

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

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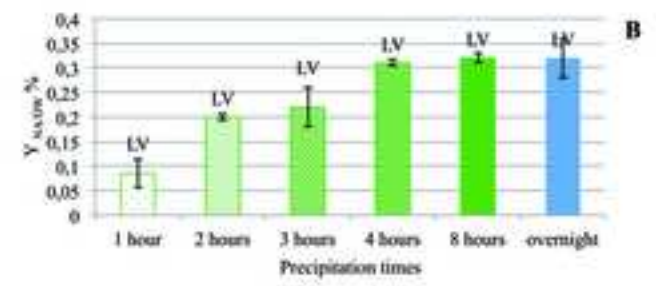
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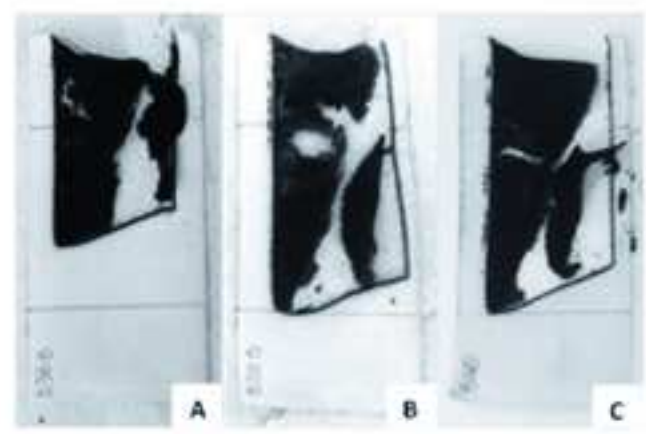
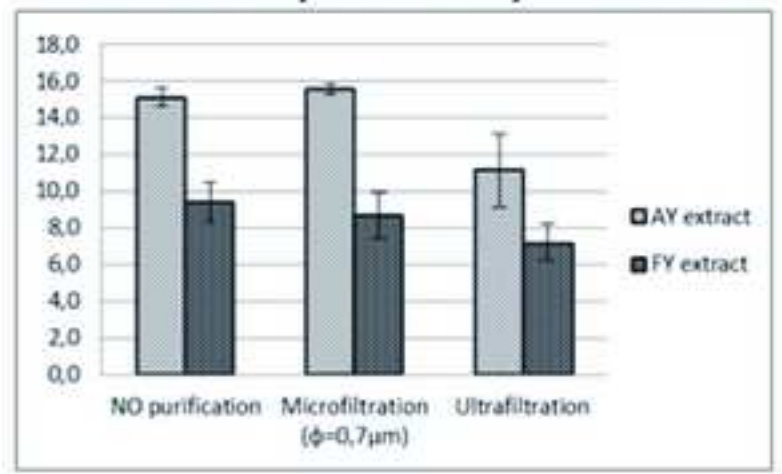
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(Article begins on next page)

### 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

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

3 *Francesca Bosco<sup>a</sup>, Annalisa Casale<sup>a,b</sup>, Giorgio Gribaudo<sup>c</sup>, Chiara Molleda<sup>a</sup>, Giulio Malucelli<sup>b\*</sup>*

4 *<sup>a</sup>Department of Applied Science and Technology, Politecnico di Torino, C.so Duca degli Abruzzi*  
5 *24, 10129 Torino, Italy.*

6 *<sup>b</sup>Department of Applied Science Technology, Politecnico di Torino, Viale T. Michel 5, 15121*  
7 *Alessandria, Italy.*

8 *<sup>‡</sup>Department of Life Sciences Systems Biology, Laboratory of Microbiology and Virology, Via*  
9 *Accademia Albertina 13, 10123 Torino, Italy.*

10 \* corresponding author. Phone: +39-0131229369; Fax: +39-0131229399 e-mail:

11 [giulio.malucelli@polito.it](mailto:giulio.malucelli@polito.it)

12 **ABSTRACT**

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

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

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

29

## 30 **1. Introduction**

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

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

41 products have to be found in order to replace the “traditional” FRs that, despite their good  
42 efficiency, may suffer from some environmental drawbacks. One of the latest approaches, which  
43 is worth of further investigation, is that of the use of biomacromolecules, such as proteins and  
44 DNA, as FRs, thanks to their chemical structures and composition. In fact, some of them have  
45 already been proven to be very effective at a lab-scale, exploiting green finishing processes (i.e.  
46 by applying aqueous solutions/dispersions directly to different textiles, using traditional  
47 processes, such as impregnation/exhaustion and spraying). Recently, low molecular weight DNA  
48 was employed as an effective intumescent flame retardant coating for cotton fabrics, since it has  
49 demonstrated to provide self-extinction in horizontal flame spread tests and a substantial  
50 reduction in the heat release rate in cone calorimetry tests (Alongi et al., 2013; Bosco et al.,  
51 2015). The DNA’s fire retardant activity has been ascribed to the simultaneous presence of  
52 phosphate groups, nitrogen bases and deoxyribose sugars in the same molecule, which makes the  
53 biomacromolecule to be considered as an intumescent-like product. On the other hand, proteins  
54 derived from milk, such as caseins and whey proteins, show a fire retardant effect when  
55 deposited on cotton fabrics. Although they cannot provide self-extinction of the flame, they are  
56 able to significantly reduce the burning rate and increase the final residue (Alongi et al., 2014;  
57 Bosco et al., 2013).

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

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

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

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

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

86 and minerals), can also be mentioned (Supphantharika et al., 2003; Podpora et al., 2015).  
87 Vegetable wastes from food industry plants are discharged in remarkable quantities. The  
88 quantities and compositions of these wastes depend, to a great extent, upon the seasonal  
89 periodicity of the agricultural productions. They are mainly utilized for the production of  
90 compost, livestock feeding and fermentation for biogas or ethanol production (Di Donato et al.,  
91 2014; Nagarajan et al., 2014; Bouallagui et al., 2005).

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

98

## 99 **2. Materials and methods**

### 100 *2.1 Materials*

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



107 courgettes, potatoes, carrots and pumpkin. The vegetable matrices considered have been  
108 indicated as follows: mixed leafy vegetables (MLV) and leafy vegetables (LV). MLV mainly  
109 comprised leafy vegetables and a small portion of vegetable pieces (about 10%), while LV  
110 matrix was only composed of leafy vegetables. The matrices were separated into small portions  
111 (500 g) and stored at +4°C (for a maximum of 48 hours) or at -20°C before use.

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

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

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

## 121 *2.2 NA extraction from the mixed vegetables*

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

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

131 Different parameters were changed, one at a time, to optimize the extraction procedure, as  
132 follows: i) the starting material was either stored at -4°C or at -20°C before extraction; ii) 99.8%  
133 ethanol was further diluted with bi-distilled water to 96 and 90% purity grade and used for the  
134 precipitation step; iii) the precipitation was carried out at two different temperatures (+4 and  
135 -20°C); iv) the precipitation was performed at -20°C for different incubation times, namely 1, 2,  
136 3, 4, 8 hours and overnight.

### 137 *2.3 NAs extraction from the spent brewer's yeast*

138 Beer slurries were stored in two different ways before NAs extraction: one batch was  
139 maintained at +4°C for more than 6 months, and, just before extraction, the yeast cells were  
140 centrifuged and washed three times with bi-distilled water (AY); in a second batch, the cells  
141 were immediately centrifuged, washed three times with bi-distilled water and the pellet stored at  
142 -20°C until they were used (FY).

143 Each of the extraction buffers listed hereafter was added at a ratio of 1 ml per 8mg of the yeast  
144 pellet weight. The mixture was frozen at -20°C for 24 hours and then thawed three times at 65°C  
145 for 15min. After thawing, cells were mechanically disrupted using 1 and 2 mm diameter glass  
146 beads (1mg:5g yeast:glass beads ratio) and the mixtures were then subjected to ball milling for  
147 10 min. Glass beads were then recovered and cell debris removed by means of centrifugation  
148 (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  $\mu\text{m}$ )  
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) TrisHCl 50 mM + NaCl 50 mM + EDTA 10 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 ( $\phi=1.2 \mu\text{m}$ ), 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

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

$$169 \quad C_{NA} (\mu g/ml) = A \cdot DF \cdot 40 \quad (\text{Eq. 1})$$

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

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

$$175 \quad Y_{NA/DW} \% = \frac{(NA(mg))}{(Starting\ dry\ weight\ material\ (mg))} 100 \quad (\text{Eq. 2})$$

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

#### 178 *2.4.2 Agarose gel electrophoresis*

179 Aliquots of the different NA samples were digested with a DNAase-free RNase (20  $\mu\text{g/ml}$ ),  
180 and then analyzed by means of gel electrophoresis on 0.8% w/v agarose gels (Sigma-Aldrich  
181 S.r.l. Milano, Italy) at 5 V/cm for 2 h in a 1  $\times$  Tris–acetate–ethylenediaminetetraacetic acid  
182 (TAE) buffer containing 0.5  $\mu\text{g/mL}$  of ethidium bromide (Sigma-Aldrich S.r.l. Milano, Italy). A  
183 1 kb molecular ruler (Thermo Scientific) was run with DNA samples in order to estimate the  
184 lengths. Gel pictures were taken using a Gel Doc XR+ Imaging System (BioRad).

185

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  $4\text{ cm}^{-1}$  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 ( $5 \times 5\text{ mm}^2$ ) 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  $\text{HNO}_3/\text{HClO}_4$  aqueous solution (4:1 molar ratio) at  $50^\circ\text{C}$ , 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 
$$AO\% = \frac{W_f - W_i}{W_i} \cdot 100 \quad (\text{Eq. 3})$$

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 ( $50 \times 100$   
224  $\text{mm}^2$ ), which were clamped in a U-shaped metallic frame, tilted  $45^\circ$  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

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

230

### 231 **3. Results and discussion**

#### 232 *3.1 NAs extraction from the mixed vegetable wastes*

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

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

240 First, a set of preliminary trials was performed to evaluate the influence of the storage  
241 conditions on the vegetable matrix. For this purpose, the NAs extraction was carried out on  
242 either frozen or fresh MLV and LV samples. Freezing is an additional step that offers the  
243 advantage of preserving vegetables for long periods before they are used. The frozen vegetable  
244 mixture was thawed, ground and fully mushed up, while the fresh material was sent directly for  
245 grinding. As reported in Table 1, the extraction yields obtained from the frozen material were  
246 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



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

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

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

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

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

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

303 Third, after extraction at 65°C for 30 minutes and filtration, the NAs were precipitated with  
304 90% ethanol for 4 hours at -20°C. These optimized conditions were adopted to process the LV  
305 matrix, in order to obtain sufficient NAs amount to impregnate the cotton fabrics. In this context,  
306 it was mandatory to scale-up the whole process by increasing the starting material five-fold. In  
307 particular, it was necessary to adjust the extraction temperature, which was raised to 68°C, and  
308 the time, which was doubled (60 minutes). However, the scale-up did not affect the recovery  
309 yields. In fact, the obtained value was equal to 0.33% and comparable with that obtained  
310 previously in small-scale experiments using the same recovery conditions. Thus, it was possible  
311 to utilize a solution with a NA concentration equal to 1.7 mg/ml and with a purity degree  
312 ( $OD_{260/280}$ ) of 1.11, suitable for impregnating the cotton fabrics.

### 313 *3.2 NAs extraction from spent brewer's yeast*

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

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

#### 329 *3.2.1 Optimization of the extraction buffer*

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

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

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

347 The different extraction buffer efficacy was evaluated for both the AY and the FY starting  
348 materials. The NAs yield (%) and purity values, calculated after the centrifugation step, are  
349 shown in Table 2. For AY, high yields were obtained with TNE 50/50/10 (13.1%), TNE  
350 50/100/10 (13.1%) and PB (13.5%). Conversely, low yields were observed for TN 50/50 and TN  
351 50/100 (11.1 and 11.6%, respectively). When the yields and purity values were combined, TNE  
352 50/100/10 and PB resulted to be the best extraction solutions. In the case of FY, the results were  
353 similar for all of the utilized extraction solutions and both the yield and purity values were lower,  
354 compared to those of AY.

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

### 358 *3.2.2 Optimization of the ball milling step*

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

### 369 *3.2.3 Optimization of post-extraction purification steps*

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

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

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

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

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

398 *3.3 Characterization of the extracted DNA*

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

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

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

420  $\text{cm}^{-1}$ , attributable to the nitrogen bases. Some absorption peaks with different intensity at about  
421  $1400 \text{ cm}^{-1}$ , 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<sup>4</sup>. 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).

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

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

470 Since phosphorus is a key component of NAs, and is necessary to provide flame retardant  
471 features, ICP-MS was exploited for assessing the phosphorous content of the cotton fabrics  
472 treated with the NA extracted from yeast. The ICP-MS measurements showed that the  
473 concentration of this element in the fabrics coated by AY- and FY-derived NA samples was 0.47  
474 wt.%, hence comparable to that obtained for the commercial DNA coating (0.5%) (Bosco et al.,  
475 2015).

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

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

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

492

#### 493 **4. Conclusion**

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

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

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

#### 509 **Conflict of interest**

510 None.

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514

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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 ( $\phi=0.7\mu\text{m}$ ), 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 ( $\phi=0.7\mu\text{m}$ ), 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 ( $\pm 0.008$ ) | 0.07 ( $\pm 0.010$ ) |
| Frozen | 0.14 ( $\pm 0.006$ ) | 0.12 ( $\pm 0.008$ ) |

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

| Extraction solutions | AY          |                                 | FY         |                                 |
|----------------------|-------------|---------------------------------|------------|---------------------------------|
|                      | Yield (%)   | Purity (OD <sub>260/280</sub> ) | Yield (%)  | Purity (OD <sub>260/280</sub> ) |
| 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

| Grinding time | Yield %     | AY           |                                    | FY           |                                    |
|---------------|-------------|--------------|------------------------------------|--------------|------------------------------------|
|               |             | Yield %      | Purity<br>(OD <sub>260/280</sub> ) | Yield %      | Purity<br>(OD <sub>260/280</sub> ) |
| 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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616 **Table 4.** Combustion data of the cotton fabrics coated with different extracted NAs obtained  
 617 from horizontal flame spread tests.

|   | Total<br>burning<br>time (s) | Char<br>length<br>(mm) | Total<br>burning rate<br>(mm/s) | Residue<br>(%) | Self-extinction |
|---|------------------------------|------------------------|---------------------------------|----------------|-----------------|
| COT   | 66                           | 100                    | 1.5                             | 0              | NO              |
| AY extract  | 51 (181)*                    | 60                     | 1.17                            | 60             | YES             |
| AY extract after<br>microfiltration ( $\phi=0.7\mu\text{m}$ ) | 65 (203)*                    | 75                     | 1.15                            | 50             | YES             |
| AY extract after<br>ultrafiltration                           | 64 (227)*                    | 75                     | 1.15                            | 47             | YES             |
| FY extract  | 95                           | 100                    | 1.05                            | 38             | NO              |
| FY extract (Celite<br>filtration)                             | 80                           | 87                     | 1.08                            | 49             | YES             |
| FY extract after<br>microfiltration ( $\phi=0.7\mu\text{m}$ ) | 61                           | 70                     | 1.15                            | 72             | YES             |
| FY extract after<br>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              |

618

619 \* Afterglow time

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

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Figure 1

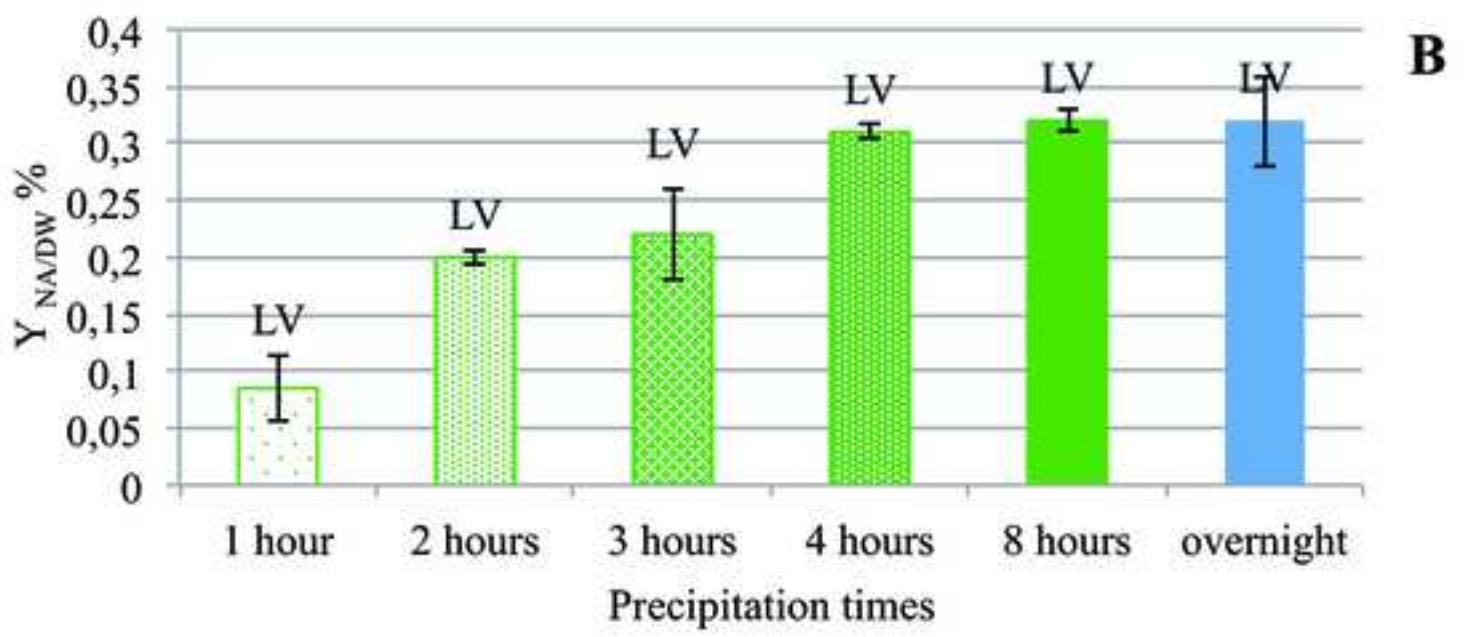
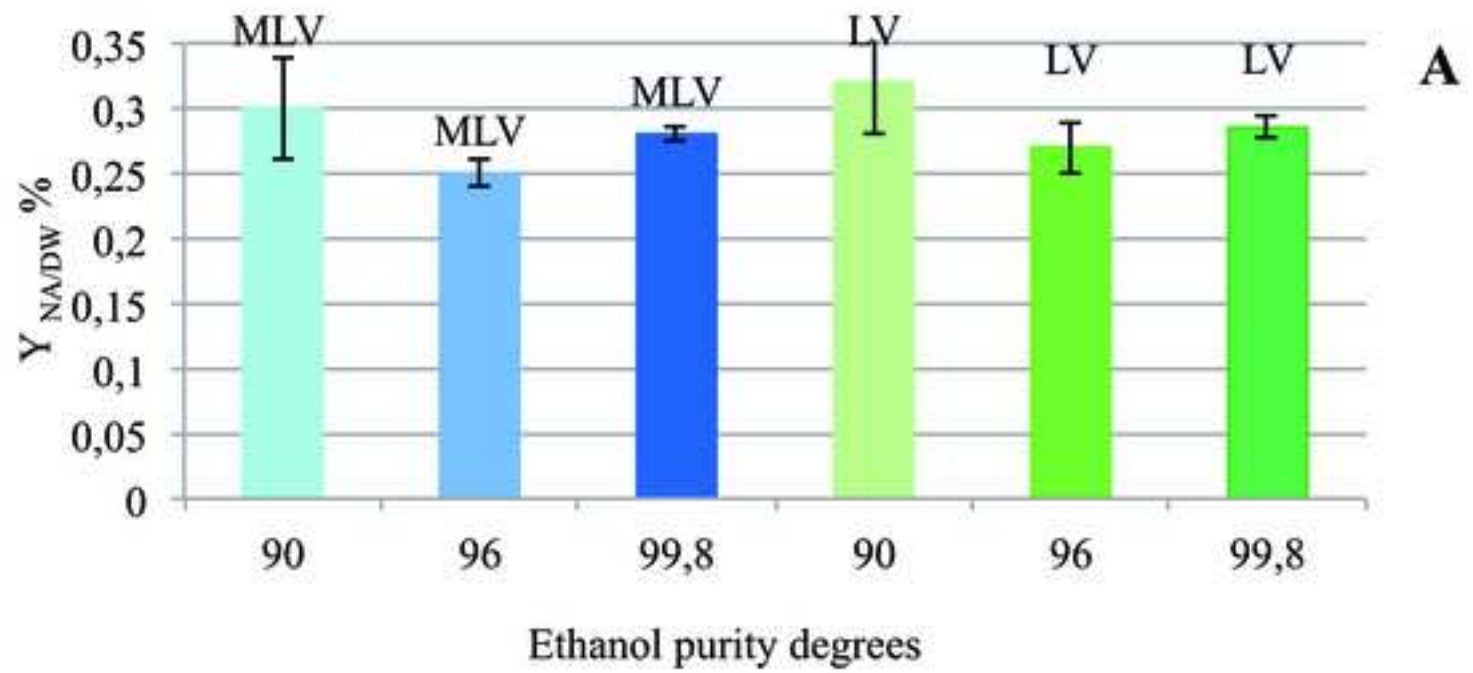


Figure 2

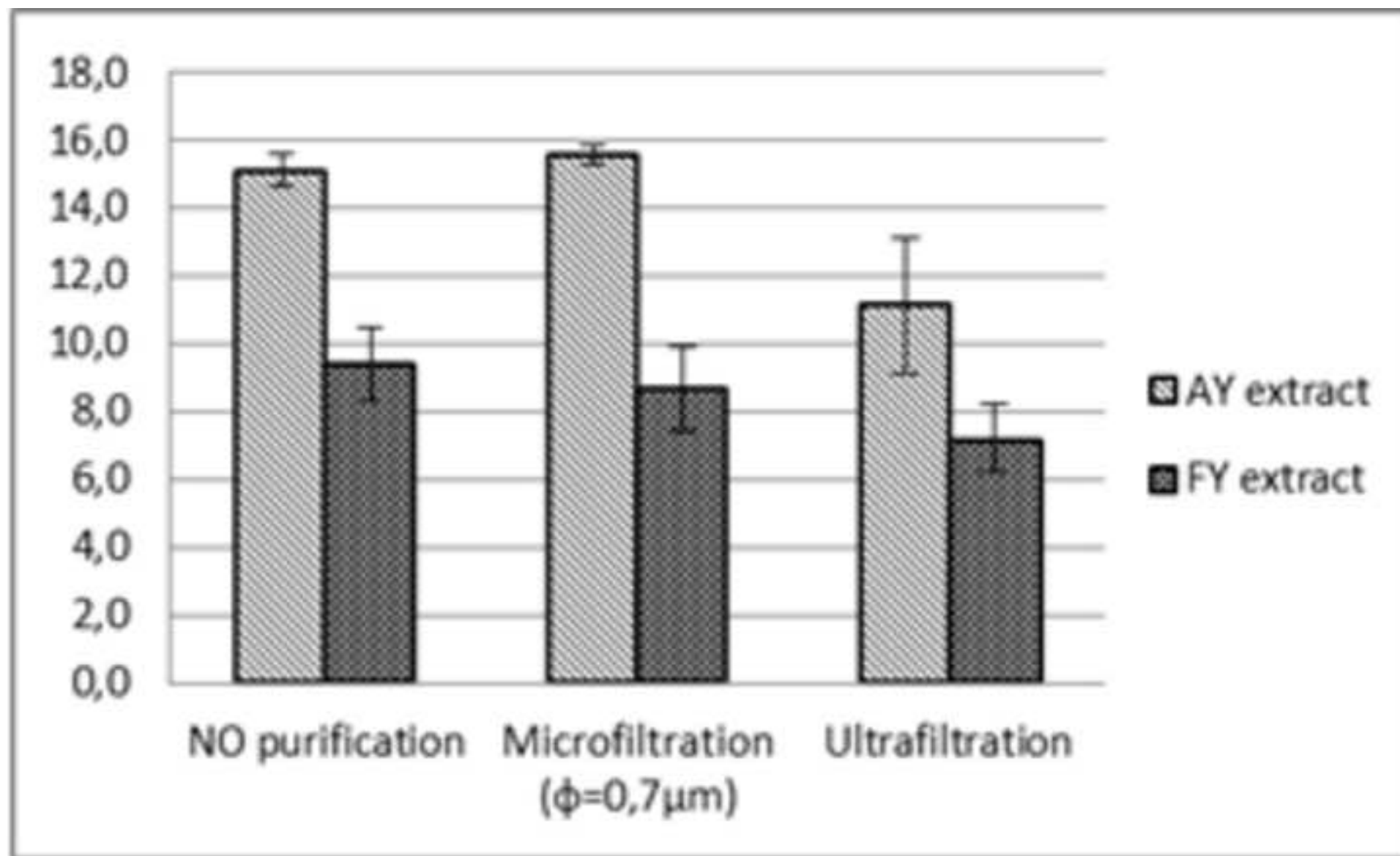


Figure 3

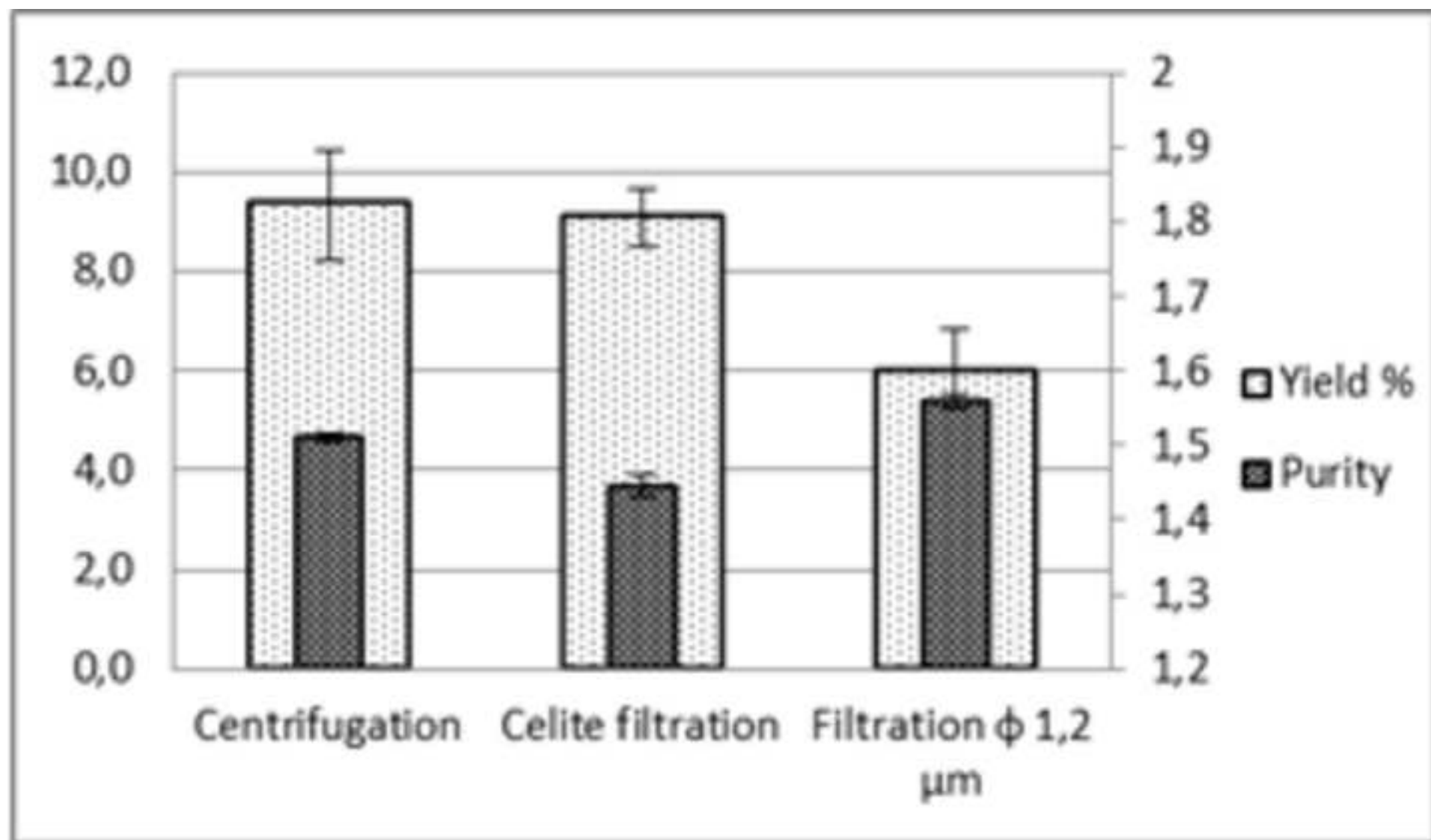




Figure 4

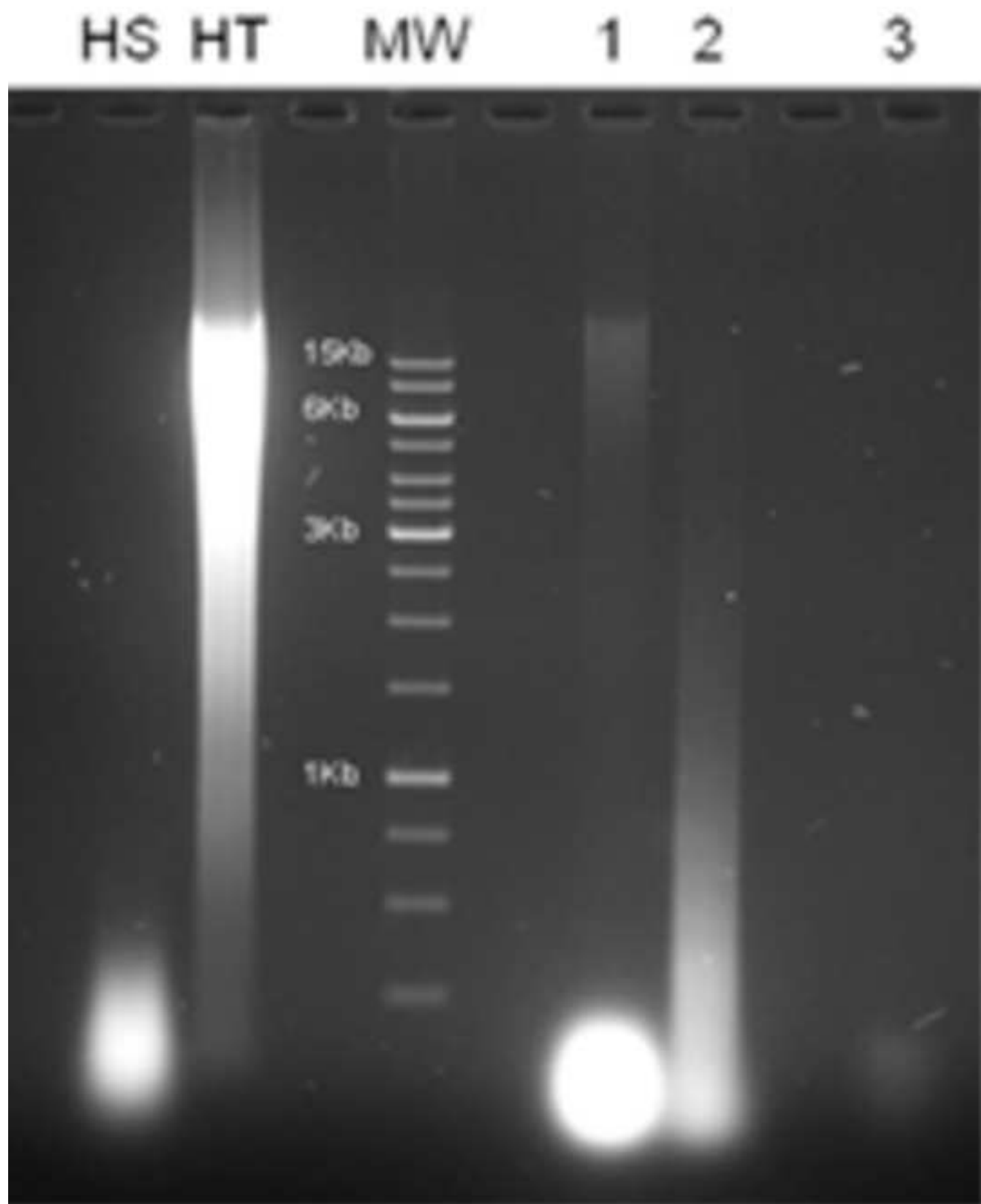


Figure 5

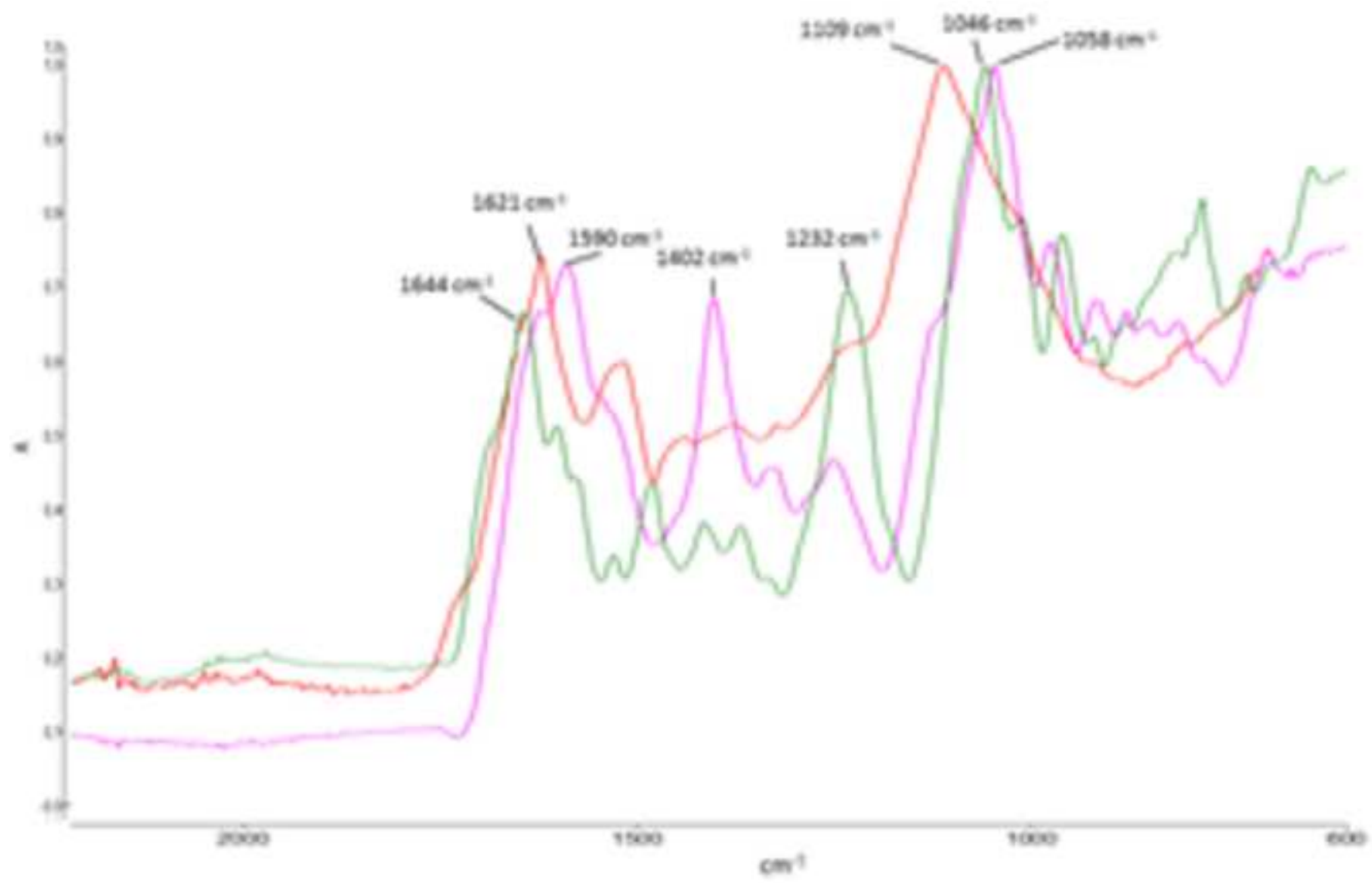


Figure 6

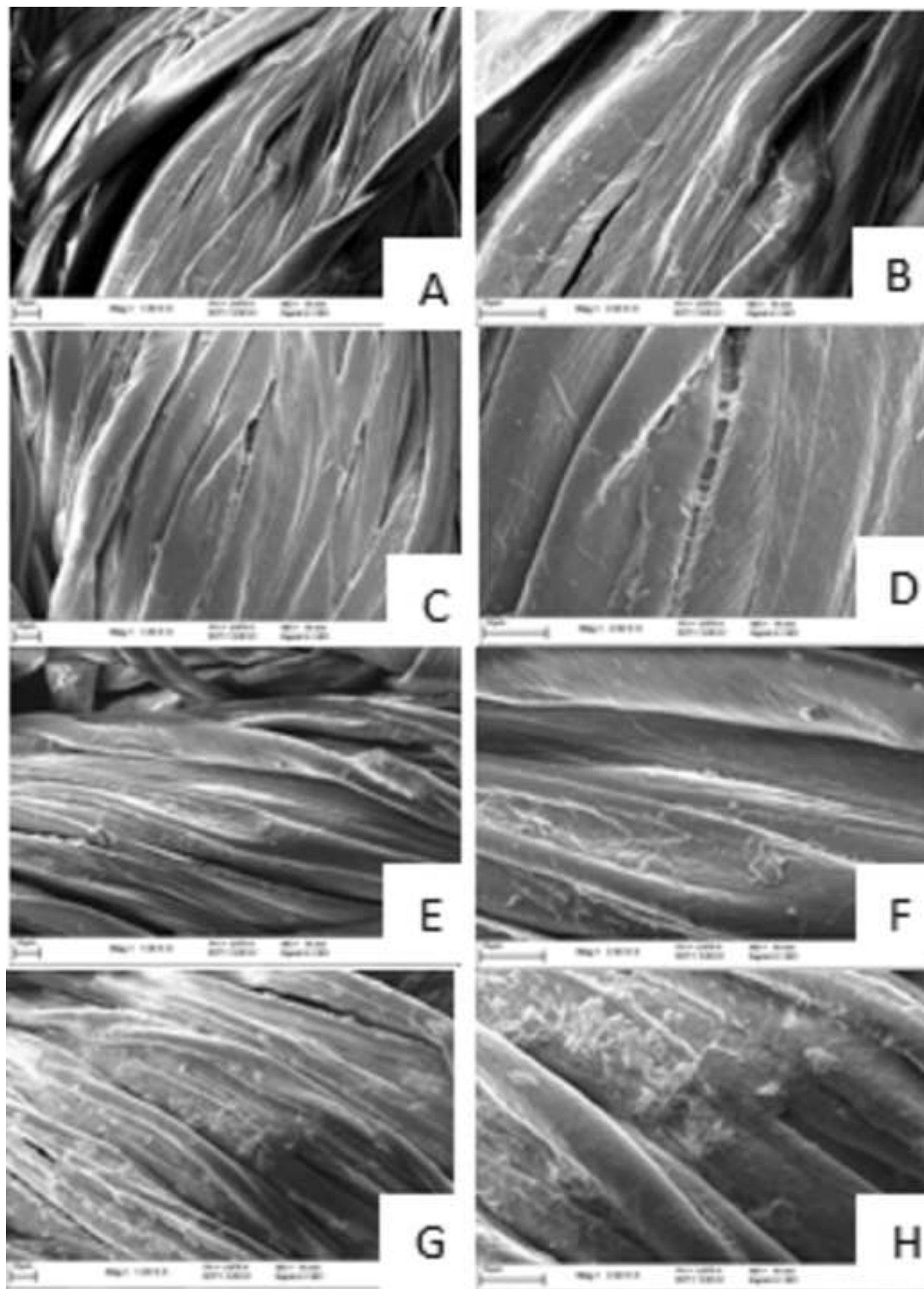


Figure 7

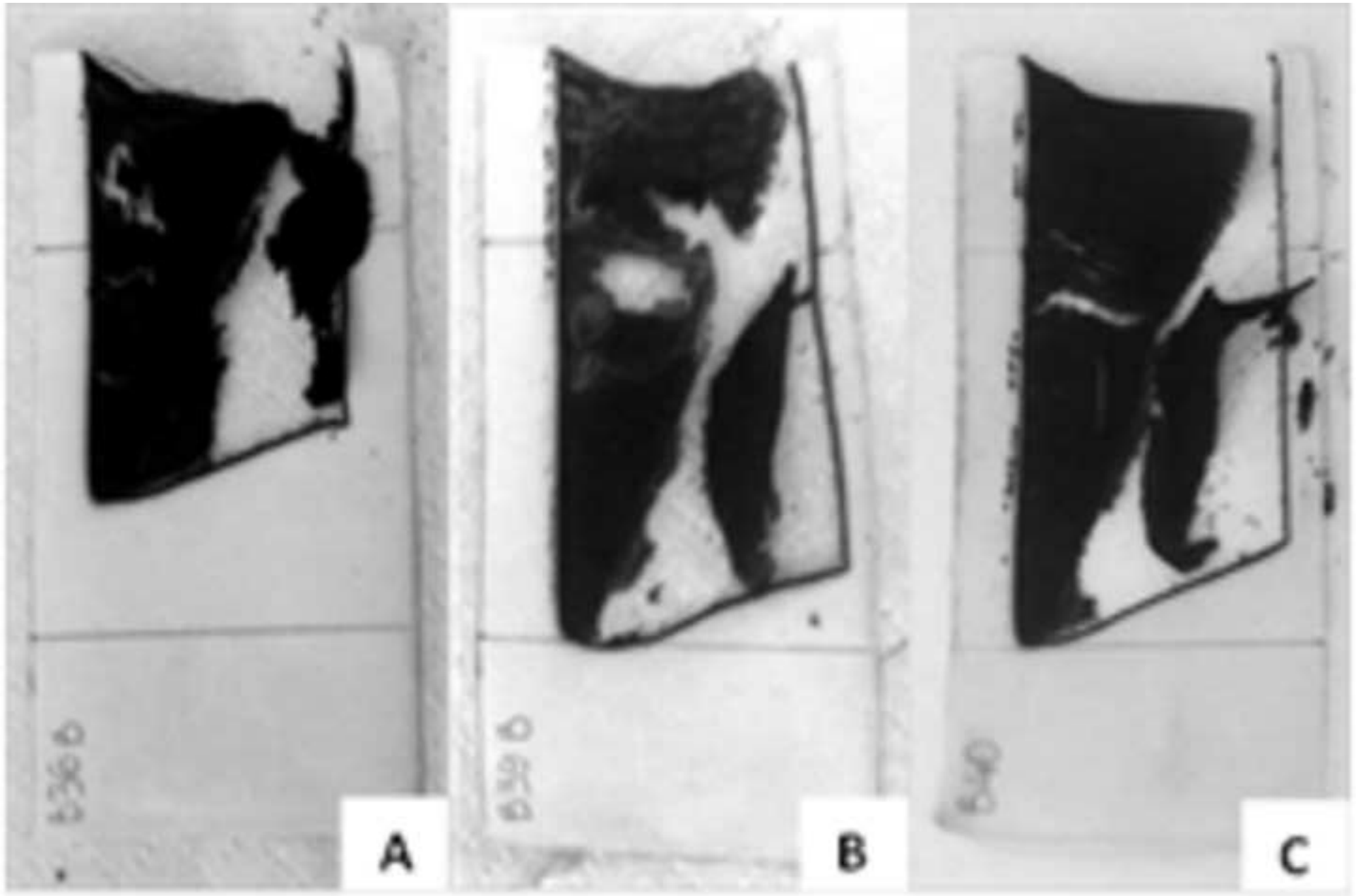


Figure 8

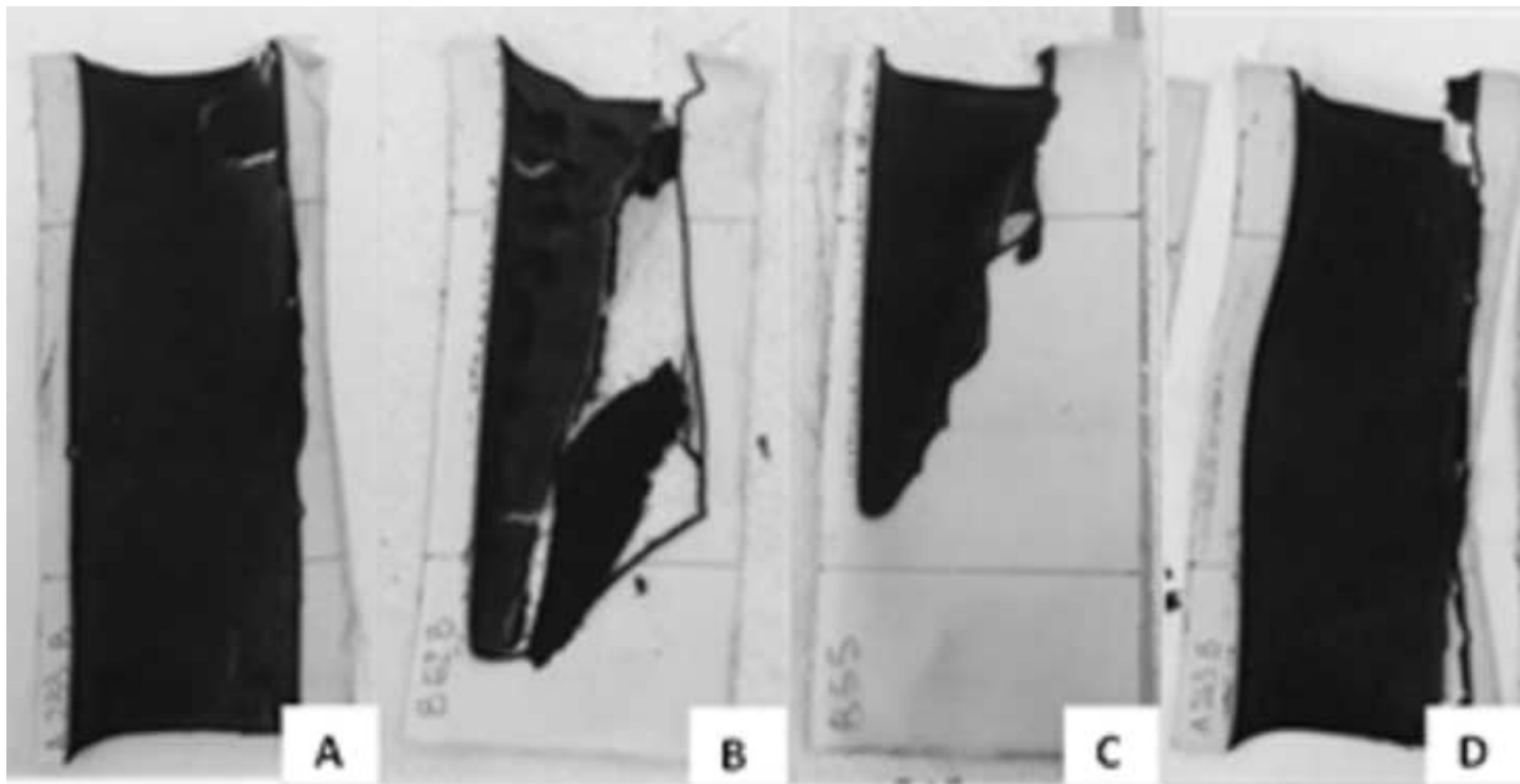


Figure 9

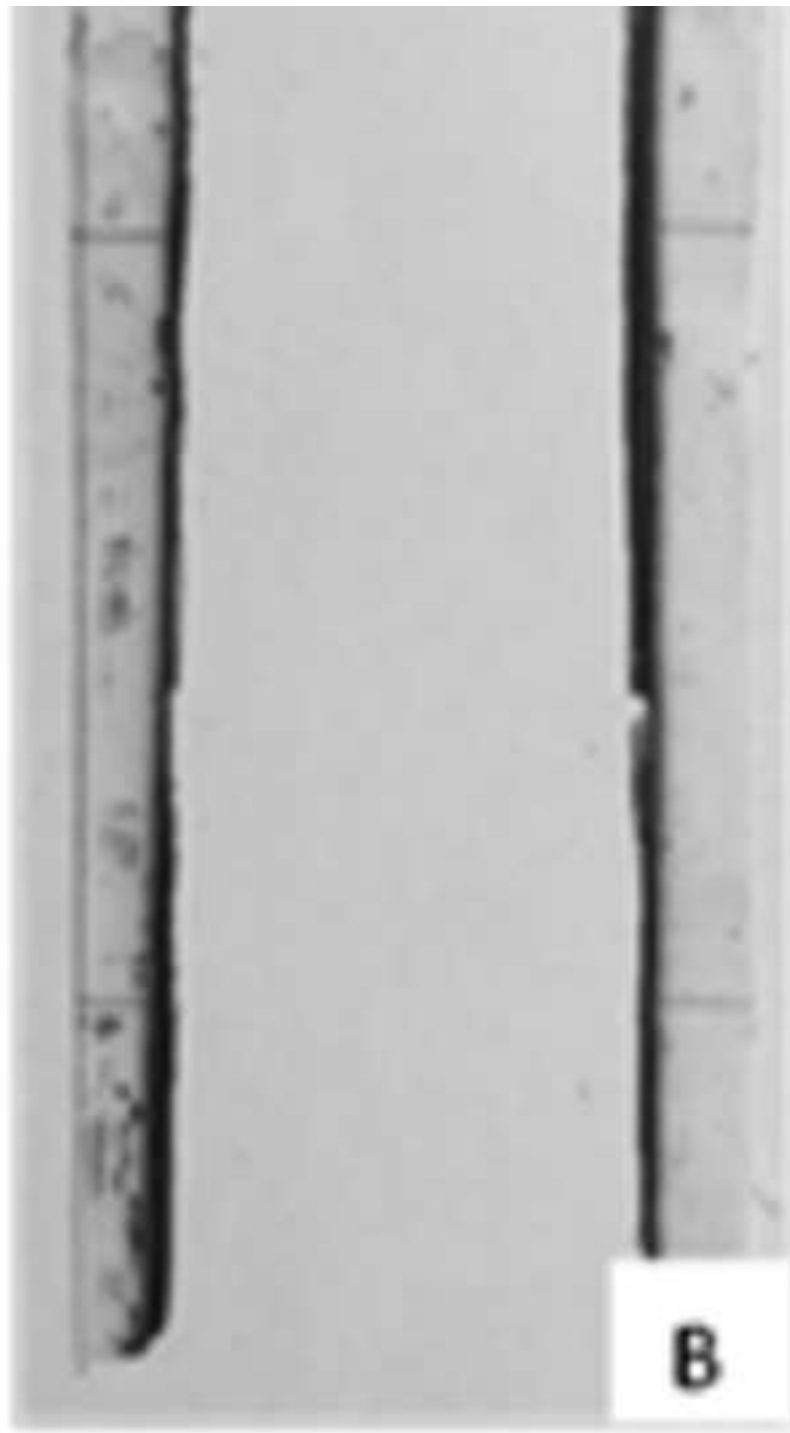
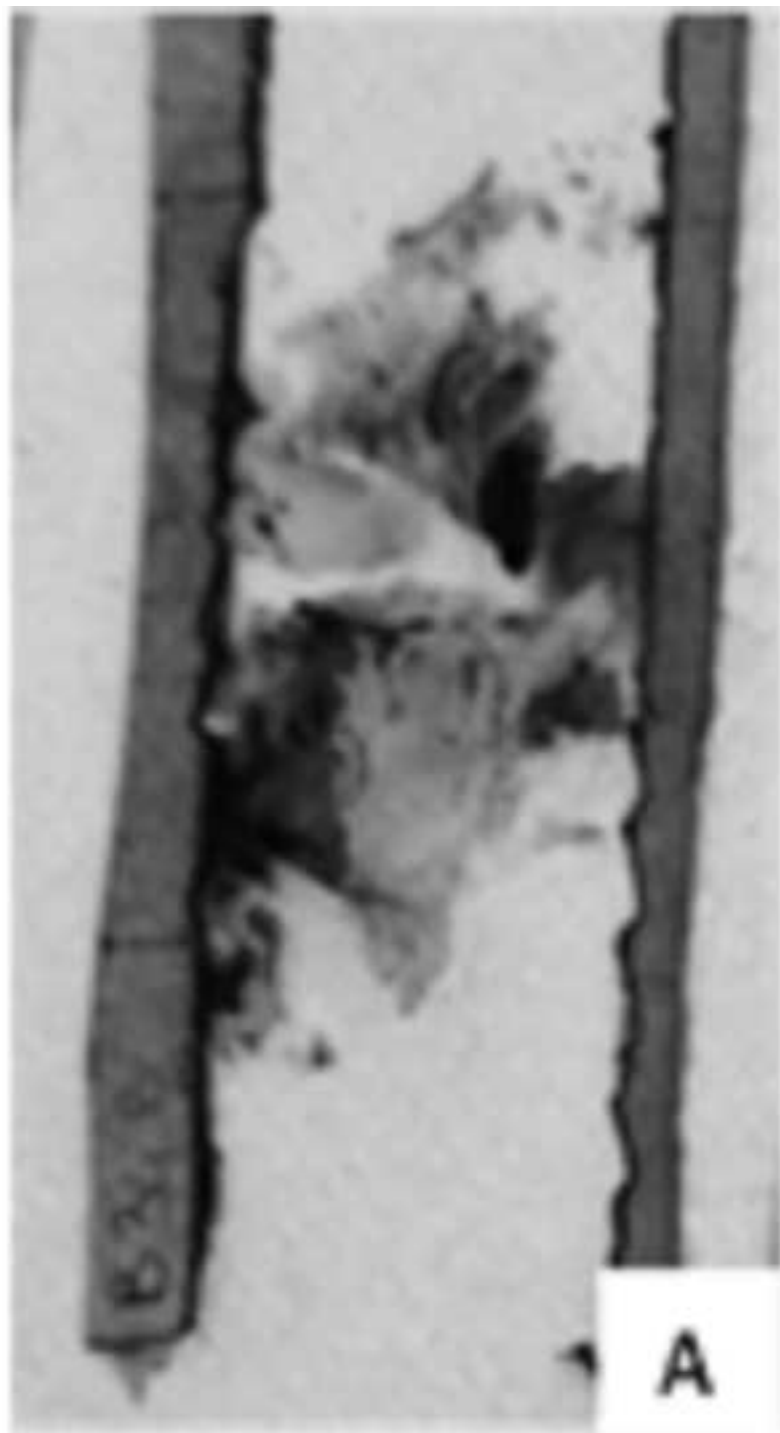


Figure 10

