Tris (hydroxymethyl) aminomethane Hydrochloride (Tris-HCl) (pH 8) and Ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich S.r.l. Milano, Italy and used as received.

2.2 NA extraction from the mixed vegetables

First, mixed vegetable wastes stored at -20°C were thawed. The vegetable portions (30 g) were then ground using a mortar and pestle or a hand blender and were then mixed with the extraction buffer (liquid detergent 10% v/v, NaCl, 10% w/v). The extraction buffer:vegetable dry weight ratio was set at 1:7. The extraction was carried out at 65°C for 30 min. Then, the waste was separated from the aqueous extract through filtration, using a nylon gauze, and then using qualitative filter paper. Cold ethanol (stored at -20°C) was then added to the aqueous fraction in

a 1:1 (v:v) ratio. Precipitation was first performed overnight at -20°C. The alcoholic upper phase, containing the precipitated nucleic acids, was recovered and centrifuged at 4100 rpm for 10 min. Finally, the precipitated pellet, containing NAs, was re-suspended with distilled water.

Different parameters were changed, one at a time, to optimize the extraction procedure, as follows: i) the starting material was either stored at -4°C or at -20°C before extraction; ii) 99.8% ethanol was further diluted with bi-distilled water to 96 and 90% purity grade and used for the precipitation step; iii) the precipitation was carried out at two different temperatures (+4 and -20°C); iv) the precipitation was performed at -20°C for different incubation times, namely 1, 2, 3, 4, 8 hours and overnight.

2.3 NAs extraction from the spent brewer's yeast

Beer slurries were stored in two different ways before NAs extraction: one batch was maintained at +4°C for more than 6 months, and, just before extraction, the yeast cells were centrifuged and washed three times with bi-distilled water (AY); in a second batch, the cells were immediately centrifuged, washed three times with bi-distilled water and the pellet stored at -20°C until they were used (FY).

Each of the extraction buffers listed hereafter was added at a ratio of 1 ml per 8mg of the yeast pellet weight. The mixture was frozen at -20°C for 24 hours and then thawed three times at 65°C for 15min. After thawing, cells were mechanically disrupted using 1 and 2 mm diameter glass beads (1mg:5g yeast:glass beads ratio) and the mixtures were then subjected to ball milling for 10 min. Glass beads were then recovered and cell debris removed by means of centrifugation (4100 rpm for 10 min). Further chemical purification steps were avoided. However, physical

149 purification was carried out through different types of filtration: microfiltration (porosity 0.7 μm)
150 and ultrafiltration (cut-off of 10 kDa).

151 Nucleic acid recovery from yeast was optimized by changing some parameters, one at a time,
152 as follows:

153 i) different extraction buffers were tested, namely:

154 1) TrisHCl50 mM + NaCl 50 mM + EDTA10 mM , pH 8 (labelled TNE 50/50/10)

155 2) TrisHCl 50 mM + NaCl 100 mM + EDTA 10 mM, pH 8 (labelled TNE 50/100/10)

156 3) TrisHCl 50 mM + NaCl 50 mM, pH 8 (labelled TN 50/50)

157 4) TrisHCl 50 mM + NaCl 100 mM, pH 8 (labelled TN 50/100)

158 5) Sodium phosphate, pH 8 (labelled PB);

159 ii) different times of ball milling (from 10 to 90 min at 150 rpm) were compared;

160 iii) to separate cell debris after the nucleic acid extraction, mixtures were centrifuged, or
161 microfiltrated (ϕ=1.2 μm), or filtrated through Celite as reported by Hatti-Kaul and Mattiasson
162 (Hatti-Kaul and Mattiasson, 2003).

163 *2.4 Characterization of extracted NAs*

164 *2.4.1 Quantitative NAs determination.*

165 The NA concentration was determined by means of a spectrophotometric method, and the
166 absorbance was measured at 260 nm wavelength. Nucleic acids (DNA and RNA) absorb UV

light at 260 nm, due to the presence of nitrogen bases. The NA concentration in the extract was determined according to the following equation (Eq.1):

$$C_{NA}\ (\mu g/ml) = A \cdot DF \cdot 40 \qquad \text{(Eq. 1)}$$

where A is the absorbance value at 260 nm, DF is the dilution factor and 40 is a constant (40 µg/ml of RNA has an absorbance value of 1 with an optical path of 1 cm). This concentration is slightly underestimated because DNA, which has a constant of 50, is also present in the extract; however, RNA should be predominant in quantity.

The NA yield ($Y_{NA/DW}$%) was calculated by means of the following equation (Eq.2):

$$Y_{NA/DW}\% = \frac{(NA(mg))}{(Starting\,dry\,weight\,material\,(mg))}100 \qquad \text{(Eq. 2)}$$

The nucleic acid purity was assessed from the absorbance ratio at 260 and 280 nm (Warburg and Christian, 1942).

2.4.2 Agarose gel electrophoresis

Aliquots of the different NA samples were digested with a DNAase-free RNase (20 µg/ml), and then analyzed by means of gel electrophoresis on 0.8% w/v agarose gels (Sigma-Aldrich S.r.l. Milano, Italy) at 5 V/cm for 2 h in a 1 × Tris–acetate–ethylenediaminetetraacetic acid (TAE) buffer containing 0.5 µg/mL of ethidium bromide (Sigma-Aldrich S.r.l. Milano, Italy). A 1 kb molecular ruler (Thermo Scientific) was run with DNA samples in order to estimate the lengths. Gel pictures were taken using a Gel Doc XR+ Imaging System (BioRad).

186 *2.4.3 ATR spectroscopy*

187 Attenuated Total Reflectance (ATR) spectroscopy of commercial herring testes DNA and of the
188 NAs extracted from FY and LV was performed using a Frontier FT-IR/FIR spectrophotometer
189 (16 scans and 4 cm^{-1} resolution), equipped with a Universal ATR Sampling Accessory (diamond
190 crystal).

191 *2.4.4 SEM microscopy*

192 The surface morphology of the NA-treated samples was studied using a LEO-1450VP
193 Scanning Electron Microscope (beam voltages: 5 kV). Fabric pieces (5×5 mm^2) were cut,
194 pinned onto conductive adhesive tape and gold-metallized.

195 *2.4.5 Determination of Phosphorus concentration*

196 The concentrations of the P element on the fabrics treated with the yeast NA extracts was
197 determined by performing ICP-MS tests. These measurements were carried out with a ICAP-Q
198 apparatus (Thermo Fisher, USA). The NA-treated fabrics (100 mg) were dissolved in 80 mL of
199 an $HNO_3/HClO_4$ aqueous solution (4:1 molar ratio) at 50°C, then cooled to room temperature;
200 bi-distilled water was added to the obtained solution until a final volume of 100 mL was
201 achieved and then diluted in order to reach a suitable concentration for the tests (between 100
202 and 1000 ppb). The apparatus had previously been calibrated considering a multi-element
203 standard for phosphorus.

204

205

206 *2.4.6 Impregnation of the cotton fabrics with extracted NAs*

207 The impregnation solution containing NAs extracted from vegetables was obtained by re-
208 suspending the nucleic acid pellet from ethanol precipitation in water.

209 The impregnation solution containing NAs extracted from yeast was obtained after the removal
210 of the cell fragments, without separating the nucleic acids from the rest of the extracted
211 components or buffer solution.

212 Cotton fabrics were immersed for 1 min in the nucleic acid solution, which was kept in static
213 conditions and at room temperature. A multiple impregnation procedure was adopted to achieve
214 the desired final dry add-on (AO%). After each impregnation step, the fabrics were dried in an
215 oven at 105°C for 30 min. The total dry add-on applied to the cotton samples was determined by
216 weighing each sample before (W_i) and after impregnation with the solution and the subsequent
217 drying (W_f), using an analytical balance (Scaltec, accuracy: $\pm 10^{-4}$ g). The uptake was calculated
218 according to the following equation (Eq.3):

219 $$AO\% = \frac{W_f - W_i}{W_i} \cdot 100 \qquad \text{(Eq. 3)}$$

220 *2.4.7 Characterization of the fire behaviour*

221 The resistance of the untreated and treated cotton fabrics to a flame application was assessed
222 by means of flammability tests in horizontal configuration. These tests were carried out by
223 applying a methane flame (25 mm length) for 3 s to the short side of the samples (50 × 100
224 mm^2), which were clamped in a U-shaped metallic frame, tilted 45° with respect to the plane
225 containing the frame. Total burning time, char length, total burning rate after the flame
226 application (calculated as the ratio between the char length and the total burning rate) and the

final residues were evaluated. The flammability tests had the aim of mimicking the procedure described in the ASTM D4804 standard, which is commonly employed for thin films, although the specimen size was different (50 x 200 mm^2 in the ASTM D4804 standard).

3. Results and discussion

3.1 NAs extraction from the mixed vegetable wastes

NAs extraction from the vegetable wastes was carried out on a mixed scrap derived from a IV range vegetable production line, as reported in the "Materials and Methods" section. Owing to the high heterogeneity of the supplied wastes, the extraction results refer to two matrices, i.e. MLV (mixed leafy vegetables) and LV (leafy vegetables).

The two matrices were extracted using the basic procedure described in Materials and Methods that relies on a detergent-mediated lysis followed by ethanol precipitation. Then, different parameters were taken into account in order to optimize the extraction procedure.

First, a set of preliminary trials was performed to evaluate the influence of the storage conditions on the vegetable matrix. For this purpose, the NAs extraction was carried out on either frozen or fresh MLV and LV samples. Freezing is an additional step that offers the advantage of preserving vegetables for long periods before they are used. The frozen vegetable mixture was thawed, ground and fully mushed up, while the fresh material was sent directly for grinding. As reported in Table 1, the extraction yields obtained from the frozen material were almost doubled, compared to those obtained with the fresh substrate (+4°C for a maximum of 48

247 hours) for the same extraction procedure. The NA purity values ($OD_{260/280}$) were almost the
248 same, and ranged from 1.12 to 1.17.

249 The main effect of freezing was a softening of the vegetable material, which became easier to
250 manage since vegetable tissues were broken down, thanks to the freezing/thawing cycle. This
251 procedure allowed higher quantities of NAs to be extracted with the same purity obtained by
252 treating the fresh material. This means that the total extracted mass of biomacromolecules (NAs
253 and proteins) was higher, but the proportion between those that absorb at 260 nm and those that
254 absorb at 280 nm remained unchanged.

255 The main parameters that influence the economy of a recovery process, at large scale, are time
256 and temperature, as well as quantities and the purity grade of the used reagents. For this reason, it
257 was important to identify the optimal combination of the parameters related to the ethanol
258 precipitation, which is one of the most frequently used NA recovery procedures. This operation
259 affects the process economy, due to the use of ethanol and the requirement of an incubation
260 period at low temperature that can be time consuming, as it is sometimes prolonged overnight
261 (Zeugin and Hartley, 1985). For this purpose, the ethanol purity grade, the temperature, and the
262 duration of the precipitation phase were changed one at a time to obtain the best extraction yield,
263 taking into account the economy of the process.

264 The ethanol precipitation step was performed at two different incubation temperatures, namely
265 +4 and -20°C. The influence of this parameter on the extraction yield was evident. For MLV,
266 higher NA yield values (0.1%) were obtained at -20°C in comparison to the significant lower
267 percentage (0.02%) extracted from the same source at +4°C. Again in this case, there were no

differences in the NAs purity ($OD_{260/280}$), which was close to 1.13. For these reasons the following extractions were performed at -20°C.

The influence of the ethanol purity grades (90, 96 and 99.8%) on the NA yields and purity was then evaluated on the two matrices. As shown in Figure 1 (a), no significant differences in the extraction yields were found for the MLV and LV matrices, when the different ethanol purity grades were used. The $Y_{NA/DW}$% values were found to be in between 0.25 and 0.32%. On the other hand, the ethanol with the lowest purity provided the highest NA yields, but with a high standard deviation value. No differences were found for the NA purity ($OD_{260/280}$), which was within 1.11 and 1.17 for all the samples. On the basis of these results, it was concluded that the ethanol with the lowest purity grade (90%) could be utilized for the precipitation step, hence achieving high extraction yields at lower cost.

Then, the effect of the incubation time on NAs precipitation was investigated. The results obtained for the LV matrix after an overnight incubation (12 hours) are compared in Figure 1(b) with those achieved for shorter times (i.e. 1-8 hours). It should be noted that the time of this step affects the extraction yields: in particular, these latter reached a plateau (at about 0.3%) after 4 hours of incubation. Furthermore, prolonging the incubation time did not provide any further increase in the extraction yields. Therefore, 4 hours were enough for the highest yield to be obtained. Purity was not affected by the time of the precipitation step: the purity values ($OD_{260/280}$) were equal to 1.1 for all of the samples.

The results of the above trials, taken together, allowed the best conditions to extract NAs from mixed vegetable wastes to be established. First, the MLV and LV sources gave comparable $Y_{NA/DW}$% and purity values for the same extraction conditions. Nevertheless, the LV source is

preferable because it is only composed of leaves that are characterized by a similar composition, irrespective of the origin of the vegetable tissues. On the other hand, the MLV source can contain small pieces of vegetables (e.g. potatoes, carrots and pumpkin), in variable quantities, which are rich in complex polysaccharides and which interfere negatively with NA extraction and recovery. In fact, they contribute to the formation of macromolecular complexes, which are able to embed the nucleic acids in a sticky and gelatinous matrix. Furthermore, polysaccharides have a viscous and glue-like texture, which makes the precipitation and separation of NAs difficult during the subsequent recovery steps (Guillemaut and Marechal-Drouard, 1992; Echevarría-Machado et al., 2005).

Second, the best results were obtained by starting from a frozen material that was subsequently thawed, mashed up and mixed with the extraction buffer in a 1:7 ratio. This ratio was chosen on the basis of the results reported in the scientific literature (Carboni, 2016); it also corresponded to the minimum buffer quantity necessary to completely soak the utilized matrices.

Third, after extraction at 65°C for 30 minutes and filtration, the NAs were precipitated with 90% ethanol for 4 hours at -20°C. These optimized conditions were adopted to process the LV matrix, in order to obtain sufficient NAs amount to impregnate the cotton fabrics. In this context, it was mandatory to scale-up the whole process by increasing the starting material five-fold. In particular, it was necessary to adjust the extraction temperature, which was raised to 68°C, and the time, which was doubled (60 minutes). However, the scale-up did not affect the recovery yields. In fact, the obtained value was equal to 0.33% and comparable with that obtained previously in small-scale experiments using the same recovery conditions. Thus, it was possible to utilize a solution with a NA concentration equal to 1.7 mg/ml and with a purity degree ($OD_{260/280}$) of 1.11, suitable for impregnating the cotton fabrics.

3.2 NAs extraction from spent brewer's yeast

The second food industry waste that was investigated as a potential source of NAs was a spent brewer's yeast. The basic extraction procedure of this matrix required the addition of a defined volume of extraction buffer, followed by a series of freeze-thaw cycles and a ball milling step, which was performed by adding glass beads to the yeast/buffer slurry. Then, after the removal of the glass beads and the centrifugation to separate cell debris, the extract was microfiltrated and ultrafiltrated to obtain an aqueous solution containing NAs.

As for vegetable wastes, different parameters of the basic procedure were changed in order to maximize yields and purity of the extracted NAs, starting from beer slurry that was pre-treated in two different ways: one batch was maintained at +4°C for more than 6 months and, just before being used, yeast cells were centrifuged and washed three times with bi-distilled water (AY); in a second batch, beer slurry cells, after their receiving from brewing industries, were immediately centrifuged to separate yeast cells that were then washed three times with bi-distilled water and stored at -20°C until their utilization (FY). On these two starting materials, the basic procedure was applied and different extraction buffers, ball milling conditions, and filtration methods were evaluated.

3.2.1 Optimization of the extraction buffer

To optimize the extraction buffer, first we considered the components required for yeast DNA purification reported in the related literature: 1) one or more detergents, such as SDS (Sodium Dodecyl Sulphate), CTAB (Cetyl Trimethyl Ammonium Bromide), and Triton™ X-100 (Octylphenol Ethylene Oxide Condensate) that are sometimes combined with enzymes, which contribute to the cell lysis and to the protein removal; 2) the pH is usually maintained at a value

of about 8.0 by means of a Tris-HCl or a Phosphate buffer; 3) a salt, e.g. NaCl, which helps during the DNA precipitation by neutralizing its negative charges; and 4) a chelating agent, e.g. EDTA, to prevent DNA damage.

To reduce the cost of extraction buffer, the use of detergents and enzymes was avoided and they were replaced by a combination of physical (freeze-thaw) and mechanical (glass beads milling) methods to pursue the yeast cell lysis step. Therefore, the optimization of the extraction buffer was investigated through three different combinations of the above remaining essential components. The first consisted of 50 mM Tris-HCl, 10 mM EDTA and 50 or 100 mM NaCl (coded as TNE 50/50/10 and TNE 50/100/10, respectively). EDTA was not included in the second formulation, which just contained Tris-HCl and NaCl, in the same concentrations as the first one (coded as TN 50/50 and TN 50/100). The third extraction solution was a phosphate buffer (PB), without any of the other components.

The different extraction buffer efficacy was evaluated for both the AY and the FY starting materials. The NAs yield (%) and purity values, calculated after the centrifugation step, are shown in Table 2. For AY, high yields were obtained with TNE 50/50/10 (13.1%), TNE 50/100/10 (13.1%) and PB (13.5%). Conversely, low yields were observed for TN 50/50 and TN 50/100 (11.1 and 11.6%, respectively). When the yields and purity values were combined, TNE 50/100/10 and PB resulted to be the best extraction solutions. In the case of FY, the results were similar for all of the utilized extraction solutions and both the yield and purity values were lower, compared to those of AY.

TNE 50/100/10 was therefore chosen for all the subsequent extraction tests. The PB solution was discarded because, as it will be later discussed, it possessed an intrinsic flame retardant effect that covered that of the extract.

3.2.2 Optimization of the ball milling step

As previously reported, a mechanical cell disruption method was employed to improve the release of the intracellular components, especially NAs. For this purpose, glass beads were added to the samples after the last freeze-thaw cycle, and a ball milling apparatus, with a rotation speed of 150 rpm, was employed. The influence of two different grinding times (namely 10 and 90 minutes) on cell disruption was evaluated for samples incubated with the TNE 50/100/10 extraction solution. The NA yield (%) and purity values, obtained for AY and FY after the centrifugation, are reported in Table 3. The treatment time was found to affect the yield %, as an increase from 13.1 to 15.5% for AY and from 5.3 to 9.4% for FY was observed as the ball milling time was increased. On the other hand, the purity was not affected substantially for both the yeasts.

3.2.3 Optimization of post-extraction purification steps

Two alternative filtration methods were applied to samples extracted with TNE 50/100/10 and then centrifuged. Microfiltration was carried out by using a glass fibre filter (Φ=0.7 μm) to remove any solid particles that might still be present in the NAs solution. Ultrafiltration was applied with a membrane cut-off of 10 kDa in order to reduce the concentration of the molecules with an MW below 10kDa. The permeate was discarded, while the retentate, rich in NAs, was recovered.

The yield % obtained for AY and FY after the two purification steps is shown in Figure 2. A decrease of the yield after ultrafiltration was particularly evident for AY (about 11%), likely due to the presence of low molecular weight NAs (Figure 4) able to pass through the membrane. However, purity of AY was further increased by the filtration steps since it reached $OD_{260/280}$ values higher than 2. This was probably ascribed to the removal of the smaller cell debris by microfiltration and to the removal of low molecular size proteins by ultrafiltration.

Conversely, the filtration steps did not significantly affect the purity of the FY extract, since an $OD_{260/280}$ value of 1.5 was found for the unpurified sample, whereas 1.6 was obtained for both the microfiltrated and ultrafiltrated samples.

Taking into account that the yields and purities of FY were lower than those of AY, further attempts were made to improve yield and purity of the FY NAs. Thus, the centrifugation was replaced by a Celite filtration or a microfiltration (Φ=1.2 μm) step. As shown in Fig. 3, Celite filtration allowed obtaining a NA solution with yield and $OD_{260/280}$ values (9.1% and 1.45, respectively), very similar to those obtained from centrifugation (9.4% and 1.50). Microfiltration, on the other hand, led to the worst results, in terms of yield (6%) and a negligible increase in purity (1.56).

From all of the obtained results with AY and FY, an optimized extraction procedure was defined according to the following conditions: extraction buffer composed by Tris-HCl 50 mM, EDTA 10 mM and NaCl 100 mM; physical cell disruption with 3 alternate freeze-thaw cycles; mechanical cell disruption by means of ball milling with glass beads for 90 minutes. Finally, post-extraction purification steps were not strictly necessary, but the most performing method implied the use of microfiltration with glass fibre filter (Φ=0.7 μm).

3.3 Characterization of the extracted DNA

NAs extracted from three different samples (FY, AY, and LV) were digested with DNase-free RNase and then characterized with agarose gel electrophoresis to establish their molecular sizes in comparison to those of commercially available herring DNA (Figure 4). The DNA extracted from FY showed a continuous smear between 3 and more than 15 kb comparable to that of commercial DNA from herring testes (HT DNA), thus indicating that FY DNA did not degrade during the extraction procedure. In contrast, the DNA obtained from AY showed a partial degradation with a smear lower than 3 kb, comparable with that of commercial DNA from herring sperm (HS DNA). This degradation is probably due to the cells autolysis occurred during the prolonged storage of the yeast at +4°C.

Conversely, DNA extracted from FY cells remained almost intact and enzyme degradation did not occur. However, for FY, harder conditions for cell disruption were needed to get good recovery of extracted DNA. Thus, FY was more sensitive to both the physical and the mechanical cell disruption processes. As shown in Table 3, the influence of the glass bead grinding time was more pronounced for FY than for AY with a 4% difference between 10 and 90 min grinding. Finally, the LV DNA sample did not show a detectable smear and only a very low quantity of oligonucleotides likely derived from the RNA degradation was visible.

FT-IR ATR spectroscopy was then used to evaluate the quality of the yeast NA (extracted from FY) and vegetable NA (extracted from LV). The corresponding spectra were compared with that of commercial herring testes DNA (Figure 5). First of all, it is noteworthy that, despite a difference in intensity, all the spectra show signals in the 1250-1000 cm^{-1} region that correspond to phosphate vibrations; furthermore, they also exhibit C=N vibrations at about 1600

420 cm^{-1}, attributable to the nitrogen bases. Some absorption peaks with different intensity at about
421 1400 cm^{-1}, at least for FY NAs, could be referred to uracil base and thus to the presence of RNA
422 in the extract (Banyay et al., 2003). The two FTIR-ATR spectra of the extracted NA show
423 similarities, notwithstanding a slight shift of the peaks for the LV extract toward higher
424 wavelengths.

425 *3.3.1 Morphology of the treated fabrics*

426 Figure 6 shows some typical pictures of the fabrics, as assessed by SEM, before and after the
427 treatment with the extracted biomacromolecules (namely, LV, FY and AY). The roughness of
428 pure cotton (Figures 6 A and B) disappears when the fabrics are treated with the extracted NAs:
429 these latter usually exhibit a smooth surface, because of the presence of a biomacromolecule
430 coating (Figures 6 C, D, E, F), which is also present in the fabric interstices, as clearly shown in
431 Figure 6 D. Specifically referring to LV NA, its coating shows a rougher surface, due to the
432 presence of impurities that tend to aggregate, hence giving rise to a "island" growth of the
433 coating (Figures 6 G and H).

434 *3.3.2 Fire retardant behaviour of the extracted NAs*

435 The extracts showing the highest NA yields and purity were applied on cotton fabrics and
436 subjected to flammability tests in horizontal configuration. Table 4 collects the obtained data;
437 Figures 7-9 show some pictures of the treated fabrics at the end of the tests.

438 As already mentioned, the fabrics treated with the phosphate buffer alone were found to be
439 self-extinguishing with only 3 AO% and therefore PB was not considered for the evaluation of
440 the flame retardant effectiveness of the NA extracts. On the other hand, the TNE 50/100/10

441 buffer alone was able to confer some flame retardant features to cotton (AO%=8): a few samples
442 self-extinguished and quite thin residues remained.

443 An add-on of 8% was also considered for yeast-derived DNA samples, as optimized in
444 previous studies[4]. In contrast, the LV-derived NA-containing extract did not allow reaching an
445 8% dry add-on, and the maximum obtained value was about 5%.

446 Interesting results were observed with the application of the AY-derived NA-containing
447 extract. In fact, all the tested samples achieved self-extinction, and the best performances were
448 obtained for the AY extract even without purification (60% residue). However, AY NA-
449 containing solution after microfiltration and ultrafiltration, provided similar flame retardant
450 features, as confirmed by almost comparable residues (50% and 47%, respectively) and burning
451 rates (1.15 mm/s) (Table 4 and Figure 7).

452 Furthermore, long afterglow combustion was observed in all of the investigated samples. The
453 good flame retardant properties of unpurified AY-derived extract are likely due to the high
454 content of low molecular weight DNA (Fig. 4), which makes the biomacromolecule more
455 effective in protecting the underlying fabric (Bosco et al., 2015).

456 Different results were obtained for fabrics treated with FY-derived NA-containing solution. In
457 particular, after removal of the cell debris by means of centrifugation, the extract showed a
458 significant decrease in the burning rate, compared to the untreated cotton (1.05 vs. 1.5 mm/s),
459 with no self-extinction of the samples (Figure 8 and Table 4). Better results, i.e. self-extinction
460 and residues as high as almost 50% were achieved with samples filtrated by Celite (Table 4).

As regards the post-extraction purification steps, the most effective treatment for fire retardancy for both AY- and FY-derived samples was microfiltration with a glass fibre filter (Φ=0.7). In this case, self-extinction was achieved, together with a high final residue (72%) and a low burning rate (1.15 mm/s). A similar behaviour, but without the flame out being reached, was found for the cotton treated with the ultrafiltrated extract.

FY was less effective than AY because of the higher molecular weight of extracted DNA (Bosco et al., 2015). Nevertheless, microfiltration probably caused a better homogenization of the extract and allowed the formation of a more protective coating, in comparison with unpurified FY-derived extract.

Since phosphorus is a key component of NAs, and is necessary to provide flame retardant features, ICP-MS was exploited for assessing the phosphorous content of the cotton fabrics treated with the NA extracted from yeast. The ICP-MS measurements showed that the concentration of this element in the fabrics coated by AY- and FY-derived NA samples was 0.47 wt.%, hence comparable to that obtained for the commercial DNA coating (0.5%) (Bosco et al., 2015).

The effect of the LV-derived extracts in the flammability tests was almost negligible: just a slight decrease in the burning rate was observed (1.3 mm/s vs. 1.5 mm/s for treated and untreated fabrics respectively). Furthermore, an incoherent and very limited final residue was observed (Figure 9). These findings could be ascribed to the low dry add-on of the biomacromolecules on the fabrics (i.e. about 5 wt.%), as compared to the other samples (8 wt.%).

SEM analyses were also exploited for assessing the morphology of the residues after the horizontal flammability tests. As clearly shown in Figure 10, the texture of all the treated fabrics

is maintained, thus indicating a significant protection exerted by the biomacromolecule coating on the underlying fibres. Furthermore, the fibres show a hollow structure (Figures 10 B and D), derived from the dehydration reaction promoted by the NA degradation, which favours the formation of a stable char (Alongi and Malucelli, 2015). Finally, as compared to the fabrics treated with the commercial DNA coating, which show the presence of numerous bubbles, as a consequence of the intumescent process occurring after the flame application (Figures 10 G and H), the residues of the substrates treated with extracted NAs display a different surface topography, characterized by the presence of several char fragments adhered to the hollow structure of the fibres.

4. Conclusion

In this work, a new green method has been designed and developed for the recovery of NAs from spent brewer's yeast and vegetable scraps. In particular, two optimized extraction procedures were found to be suitable for recovering NAs with acceptable yields, and molecular sizes comparable with commercially available DNAs. Solutions containing NAs from agri-food wastes were then exploited as green flame retardants for cotton fabrics. To this aim, the fabrics were treated with extracted NAs (8 wt.% final dry add-on), and subjected to flammability tests in horizontal configuration. The most performing NAs were extracted from spent brewer's yeast, which provided self-extinction to most of the treated fabrics. On the other hand, the NAs recovered from vegetable waste were not as effective as their yeast counterpart, likely due to the lower final dry add-on achieved (i.e. about 5 wt.%).

In conclusion, the proposed NA extraction methodologies from agri-food wastes, which do not

require the use of toxic reagents, may represent, as a whole, a new simple and gree approach to face the current challenges related to rational environmentally friendly valorisation of wastes from agriculture and food industry. Finally, the simplicity of the proposed method seems to make it quickly upscalable for larger scale exploitation.

Conflict of interest

None.

Acknowledgements

The authors thank H2020 DAFIA - Biomacromolecules from municipal solid bio-waste fractions and fish waste for high added value applications project for financial support.

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569 **Captions for Figures**

570 **Fig. 1**. Extraction yields ($Y_{NA/DW}$ %) obtained with ethanol at different degrees of purity (a).
571 Effect of the precipitation time (b).

572 **Fig. 2**. Extraction yields (%) obtained for different purification steps.

573 **Fig. 3**. Extraction yields (%) (left axis) and purity (right axis) data obtained with different cell
574 debris separation methods.

575 **Fig. 4**. Agarose gel electrophoresis analysis of : HS) low MW DNA from herring sperm (Sigma-
576 Aldrich S.r.l.); HT) high MW DNA from herring testes (Sigma-Aldrich S.r.l.); MW) ladder; 1)
577 DNA from FY; 2) DNA from AY; 3) DNA from LV

578 **Fig. 5**. FT-IR ATR spectroscopy of extracted NAs: NA from FY (purple line); NA from LV (red
579 line) commercial DNA from herring testes (green line).

580 **Fig. 6**. SEM micrographs of treated and untreated fabrics: A,B) untreated cotton; C,D) cotton
581 treated with AY NA; E,F) cotton treated with FY NA; G,H cotton treated with LV NA.

582 **Fig. 7**. Residues after flammability tests (horizontal configuration) of autolysed yeast: A)
583 Extract, B) Extract after microfiltration (ϕ=0.7μm), C) Extract after ultrafiltration.

584 **Fig. 8**. Residues after flammability tests (horizontal configuration) of frozen yeast: A) Extract,
585 B) Extract with Celite filtration, C) Extract with microfiltration (ϕ=0.7μm), D) Extract with
586 ultrafiltration.

587 **Fig. 9**. Residues after flammability tests (horizontal configuration) of A) vegetables extracts; B)
588 untreated cotton.

589 **Fig. 10**. SEM micrographs of residues after horizontal flammability tests: A,B) cotton treated
590 with AY NA; C,D) cotton treated with FY NA; E,F) cotton treated with LV NA; G,H) cotton
591 treated with commercial DNA from herring testes.

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598 **Table 1.** Extraction yields ($Y_{NA/DW}$ %) obtained with fresh or frozen vegetable material. The

599 standard deviation values are indicated in brackets.

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	MLV	LV
Fresh	0.07 (±0.008)	0.07 (±0.010)
Frozen	0.14 (±0.006)	0.12 (±0.008)

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605 **Table 2.** Extraction yields (%) and purity data obtained for different extraction solutions of spent

606 brewer's yeast

	AY		FY	
Extraction solutions	Yield (%)	Purity ($OD_{260/280}$)	Yield (%)	Purity ($OD_{260/280}$)
TNE 50/50/10	13.1 (±0.9)	1.85 (±0.01)	5.5 (±0.3)	1.57 (±0.01)
TNE 50/100/10	13.1 (±0.8)	1.95 (±0.01)	5.3 (±0.2)	1.60 (±0.01)
TN 50/50	11.1 (±0.5)	1.90 (±0.02)	5.6 (±0.2)	1.65 (±0.01)
TN 50/100	11.6 (±0.5)	1.95 (±0.01)	5.4 (±0.3)	1.60 (±0.02)
PB	13.5 (±1)	1.95 (±0.02)	5.7 (±0.3)	1.50 (±0.02)

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611 **Table 3.** Spent brewer's yeasts: extraction yields (%) and purity data obtained for the different

612 ball grinding times

	AY		FY	
Grinding time	Yield %	Purity ($OD_{260/280}$)	Yield %	Purity ($OD_{260/280}$)
10 min	13.1 (±0.8)	1.95 (±0.01)	5.3 (±0.2)	1.60 (±0.01)
90 min	15.5 (±0.5)	2.00 (±0.01)	9.4 (±0.9)	1.50 (±0.01)

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Table 4. Combustion data of the cotton fabrics coated with different extracted NAs obtained from horizontal flame spread tests.

	Total burning time (s)	Char length (mm)	Total burning rate (mm/s)	Residue (%)	Self-extinction
COT	66	100	1.5	0	NO
AY extract	51 (181)*	60	1.17	60	YES
AY extract after microfiltration (ϕ=0.7μm)	65 (203)*	75	1.15	50	YES
AY extract after ultrafiltration	64 (227)*	75	1.15	47	YES
FY extract	95	100	1.05	38	NO
FY extract (Celite filtration)	80	87	1.08	49	YES
FY extract after microfiltration (ϕ=0.7μm)	61	70	1.15	72	YES
FY extract after ultrafiltration	83	100	1.2	34	NO
PB	71	69	1	69	YES
TNE 50/100/10	70	83	1.2	45	YES-NO**
LV extract	77	100	1.3	3	NO

* Afterglow time

** Only 50% of the samples showed self-extinction

Figure 1

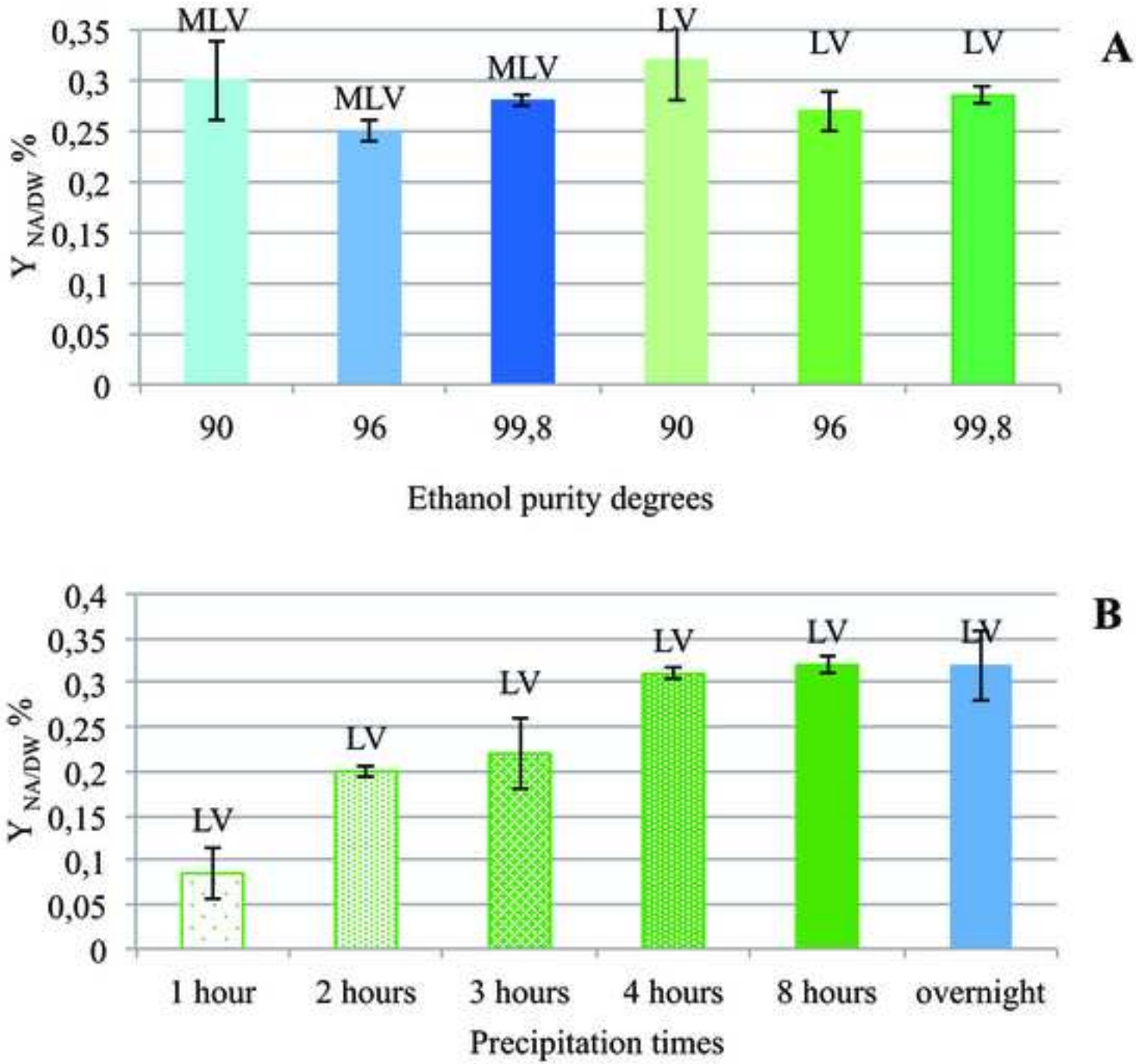
A
MLV
MLV
MLV
LV
LV
LV
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Y NA/DW %
90
96
99,8
90
96
99,8
Ethanol purity degrees
B
LV
LV
LV
LV
LV
LV
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Y NA/DW %
1 hour
2 hours
3 hours
4 hours
8 hours
overnight
Precipitation times

Figure 2

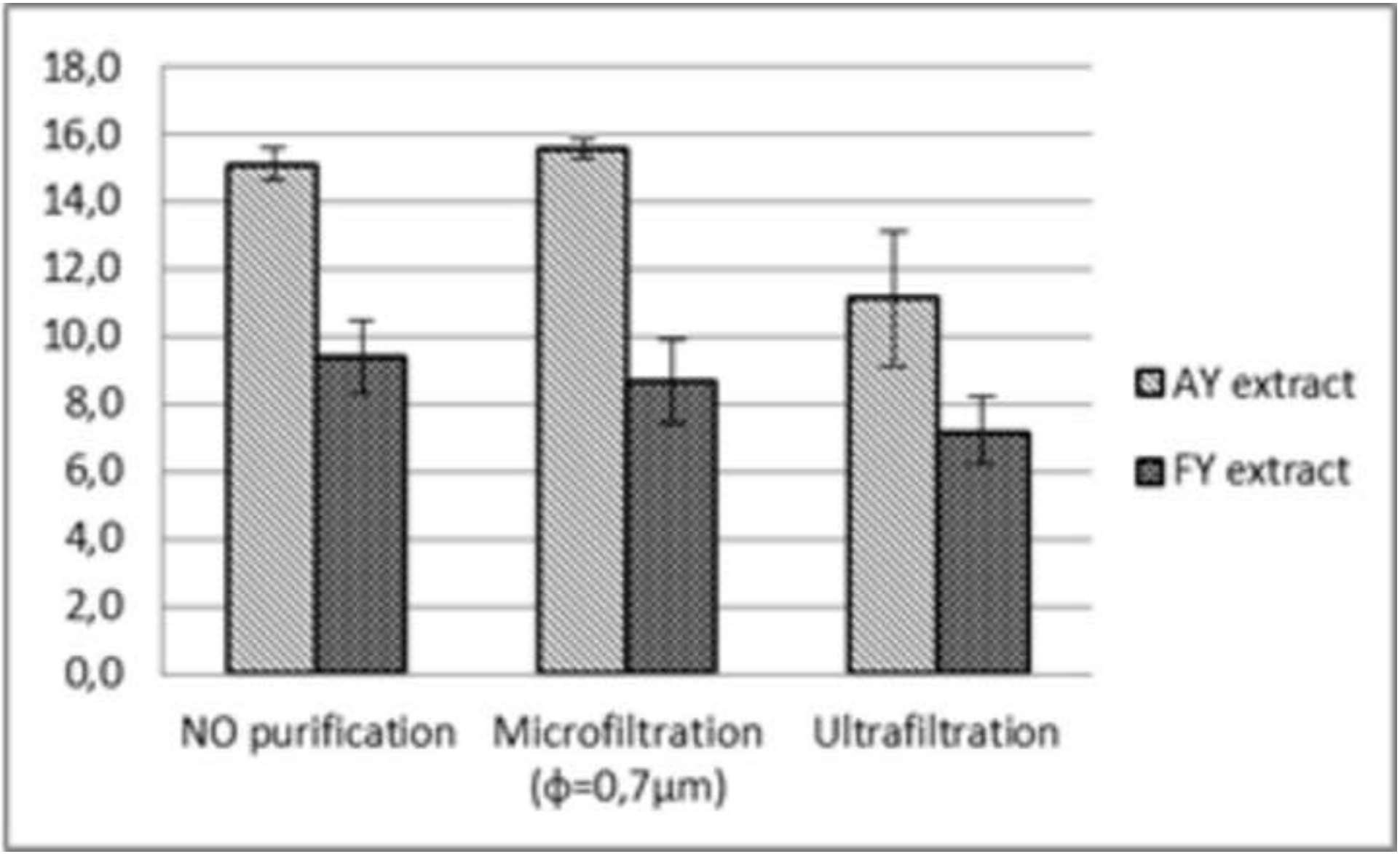
18,0
16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
AY extract
FY extract
NO purification
Microfiltration (ϕ=0,7µm)
Ultrafiltration

Figure 3

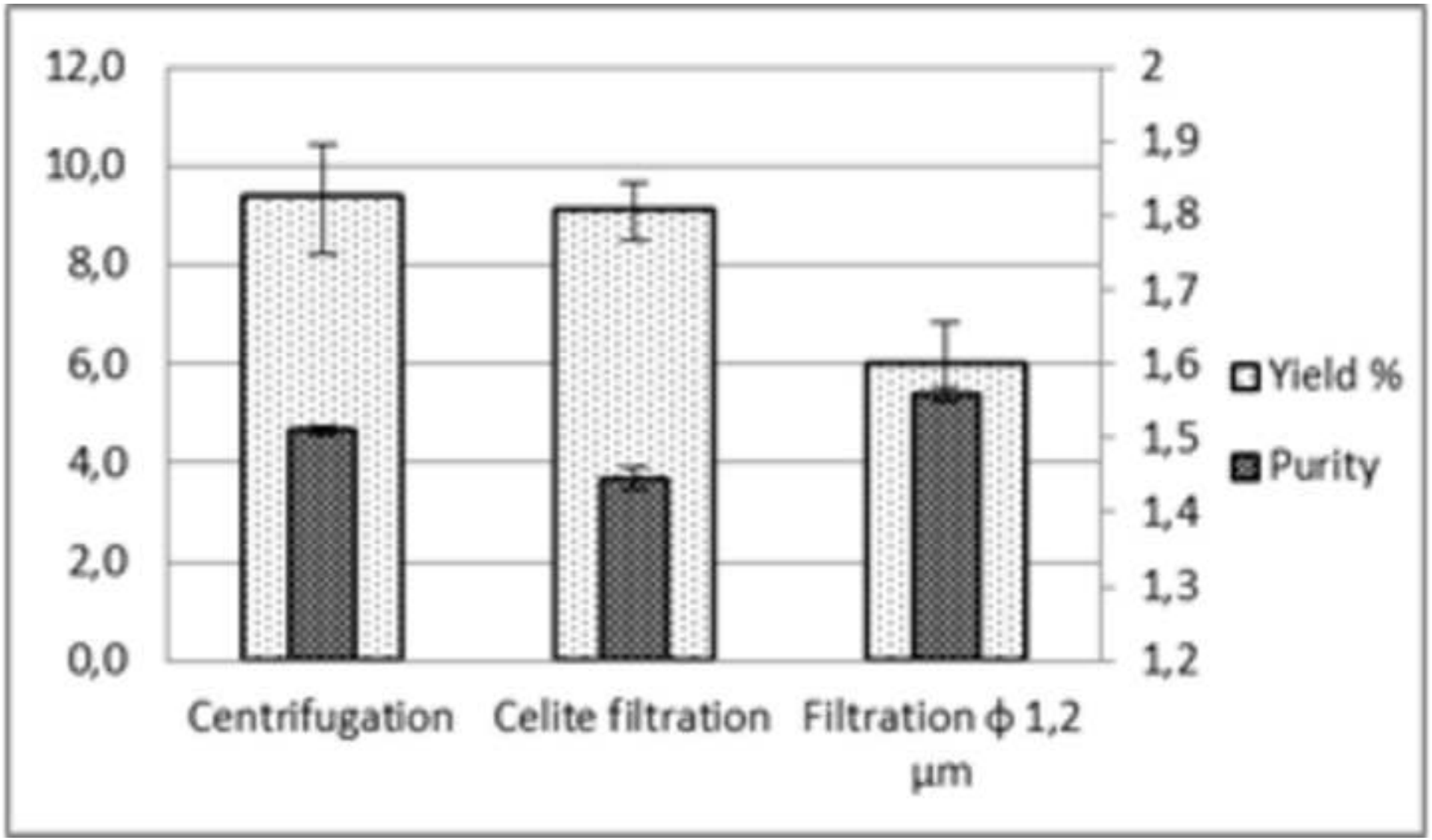
12,0
10,0
8,0
6,0
4,0
2,0
0,0
2
1,9
1,8
1,7
1,6
1,5
1,4
1,3
1,2
Yield %
Purity
Centrifugation
Celite filtration
Filtration ϕ 1,2 µm

Figure 4

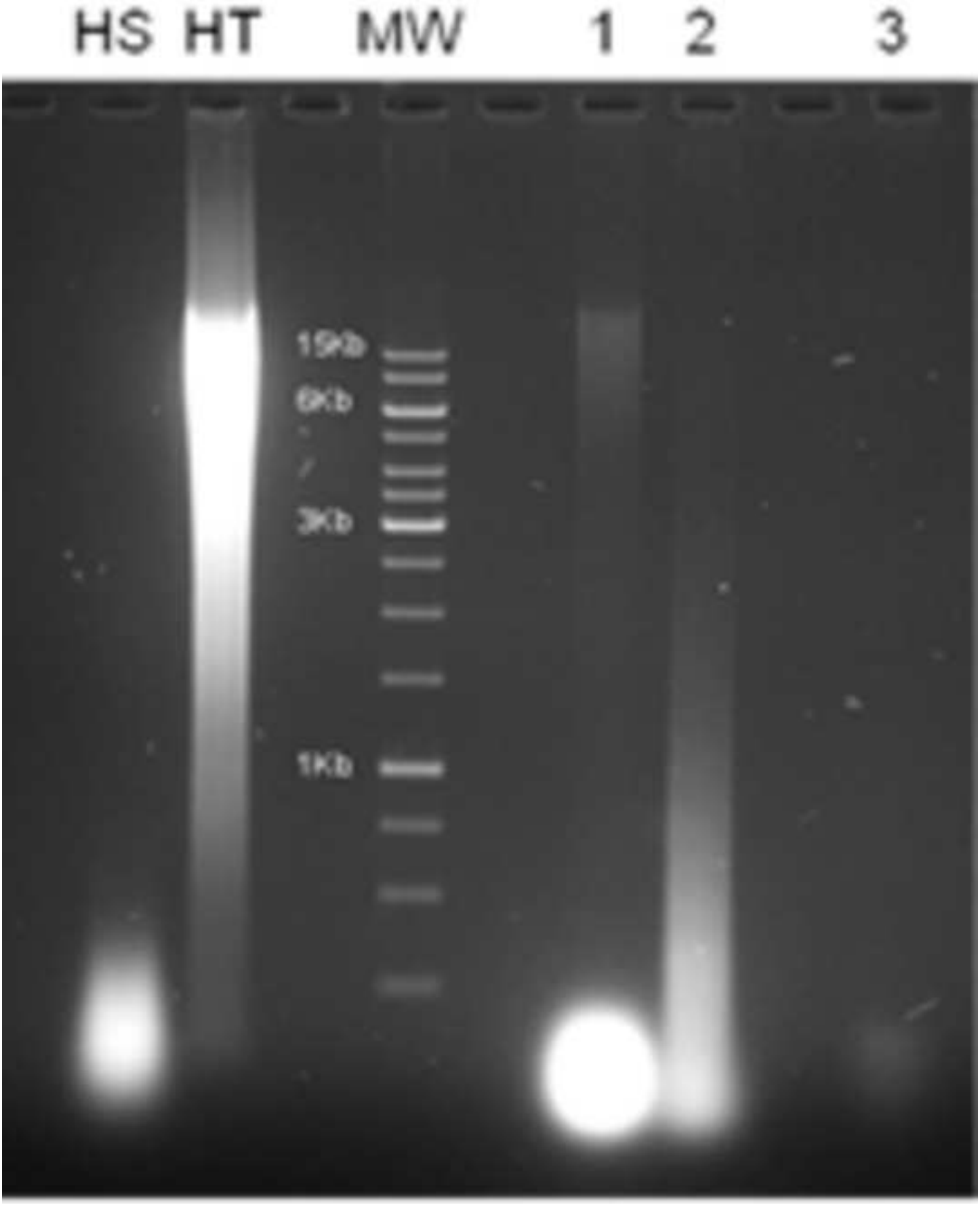

Figure 5

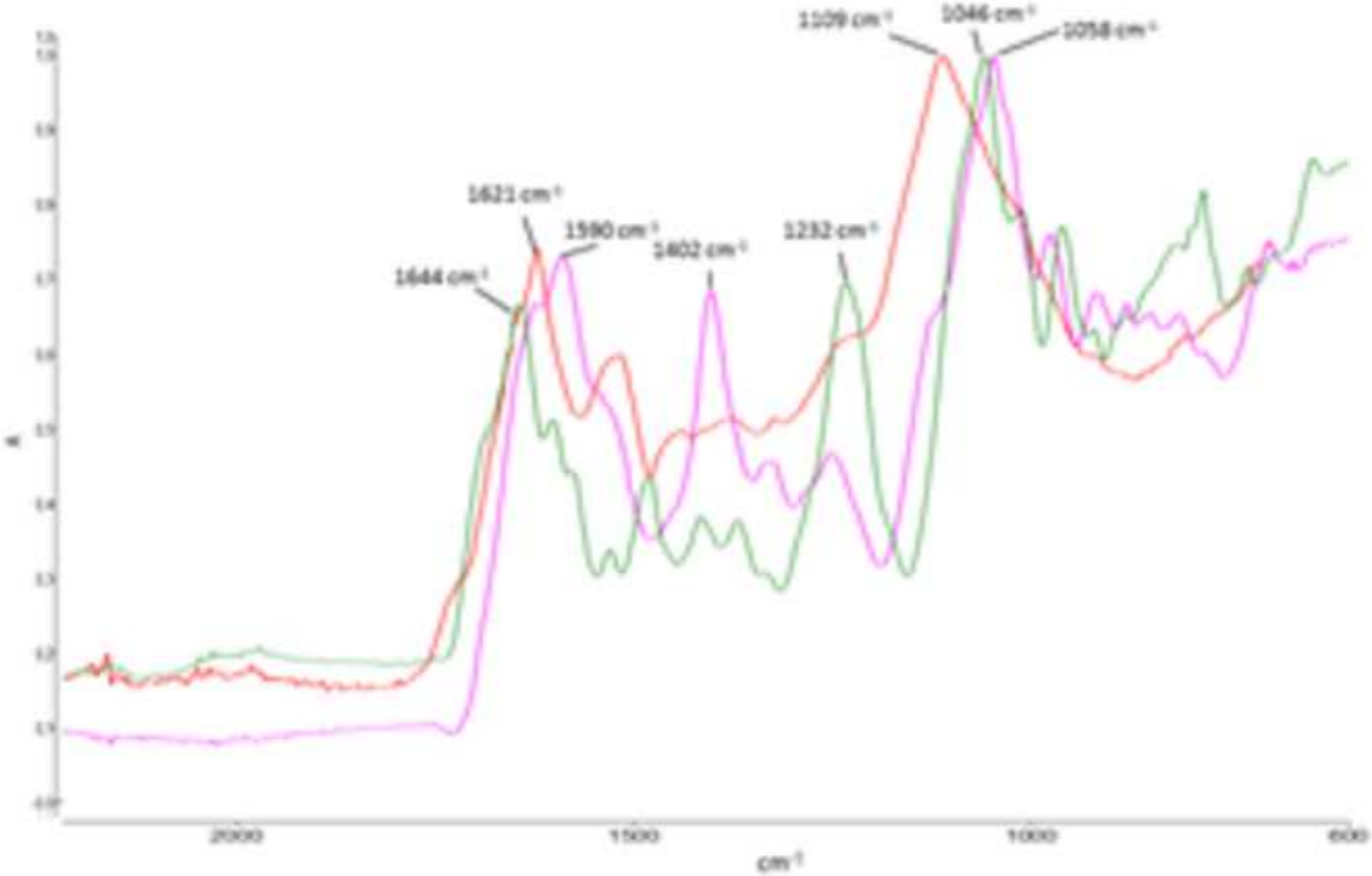
1644 cm⁻¹
1621 cm⁻¹
1590 cm⁻¹
1402 cm⁻¹
1232 cm⁻¹
1109 cm⁻¹
1046 cm⁻¹
1058 cm⁻¹
2000
1500
1000
cm⁻¹

Figure 6

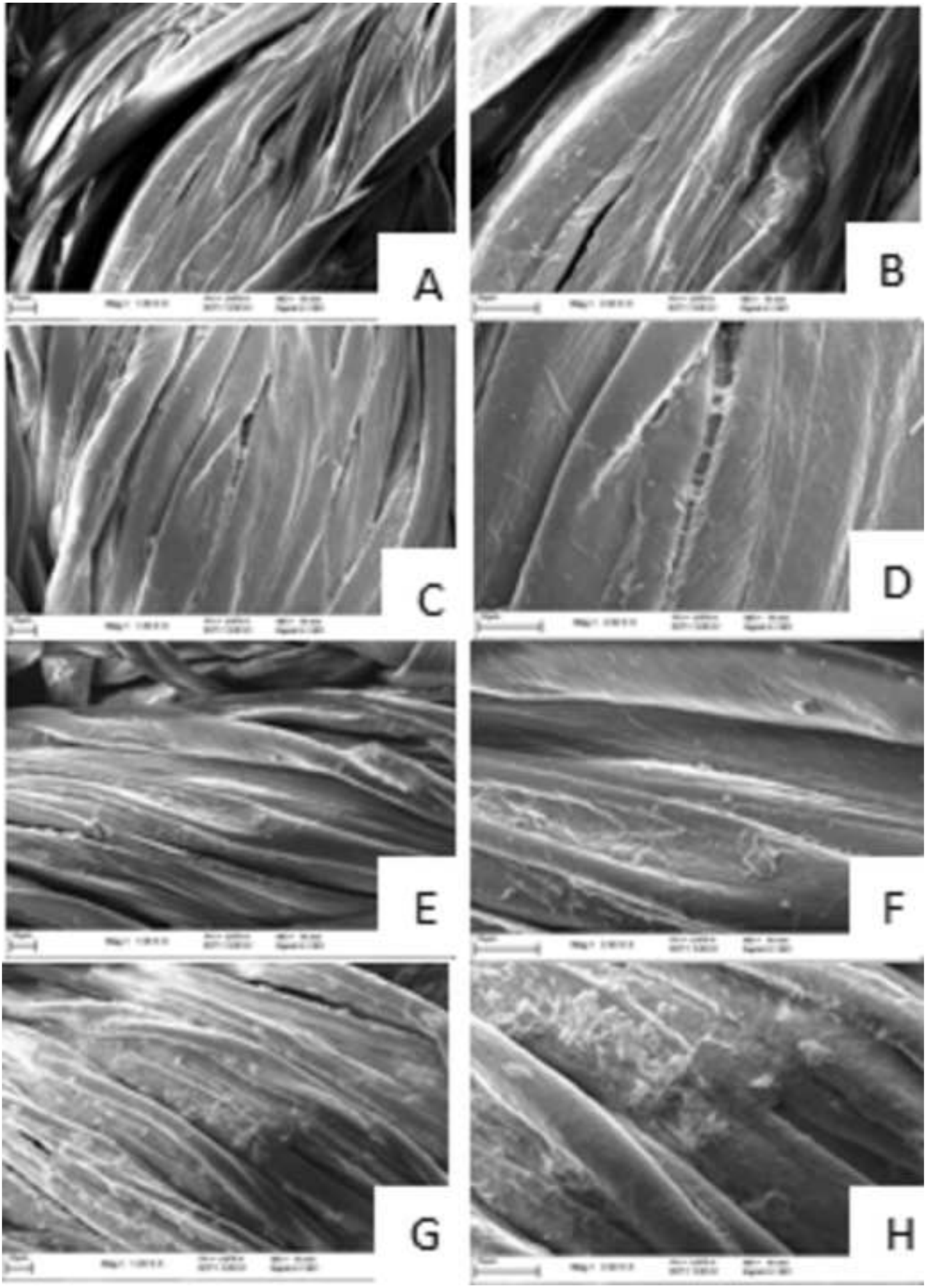

Figure 7

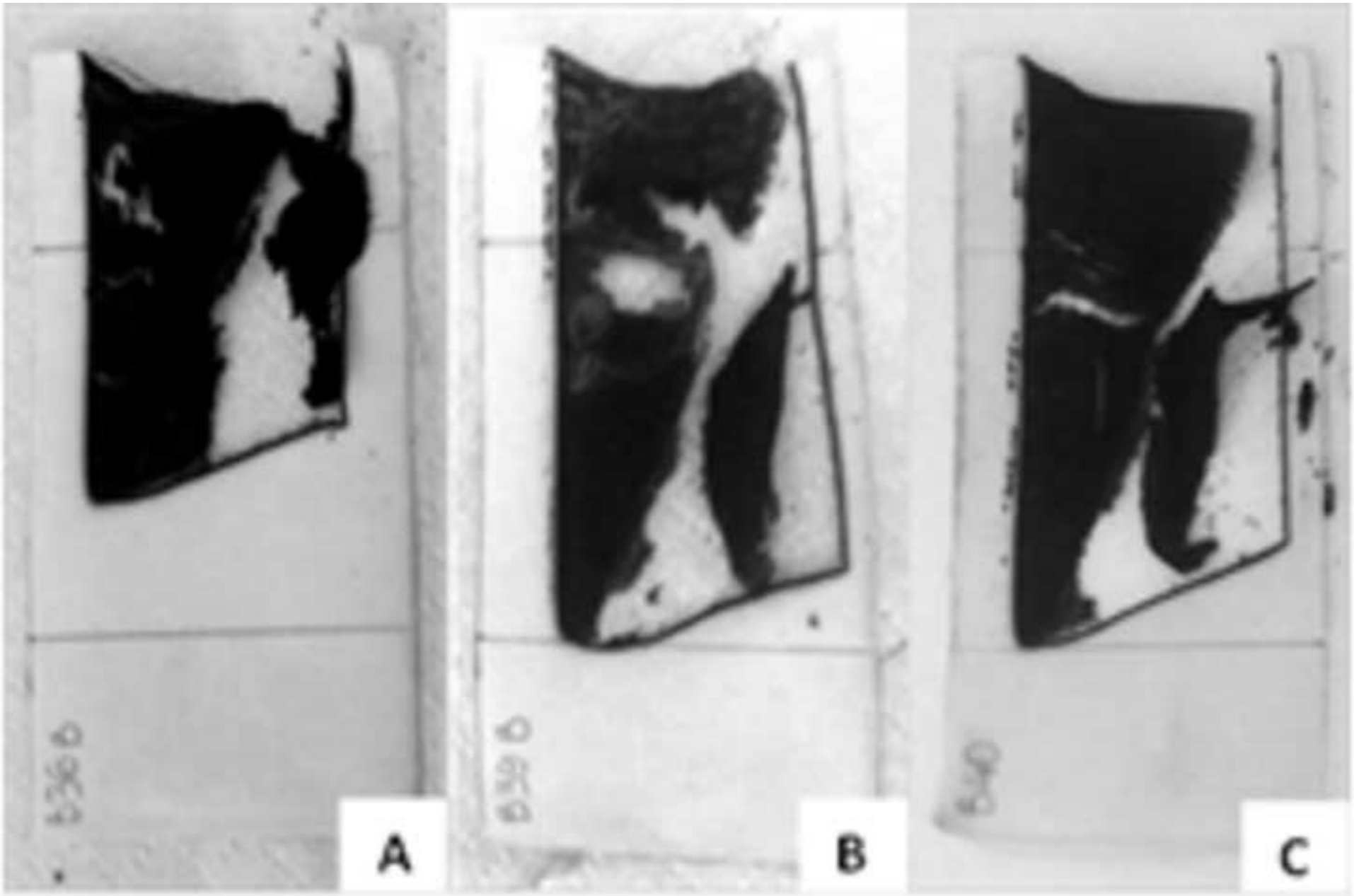

Figure 8

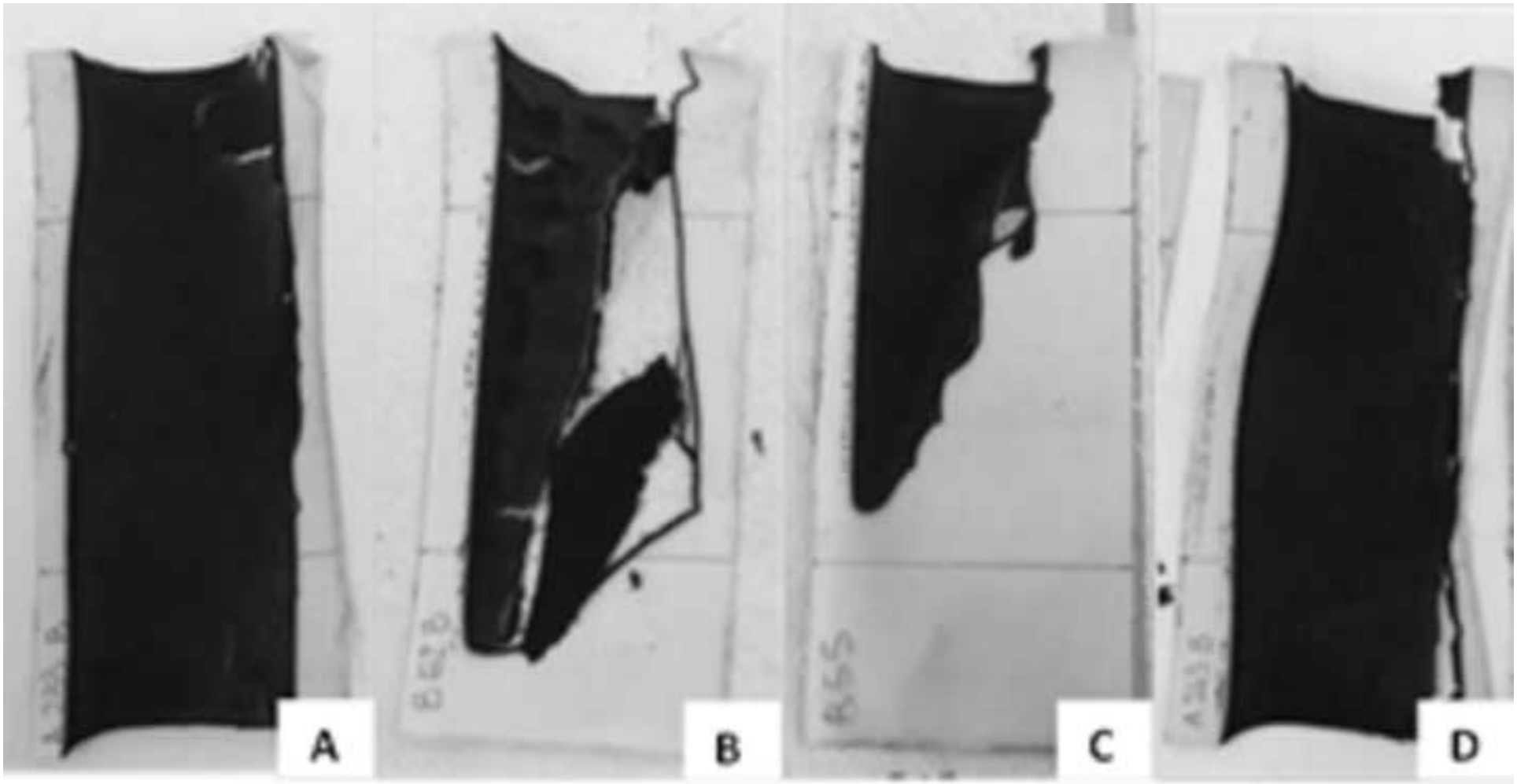

Figure 9

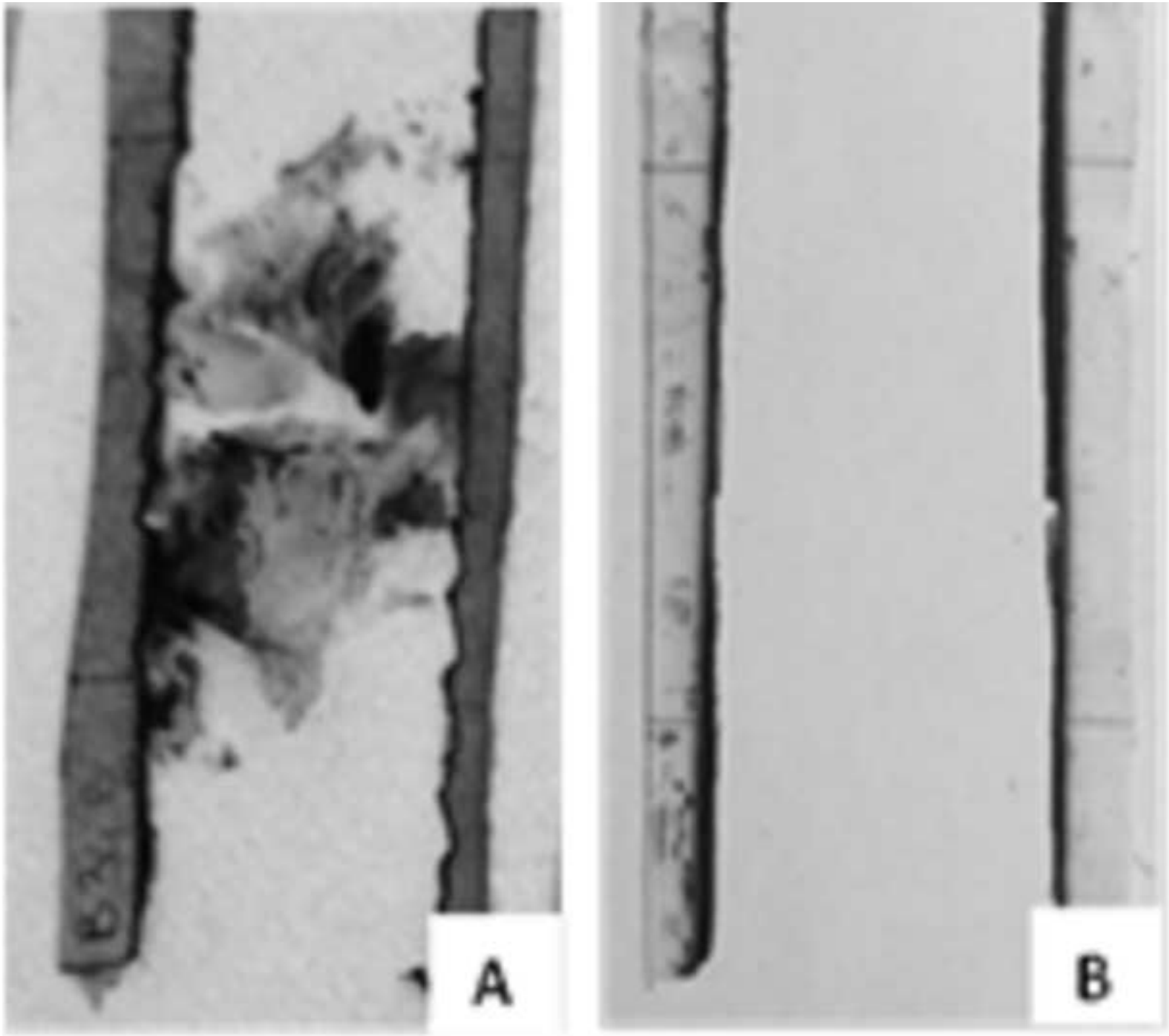

Figure 10