Optimizing a sustainable ultrasound assisted extraction method for the recovery of polyphenols from lemon by-products: comparison with hot water and organic solvent extractions

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Abstract

Response surface methodology (RSM) based on a three-factor and three-level Box-Behnken design was employed for optimizing the aqueous ultrasound-assisted extraction (AUAE) conditions, including extraction time (35-45 min), extraction temperature (45-55 °C) and ultrasonic power (150-250W), for the recovery of total phenolic content (TPC) and rutin from lemon by-products. The independent variables and their values were selected on the basis of preliminary experiments, where the effects of five extraction parameters (particle size, extraction time and temperature, ultrasonic power and sample-to-solvent ratio) on TPC and rutin extraction yields were investigated. The yields of TPC and rutin were studied using a second-order polynomial equation. The optimum AUAE conditions for TPC were extraction time of 45min, extraction temperature of 50°C and ultrasonic power of 250W with a predicted value of 18.10±0.24 mg GAE/g dw, while the optimum AUAE conditions for rutin were extraction time of 35min, extraction temperature of 48°C and ultrasonic power of 150W with a predicted value of 3.20±0.12 mg/g dw. The extracts obtained at the optimum AUAE conditions were compared with those obtained by a hot water and an organic solvent conventional extraction in terms of TPC, total flavonoid content (TF) and antioxidant capacity. The extracts obtained by AUAE had the same TPC, TF and ferric reducing antioxidant power as those achieved by organic solvent conventional extraction. However, hot water extraction led to extracts with the highest flavonoid content and antioxidant capacity. Scanning electron microscopy analysis showed that all the extraction methods led to a cell damage to varying extents.

Keywords: citrus peels; polyphenols; ultrasound; antioxidant capacity; advanced extraction; flavonoids

Introduction

Citrus is one of the most important horticultural crops in the world, with a worldwide production exceeding 121 million tons (Data 2013/14) [1]. Juice industry uses about 34% of citrus production and high amounts of by-products are generated during this procedure. Peel and seed residues are the main by-product and may account for up to 50% of the total fruit weight [2]. Citrus peel has been shown to be a good source of phenolic compounds, including phenolic acids and flavonoids (flavanones, flavones, and flavonols) [3], which have been linked to antiradical activities (*in vitro*) [4], antifungal activities against plant pathogens (*in vivo* and *in vitro*) [5,6], as well as anticancer activities (*in vivo* and *in vitro*) [7].

Rutin (quercetin-3-O-rutinoside) is a flavonoid glucoside which is found in lemon rinds [3]. Rutin is a compound of a high commercial value due to its potential health benefits. *In vitro* experiments have shown that rutin exhibits antiradical activity and may inhibit lipid peroxidation [8], while *in vivo* experiments in rats revealed the protective effects of rutin against histopathological changes of kidney induced by chemotherapeutic agents [9].

Extraction is the first step in the recovery of polyphenols with the solvent type being considered as one of the most important parameters affecting their recovery [10]. Organic solvents, including methanol, ethanol, acetone and their mixtures with water are commonly used for the recovery of polyphenols from citrus wastes [3]. However, their use should be reconsidered due to their high toxicity which negatively affects human health and environment. Water should be considered as an alternative solution, however, it can lead to lower polyphenol recovery yields compared to those obtained by organic solvents [11]. The greater polyphenol extraction yields obtained by the use of organic solvents have been attributed to their polar organic character which successfully solvates a wide range of compounds and their ability to limit polyphenol oxidase (PPO) activity which is an enzyme responsible for the oxidation of phenolic compounds [12].

77 Phenolic compounds are confined to the plant vacuole, thereby their recovery yields are
78 promoted by the disruption of cell walls of the plant matrix [13]. Ultrasound-assisted extraction
79 is considered as an advanced extraction technique, leading to high recovery yields of bioactive
80 compounds due to cavitation, which causes the breakdown of cell walls, improving diffusion
81 rates [14]. However, during ultrasound extraction process a considerable amount of
82 polyphenols might be degraded due to undesirable extraction conditions (extraction time,
83 extraction temperature, ultrasonic power, etc.), resulting in the loss of polyphenol beneficial
84 properties [15,16]. Therefore, by optimizing the ultrasound extraction conditions, high-quality
85 polyphenol extracts could be obtained for further utilization.
86 Optimization can be performed either by examining the effect of one factor at a time on
87 a dependent variable (known as one-variable-at-a-time technique) or by using multivariate
88 statistical techniques, such as response surface methodology (RSM) [17,18]. RSM is used for
89 optimizing the levels of different parameters at the same time, offering information about
90 interaction or quadratic effects of the independent variables on the dependent variables [19,20].
91 The aims of this study were to: i) investigate the effect of different ultrasonic parameters
92 (particle size, extraction temperature and time, ultrasonic power and sample-to-solvent ratio)
93 on the recovery yields of total phenolic content (TPC) and rutin from lemon by-products, ii)
94 optimize the aqueous ultrasonic assisted extraction conditions for the recovery of TPC and rutin
95 using RSM, iii) compare the polyphenol content and antioxidant capacity of the extracts
96 obtained by the optimized aqueous ultrasound-assisted extraction conditions (AUAE), with
97 those obtained by an optimized hot water extraction method [21] and an organic solvent
98 conventional extraction method [4] in terms of TPC, total flavonoid content (TF), as well as
99 antioxidant capacity, and iv) provide information about cell damage as a result of the different
100 extraction methods.
101

Materials and methods

Chemicals

All chemicals were of analytical grade. Folin–Ciocalteu phenol reagent, sodium carbonate (Na_2CO_3) anhydrous, sodium nitrite ($NaNO_2$), hydrochloric acid (HCl), ferric chloride ($FeCl_3$), gallic acid, catechin, rutin, formic acid, copper (II) chloride ($CuCl_2$), ammonium acetate (NH_4Ac), neocuproine, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Pty Ltd (Castle Hill, Sydney, Australia). Aluminium chloride ($Al_2Cl_3 \cdot 6H_2O$) was obtained from J. T. Baker Chem. Co. (Zedelgem, Belgium). Sodium hydroxide (NaOH) was purchased from Ajax Chem. (NSW, Australia). Sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$) was purchased from Government Stores Department (Sydney, Australia). Glacial acetic acid was obtained from BDH Laboratory Supplies (Poole, UK). Methanol, ethanol and acetonitrile were purchased from Merck (Darmstadt, Germany).

Materials

Lemon (*Citrus limon* L.) by-products, including endocarp residual membranes, seeds, and exocarp, was kindly provided by Eastcoast Beverages, a commercial juicing manufacturer in Kulnura, NSW, Australia. After seed removal, the remaining waste with a moisture content of 85% ± 1% (mean ± standard deviation) was stored at −18 °C until use. Lemon by-products were dried by freeze-drying for 48 h (FD3 freeze dryer; Thomas Australia Pty. Ltd., Seven Hills, Australia) [22]. The dried by-products was ground using a commercial blender (Waring 2-speed blender, John Morris Scientific, Chatswood, Australia) and sieved using steel mesh sieves of three different sizes (1.40, 2.00, 2.80 mm) (EFL 2000; Endecotts Ltd., London, England). The ground lemon waste was then sealed and stored at −18 °C for further analysis.

127 The water activity (a_w) of the dried lemon waste was 0.17 ± 0.01 (mean ± standard deviation)
128 at 23.2 °C and the residual moisture content was 7.6% ± 0.6% (mean ± standard deviation).
129
130 **Experimental design**
131 Before optimizing the AUAE conditions, the effects of five individual parameters
132 (particle size of sample, extraction time, extraction temperature, ultrasonic power and sample-
133 to-solvent ratio), on the recovery yields of total phenolic content (TPC) and rutin were
134 investigated. When one parameter was examined, the others were kept constant (Fig. 1). For
135 eliminating some of the independent parameters from the design, thus to reduce the number of
136 experimental points, the particle size of 1.40 mm and the sample-to-solvent ratio of 2g/100mL
137 of water were selected according to the preliminary experiments (Table 1) and a response
138 surface methodology (RSM) using JMP software (version 11) was then applied to design and
139 optimize the AUAE conditions (extraction time, extraction temperature, and ultrasonic power)
140 for the recovery of TPC and rutin. The greater extraction yields obtained during ultrasound
141 extraction have been attributed to the acoustic cavitation phenomena which are affected by the
142 ultrasonic power, the extraction temperature, the extraction time and the frequencies [23].
143 Therefore, the ultrasonic power, the extraction temperature, and the extraction time were
144 selected to be optimized. RSM is a multivariate strategy offering a large amount of information
145 from a small number of experiments [19]. A three-factor and three-level Box-Behnken design
146 consisting of fifteen experimental runs was employed, including three central points, since
147 Box-Behnken is an economical and efficient design [17]. The linear, quadratic and interaction
148 effects of extraction time (X_1: 35-45 min), extraction temperature (X_2: 45-55 °C) and ultrasonic
149 power (X_3: 150-250 W) were evaluated (Table 2).
150 The yields of TPC and rutin were studied using a complete second-order polynomial equation
151 (Eq. (1)).

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \quad \text{(Eq. (1))}$$

where Y is the predicted response (TPC or rutin), β_0 is the constant term, β_i, β_{ii} and β_{ij} represent the coefficients of the linear, quadratic and interaction effects, respectively, whereas X_i and X_j are the independent variables [19].

The fit of the model was evaluated by R^2, *P*-value of the model, lack of fit and root mean squared error (RMSE). The validation of the model was performed by applying the optimized extraction conditions of the independent variables and comparing them with the predicted values.

The AUAE method was compared with an optimized hot water extraction (HWE) method [21] and an organic solvent conventional extraction (OSCE) method [4], in terms of TPC, TF, and antioxidant capacity.

Extraction process

Aqueous ultrasound-assisted extraction (AUAE)

The (AUAE) of phenolic compounds was conducted by using a 20 L ultrasonic bath operating at a frequency of 43 kHz ± 2 kHz (Soniclean, Soniclean Pty Ltd., Thebarton, Australia). Distilled water was used as a solvent.

Hot water extraction (HWE)

The optimized extraction procedure described by Papoutsis et al. [21] was employed, with some modifications. Briefly, dried lemon by-product (1 g) was mixed with 100 mL of distilled water and placed in a water bath (Labec Laboratory equipment Pty. Ltd., Marrickville, NSW, Australia) at 95 °C for 15 min. During extraction, the tubes were wrapped with parafilm and aluminum foil for minimizing evaporation. After extraction, the extracts were filtered at ambient temperature using Whatman filter paper number 1.

177 **Organic solvent conventional extraction (OSCE)**

178 The extraction was performed at ambient temperature for 1 h. Briefly, dried lemon by-
179 product (3 g) was mixed with 30 mL of 50 % (v/v) ethanol and left at ambient temperature for
180 1 h [4]. After extraction, the extracts were filtered using Whatman filter paper number 1.

181

182 **Phytochemical analysis**

183 **Total phenolic content (TPC)**

184 TPC was determined according to Papoutsis et al. [21]. Briefly, 5 mL of 10% (v/v) Folin–
185 Ciocalteu reagent were mixed with 1 mL of sample. After 3 minutes incubation, 4 mL of 7.5%
186 (w/v) Na_2CO_3, were added to the mixture and incubated in the dark at room temperature for 1
187 h. The absorbance of the solution was measured at 760 nm using a UV spectrophotometer
188 (Varian Australia Pty. Ltd., Vic., Melbourne, Australia). The results were expressed as mg of
189 gallic acid equivalents per g of sample dry weight (mg GAE/g dw).

190

191 **Total flavonoid content (TF)**

192 TF was determined according to Papoutsis et al. [21]. Briefly, 0.5 mL of sample was
193 mixed with 2 mL of H_2O and 0.15 mL of 5% (w/v) $NaNO_2$ and incubated at room temperature
194 for 6 min. Then, 0.15 mL of 10% (w/v) $AlCl_3$ was added and left at room temperature for 6
195 min. Subsequently, 2 mL of 4% (w/v) NaOH and 0.7 mL of H_2O were added and the mixture
196 was left at room temperature for further 15 min. The absorbance was measured at 510 nm using
197 a UV spectrophotometer (Varian Australia Pty. Ltd., Vic., Melbourne, Australia) and the
198 results were expressed as mg of catechin equivalents per g of sample dry weight (mg CE/g dw).

199

200 **Rutin determination**

The determination of rutin was performed by using high-performance liquid chromatography (HPLC) (Shimadzu LC-20AD, Rydalmere, NSW, Australia). A photodiode array detector (Shimadzu SPD-M20A, Rydalmere, NSW, Australia) was employed for the detection. Before HPLC analysis, standards and samples were filtrated through a 0.45 μm nylon filter. C_{18} reversed-phase column (Gemini 110A 5 μm, 150 × 4.6 mm Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia) supplied with a guard column (Gemini C_{18}, 4 × 3.0 mm) was used and the injection volume for samples and standards was 50 μL. The column temperature was maintained at 30 °C using an oven (Shimadzu CTO-20AC, Rydalmere, NSW, Australia). The mobile phase contained water: acetonitrile: formic acid, 95:4:1 (v:v:v) (Mobile Phase A) and acetonitrile (Mobile Phase B). The flow rate of the solvents was 1 mL/min and the following gradient solution was used: 0 min 5% B; 15 min, 20% B; 35 min, 100% B; 40 min, 5% B; 50 min, 50% B. The analysis was stopped after 60 min. The system was equilibrated between runs for 10 min using 5% B.

Rutin content was calculated from the peak area of 280 nm by the external standard method, using a calibration curve (R^2=0.999). Rutin standards were prepared by dissolving standard compounds in methanol at a concentration of 200 μg/mL. The results were expressed as mg/g dw.

Antioxidant capacity

Cupric Reducing Antioxidant Capacity (CUPRAC) assay

CUPRAC assay was determined as described by Papoutsis et al. [21]. Briefly, 1 mL of $CuCl_2$ (10 mM) was mixed with 1 mL of neocuproine (7.5 mM) and 1 mL of NH_4Ac (pH 7.0). Then, 1.1 mL of sample was added to this mixture. The mixture was left at ambient temperature for 1.5 h before the absorbance was measured at 450 nm using a UV spectrophotometer (Varian

Australia Pty. Ltd., Vic., Melbourne, Australia). The results were expressed as mg Trolox equivalents per g of sample dry weight (mg TE/g dw).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH assay was determined as described by Papoutsis et al. [21]. A stock solution was prepared by dissolving 24 mg DPPH in 100 mL methanol and then stored at −20 °C until use. For the preparation of working solution 10 mL of stock solution were mixed with 45 mL methanol to obtain an absorbance of 1.1 ± 0.02 at 515 nm. Subsequently, 2.85 mL of working solution were mixed with 0.15 mL of sample and left under darkness at room temperature for 30 min before measuring the absorbance at 515 nm using a UV spectrophotometer (Varian Australia Pty. Ltd., Vic., Melbourne, Australia). The results were expressed as mg Trolox equivalents per g of sample dry weight (mg TE/g dw).

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP assay was determined as described by Papoutsis et al. [21]. A working FRAP solution was prepared by mixing 300 mM acetate buffer with 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM $FeCl_3$ in the ratio of 10:1:1. The working solution was warmed at 37 °C in a water bath (Ratek Instruments Pty. Ltd., Boronia,Vic., Australia). Subsequently, 2.85 mL of FRAP working solution was mixed with 0.15 mL of sample and incubated at room temperature in the dark for 30 min before its absorbance was measured at 593 nm using a UV spectrophotometer (Varian Australia Pty. Ltd., Vic., Melbourne, Australia). The results were expressed as mg Trolox equivalents per g of sample dry weight (mg TE/g dw).

Scanning electron microscopy (SEM)

SEM was employed for observing the morphology of lemon by-product residues after applying three different extraction techniques (optimized HWE, optimized AUAE and OSCE) using ZEISS SIGMA VP microscope. Freeze dried lemon by-product was used as a control. After extraction lemon residues were dried at 60 °C until constant weight. Samples were gold coated before the images were taken using a secondary electron detector. Trying to avoid the charging issue, we used backscatter detector in case of AUAE residues.

Statistical analysis

In the optimization experiment, each run was conducted in triplicate and the results were expressed as mean ± standard deviation. JMP software (version 11) was applied to design and optimize the conditions for the AUAE of TPC and rutin from lemon by-product. The effect of different factors on TPC yields in the preliminary experiments was investigated by one-way ANOVA and Duncan's post hoc multiple comparison test, using SPSS statistical software (version 23, IBM, Crop., NY, USA) at $P<0.05$. The *t*-test was employed for the comparison of the predicted TPC and rutin values with the observed ones ($P<0.05$). The comparison of the different extraction methods was performed by one-way ANOVA, and the Duncan's post hoc multiple comparison test was employed for the determination of significance among the different means, at a significance level of $P<0.05$. Before ANOVA application the assumptions of: i) homogeneity of variances (using Levene's test) and ii) normal distribution of variables (using Shapiro-Wilk test) were evaluated and satisfied. Each extraction run and analysis was performed in triplicate. The Pearson's correlation test was employed for the determination of correlation coefficients among TPC, TF and antioxidant assays at $P<0.01$.

Results and discussion

Preliminary experiments

Five preliminary experiments were conducted before optimization, for monitoring the effect of individual parameters on TPC and rutin yields and the results can be seen in Table 1.

The effect of three different particle sizes was examined in the preliminary experiment 1 since particle size is considered as an important parameter affecting the recovery yields of polyphenols from plant tissues [24,25]. The results showed that as the particle size increased from 1.40 mm to 2.00 mm the TPC yields significantly decreased ($P<0.05$). However, higher rutin yields were achieved with the particle sizes of 2.00 and 1.40 mm. These results are in agreement with Lee et al. [25] who found that the recovery yields of two polymethoxyflavonoids (nobiletin and tangeretin) increased when the particle size of orange peel decreased. The higher TPC yields obtained by the smaller particle size could be due to the larger surface area being exposed to water and ultrasonic power, facilitating a higher mass transfer of analytes from dried lemon by-products to water. The particle size of 1.40 mm was thus selected for the next preliminary and optimization experiments since with this particle size, high TPC and rutin yields were achieved. In the second preliminary experiment, the effect of extraction time was examined. As the extraction time increased from 30 to 40 min the recovery yields of TPC significantly increased and then levelled off ($P<0.05$) (Table 1). These results are in agreement with Dahmoune et al. [11] who mentioned that extraction time significantly affected the recovery of TPC from lemon by-products under ultrasound-assisted extraction. However, the extraction time employed in the previous study was shorter than in the present experiment. The difference in the extraction time between the two studies could be attributed to the different solvents that were used. It has been previously mentioned that the mixture of ethanol with water leads to higher recovery yields of polyphenols from plant tissues compared to pure water in shorter time [26]. The organic solvent may facilitate the extraction of polyphenols by enhancing the collapse of cell walls and diffusion of polyphenols to the solvent. Extraction time had no effect on rutin yields ($P>0.05$), however, an extraction time

300 greater than 40 min seems to negatively affect rutin recovery. Therefore, an extraction time
301 ranging between 35-45 min was selected for the optimization experiment. In the third
302 preliminary experiment, the effect of extraction temperature was investigated. The recovery
303 yields of TPC and rutin increased when the extraction temperature increased from 23 °C
304 (ambient temperature) to 50 and 40 °C, respectively (P<0.05) (Table 1). Higher extraction
305 temperatures might lead to higher recovery yields of TPC and rutin by increasing their
306 solubility and weakening cell wall structure by enhancing the activity of some enzymes [27].
307 Therefore, an extraction temperature ranging between 45-55 °C was selected for the
308 optimization experiment. In the fourth preliminary experiment, the effect of ultrasonic power
309 was examined. Ultrasonic power had no effect on either TPC or rutin yields, at the operating
310 conditions that were applied (extraction time of 20 min and temperature of 30 °C) (P>0.05)
311 (Table 1). However, ultrasonic power ranging between 150 and 250W was used for
312 optimization, since interactions with other extraction parameters may occur and affect the
313 extraction yields of TPC and rutin. Finally, in the fifth preliminary experiment, the effect of
314 sample-to-solvent ratio on TPC and rutin yields were examined. Sample-to-solvent ratio
315 significantly affected the recovery yields of TPC (P<0.05), while it did not affect rutin yields
316 (P>0.05). As the sample-to-solvent ratio increased from 1 g/100mL to 4 g/100mL the TPC
317 significantly decreased and then levelled off (Table 1). These findings are in agreement with a
318 previous study which mentioned that the recovery yields of some phenolic compounds from
319 *Citrus reticulata* fruit increased as the sample-to-solvent ratio decreased until an optimum
320 level, and then levelled off [28]. When a lower sample-to-solvent ratio is used, an increase in
321 the diffusion ratio is observed, due to a greater concentration gradient [29]. Considering solvent
322 consumption, the ratio of 2 g/100mL was selected for the optimization experiments.
323

Optimization of aqueous ultrasound-assisted extraction (AUAE) conditions for the recovery of TPC and rutin from lemon by-products

The prediction model for TPC was significant (P<0.05) with a non-significant lack of fit (P>0.05) and a low RMSE value (0.11), implying that the model adequately fits the data and can be used for prediction. This observation was further supported by the actual vs predicted plot (Fig. 2A), where the points are close to the fitted line. The coefficient of multiple determination (R^2) was 0.90, implying that 90% of the variation was explained by the model (Fig. 2A). The predicted optimum AUAE conditions for TPC were obtained by using the prediction profiler and the surface plots and were: extraction time of 45 min, extraction temperature of 50 °C and ultrasonic power of 250W with the predicted value of 18.10±0.24 mg GAE/g dw. For validating the accuracy of the model the extraction was performed at the optimum conditions and the observed value for TPC was 17.97±0.21 mg GAE/g dw which was not significantly different from the predicted value (P>0.05) (Table 2).

The effect of different parameters on TPC yields can be seen in Table 3 and Fig. 2B (Pareto plot). Ultrasonic power and extraction time had a significant positive linear effect on TPC of lemon by-products (P<0.05) (Table 3), implying that higher yields of TPC can be achieved by increasing ultrasonic power and extraction time (Fig. 3). The positive effect of ultrasonic power and extraction time on the recovery of phenolic compounds has been previously reported [30,11]. However, these results are in contrast with our preliminary experiments, where ultrasonic power did not affect the TPC yields. This difference could be attributed to: i) the shorter extraction time (20 min) and ii) lower extraction temperature (30 °C) used in the preliminary experiments when the effect of ultrasonic power was examined, compared to the optimization trials. Extraction temperature had a significant negative quadratic effect (P<0.05) on TPC yields (Table 3 and Fig. 2B, Pareto plot), implying that an extraction temperature higher than the optimum, results in the decrease of TPC yields. Although the Folin

Ciocalteu assay may have the interference of ascorbic acid for the quantification of total phenols, our results suggest that the degradation of cell walls may increase as the temperature increases, resulting in the release of both phenolic compounds and enzymes implicated in polyphenol oxidation. At the same time, the activity of the released enzymes (i.e. peroxidase, polyphenol oxidase) implicated in polyphenol degradation might be enhanced by the temperature applied [31,12], leading to lower polyphenol yields. Although the interaction between temperature and ultrasonic power was not significant (P>0.05), from Pareto plot (Fig. 2B), it could be concluded that the interaction between temperature and ultrasonic power may negatively affect TPC yields. Considering that the interaction between temperature and power negatively affected the antioxidant capacity of extracts measured by DPPH (Fig. 2E, Pareto plot), it could be concluded that increasing both temperature and power, some phenolic compounds with antiradical capacity might be degraded because of thermal oxidation and/or scavenging of free radicals created due to the ultrasonic power. Ma et al. [32] reported that increasing simultaneously both temperature and power, some phenolic compounds with antiradical capacity might be degraded because of thermal oxidation and/or scavenging of free radicals created due to ultrasonic power. On the other hand, the interaction between temperature and power positively affected rutin yields (Fig. 2D, Pareto plot) and antioxidant capacity measured by CUPRAC (Fig. 2F, Pareto plot), implying that the recovery of phenolic compounds exhibiting reducing potency can be enhanced by increasing both temperature and power.

The prediction model for rutin yields was significant (P<0.05) with a non-significant lack of fit (P>0.05) and a low RMSE value of 0.05, implying that the model adequately fits the data and can be used for prediction. This observation was further supported by the actual vs predicted plot (Fig. 2C), where the points are close to the fitted line. The coefficient of multiple determination (R^2) was 0.90, implying that 90% of the variation was explained by the model

374 (Fig. 2C). The predicted optimum AUAE conditions for rutin were obtained by using the
375 prediction profiler and the surface plots and were: extraction time of 35 min, extraction
376 temperature of 48 °C and ultrasonic power of 150W with the predicted value of 3.20±0.12 mg/g
377 dw. For validating the accuracy of the model the extraction was performed at the optimum
378 conditions and the observed value for rutin was 3.13±0.03 mg/g dw, which was not
379 significantly different from the predicted value (P>0.05) (Table 2).

380 The effect of different parameters on rutin yields can be seen in Table 3 and Fig. 2D
381 (Pareto plot). Extraction temperature had a significant negative quadratic effect (P<0.05) on
382 rutin yields, implying rutin degradation at temperatures higher than the optimum. A similar
383 trend was observed in the preliminary experiment where the rutin yield increased as the
384 extraction temperature increased until an optimum point and then levelled off. These findings
385 are in agreement with Ma et al. [32] who showed that temperature is a sensitive variable for
386 extracting polyphenols from citrus using ultrasound-assisted extraction. The interaction
387 between temperature and power had a significant positive effect on rutin yields (P<0.05),
388 implying that aqueous extracts with high rutin content could be achieved by increasing
389 simultaneously extraction temperature and ultrasonic power (Fig. 3). Extraction time and
390 power might negatively affect rutin yields, since both parameters had a non-significant negative
391 linear effect on rutin recovery (P>0.05) (Table 3 and Fig. 2D, Pareto plot), implying rutin
392 degradation probably due to the free radical formation which is promoted by high ultrasound
393 power in aqueous solutions [15].

394

395 **Comparison between different extraction methods**

396 The TPC of the extracts obtained by AUAE was not significantly different compared to
397 those obtained by HWE and OSCE (P>0.05) (Table 4). However, extracts obtained by HWE
398 showed higher TF than those achieved by AUAE and OSCE (P<0.05) (Table 4). High

399 temperature leads to higher polyphenol yields by enhancing the solubility of bioactive
400 compounds, as well as by increasing the diffusion of compounds from plant matrix into the
401 solvent [33]. The lower TF values of extracts obtained by AUAE could be explained by the
402 reaction of some flavonoids with the free radicals produced in the aqueous solution due to the
403 ultrasonic power [15], since flavonoids act as hydrogen donors and singlet oxygen quenchers
404 due to their high redox potential [34], which could be attributed to the low bond dissociation
405 enthalpy of the phenolic O-H group [35]. In case of OSCE, the lower TPC and TF values could
406 be attributed to: i) the degradation of some phenolic compounds to extended extraction time,
407 and ii) the reduced diffusion of polyphenols from the dried lemon by-product to the solvent,
408 because of dehydration and collapse of lemon by-product cells [36].

409 Two different assays (CUPRAC and FRAP) were used for the comparison of the
410 antioxidant capacity of the extracts, since different reactions between polyphenols and
411 antioxidant assays may take place. CUPRAC assays both hydrophilic and lipophilic
412 antioxidants, while FRAP mainly assays hydrophilic antioxidants [37]. Extracts obtained by
413 HWE exhibited the highest antioxidant capacity measured by both CUPRAC and FRAP (Table
414 4). The lower antioxidant capacity of extracts obtained by AUAE and OSCE could be attributed
415 to the lower flavonoid content in these extracts since a high correlation between TF and
416 antioxidant assays was observed (Table 4). HWE was a more efficient technique for the
417 recovery of hydrophilic compounds with ferric reducing antioxidant power compared to
418 AUAE.

419 SEM analysis was employed for observing the morphology of lemon by-product residues
420 after applying the three extraction techniques, and the images showed that all the extraction
421 methods led to cell damage to a different extent (Fig. 4). OSCE caused cell wall destruction,
422 dehydration and shrinkage (Fig. 4C) in comparison with the freeze dried by-product which was
423 not subjected to extraction where no destruction was observed (Fig. 4A). Fig. 4B shows that

HWE resulted in a disruption of the cell walls of lemon by-product which could be due to the denaturation of cell wall membranes [38]. According to a previous study, dried lemon residues are resistant to ultrasound energy [11]. However, in this study, a cell wall damage was observed in the lemon residues after AUAE (Fig. 4D). This difference could be due to the higher extraction temperatures applied in this study, as well as to the different physical parameters of the solvents that were employed. High extraction temperatures might lead to the weakening of cell walls by enhancing the activity of some enzymes [27], facilitating cell wall damage due to cavitation phenomena. Moreover, high temperatures can increase the number of cavitation bubbles facilitating greater cell wall disruption [23].

Overall, HWE and AUAE extractions required shorter extraction time than the OSCE method for the preparation of extracts with high polyphenol content and antioxidant capacity. Indeed, OSCE required less solvent volume than the two other extraction techniques. However, some disadvantages of organic solvent use should be taken into consideration in the selection of the extraction method, including: i) toxicity of organic solvent for human health and environment, ii) solvent purchase and disposal costs, iii) special storage requirements, and iv) solvent residues in the final product. Therefore, from an economical and environmental point of view, both HWE and AUAE, should be considered for the preparation of high-quality polyphenol extracts from lemon by-products.

Conclusions

A Box-Behnken design was effectively employed for optimizing the recovery of TPC and rutin from lemon by-products. The recovery of TPC was positively affected by the ultrasonic power and extraction time (linear effect), while extraction temperature had a negative quadratic effect on TPC yields. Rutin recovery was mainly affected by the interaction between extraction temperature and ultrasonic power (positive effect) and extraction

temperature (negative quadratic effect). HWE resulted in extracts with higher TF and antioxidant capacity compared to AUAE and OSCE, in a considerably shorter time. Comparing AUAE with the OSCE, it could be concluded that AUAE could be effectively employed for the recovery of polyphenols from lemon by-products, since, it leads in shorter time to extracts with approximately the same TPC, TF and antioxidant capacity, compared to those obtained by OSCE. SEM analysis showed that all the extraction methods led to a cell damage to a different extent.

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Compliance with ethical standards

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Conflict of interest statement The authors declare no conflict of interest.

Compliance with ethics requirements Research does not involve any human participants and/or animal.

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570

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Table 1. Effect of single factors (particle size of sample (mm), extraction time (min), extraction temperature (°C), ultrasonic power (W) and sample-to-solvent ratio (g/mL) on total phenolic content (TPC) and rutin of lemon (*Citrus limon* L.) by-product aqueous extracts. Data are expressed as mean ± standard deviation (n=3).

Preliminary experiment 1 Particle size			Preliminary experiment 2 Extraction time			Preliminary experiment 3 Extraction temperature			Preliminary experiment 4 Ultrasonic Power			Preliminary experiment 5 Sample-to-solvent		
	TPC	Rutin		TPC	Rutin		TPC	Rutin		TPC	Rutin		TPC	Rutin
mm	mg GAE/g dw	mg/g dw	min	mg GAE/g dw	mg/g dw	°C	mg GAE/g dw	mg/g dw	W	mg GAE/g dw	mg/g dw	g/mL	mg GAE/g dw	mg/g dw
1.40	17.0±0.2^{a*}	2.28±0.14^{ab}	10	16.9±0.1^{b}	2.47±0.14^{a}	Ambient	17.61±0.25^{bc}	2.53±0.15^{b}	150	16.64±0.14^{a}	2.42±0.23^{a}	1/100	17.08±0.17^{a}	2.42±0.14^{a}
2.00	15.87±0.03^{b}	2.35±0.14^{a}	20	17.07±0.32^{b}	2.37±0.03^{a}	30	17.55±0.12^{c}	2.60±0.07^{b}	200	16.38±0.67^{a}	2.53±0.29^{a}	2/100	16.64±0.22^{ab}	2.35±0.07^{a}
2.80	15.45±0.27^{b}	2.04±0.08^{b}	30	17.09±0.06^{b}	2.41±0.11^{a}	40	18.01±0.07^{b}	2.86±0.09^{a}	250	16.96±0.22^{a}	2.68±0.25^{a}	4/100	16.34±0.29^{b}	2.19±0.06^{a}
			40	17.8±0.2^{a}	2.49±0.08^{a}	50	18.54±0.17^{a}	2.82±0.02^{a}				5/100	16.31±0.12^{b}	2.15±0.16^{a}
			50	17.43±0.31^{ab}	2.29±0.11^{a}									
			60	17.35±0.08^{ab}	2.22±0.13^{a}									

*Values followed by different superscript letter within the same column are significantly different at $P<0.05$, according to ANOVA and Duncan's test.

Table 2. Box–Behnken design and results for total phenolic content (TPC), rutin and antioxidant capacity (measured by CUPRAC and DPPH) of lemon (*Citrus limon* L.) by-product aqueous extracts. Data are expressed as mean ± standard deviation (n=3). Validation of the predicted values for TPC and rutin.

Run	Pattern	Experimental conditions			Experimental results			
		Independent variables			Dependent variables			
		Extraction time	Extraction temperature	Ultrasonic power	TPC	Rutin	CUPRAC	DPPH
		min	°C	W	mg GAE/g dw	mg/g dw	mg TE/g dw	mg TE/g dw
1	++0	45	55	200	17.75±0.18	3.03±0.16	40.78±0.16	0.119±0.003
2	0-+	40	45	250	17.76±0.32	2.86±0.17	40.05±0.07	0.108±0.002
3	000	40	50	200	17.71±0.11	3.14±0.06	41.3±0.2	0.110±0.006
4	--0	35	45	200	17.62±0.16	3.01±0.01	40.59±0.28	0.114±0.003
5	000	40	50	200	17.67±0.07	3.17±0.04	41.43±0.45	0.111±0.006
6	-0-	35	50	150	17.63±0.04	3.17±0.06	40.31±0.23	0.118±0.002
7	-+0	35	55	200	17.30±0.02	3.03±0.06	40.01±0.38	0.115±0.002
8	+0-	45	50	150	17.6±0.2	3.10±0.13	39.94±0.48	0.112±0.002
9	+-0	45	45	200	17.69±0.11	2.98±0.01	41.13±0.36	0.113±0.003
10	0++	40	55	250	17.64±0.14	3.16±0.05	41.72±0.01	0.118±0.002
11	0--	40	45	150	17.2±0.1	3.15±0.09	40.47±0.78	0.095±0.006
12	000	40	50	200	17.82±0.11	3.14±0.03	41.12±0.25	0.109±0.003
13	+0+	45	50	250	18.07±0.29	3.16±0.02	41.5±0.2	0.111±0.001
14	0+-	40	55	150	17.62±0.13	3.04±0.05	41.10±0.36	0.117±0.003
15	-0+	35	50	250	17.76±0.44	3.21±0.09	41.38±0.64	0.117±0.002

Validation of the predicted values

Dependent Variables	Predicted values	Observed values
TPC (mg GAE/g dw)	18.10±0.24[a*]	17.97±0.21[a]
Rutin (mg/g dw)	3.20±0.12[a]	3.13±0.03[a]

*Values followed by the same superscript letter at the same row are not significantly different at $P<0.05$, according to *t*-test.

Table 3. Regression coefficients of the fitted polynomial equation (Eq. (1)) for total phenolic content and rutin content of lemon (*Citrus limon* L.) by-product aqueous extracts.

Regression coefficients	Total phenolic content				Rutin content			
	Estimate	Sum of squares	F-value	*P*-value	Estimate	Sum of squares	F-value	*P-value*
Intercept	17.733333				3.1511898			
Linear effect								
Time	0.1025	0.08405	7.3321	0.0424*	-0.017141	0.00235040	0.9302	0.3791
Temperature	0.005	0.00020	0.0174	0.9001	0.031052	0.00771381	3.0530	0.1410
Ultrasonic power	0.145	0.16820	14.6729	0.0122*	-0.007649	0.00046805	0.1852	0.6848
Quadratic effect								
Time × Time	0.0358333	0.00474	0.4136	0.5485	-0.014099	0.00073401	0.2905	0.6130
Temperature × Temperature	-0.179167	0.11853	10.3395	0.0236*	-0.124214	0.05696874	22.5473	0.0051*
Ultrasonic power × power	0.0008333	0.00000256	0.0002	0.9886	0.0248608	0.00228206	0.9032	0.3856
Interaction effect								
Time × Temperature	0.095	0.03610	3.1492	0.1361	0.0083893	0.00028152	0.1114	0.7521
Time × Ultrasonic power	0.08	0.02560	2.2332	0.1953	0.0040648	0.00006609	0.0262	0.8778
Temperature × Ultrasonic power	-0.135	0.07290	6.3594	0.0531	0.1013224	0.04106493	16.2528	0.0100*

*Significance at $P<0.05$.

Table 4. Comparison of three different extraction methods (aqueous ultrasound-assisted extraction (AUAE), hot water extraction (HWE) and organic solvent conventional extraction (OSCE)) in terms of TPC, TF and antioxidant capacity (measured by CUPRAC and FRAP) of lemon by-products (*Citrus limon* L.). Data are expressed as mean ± standard deviation (n=3).

Extraction method	TPC	TF	CUPRAC	FRAP
	mg GAE/g dw	mg CE/g dw	mg TE/g dw	mg TE/g dw
AUAE	17.97±0.21^{ab*}	4.85±0.03^{b}	40.73±0.48^{c}	9.4±0.3^{b}
HWE	18.3±0.4^{a}	5.45±0.06^{a}	46.31±0.72^{a}	10.83±0.26^{a}
OSCE	16.96±0.55^{b}	5.11±0.13^{b}	43.74±1.37^{b}	9.23±0.05^{b}
Correlation				
CUPRAC	r=0.36, P=0.342	r=0.86, P=0.003		
FRAP	r=0.66, P=0.05	r=0.87, P=0.002		

*Values followed by different superscript letter within the same column are significantly different at $P<0.05$, according to ANOVA and Duncan's test.

Figure captions

Fig. 1. Experimental design of the experiment.

Fig. 2. A) Actual vs predicted plot for total phenolic content (TPC), B) Pareto plot for TPC, C) Actual vs predicted plot for rutin, D) Pareto plot for rutin, E) Pareto plot for antioxidant capacity measured by DPPH, F) Pareto plot for antioxidant capacity measured by CUPRAC.

Fig. 3. A) Surface plot for the effect of extraction temperature (°C) and time (min) on total phenolic content (TPC), B) Surface plot for the effect of extraction temperature (°C) and ultrasonic power (W) on TPC, C) Surface plot for the effect of ultrasonic power (W) and extraction time (min) on TPC, D) Surface plot for the effect of extraction temperature (°C) and time (min) on rutin yields, E) Surface plot for the effect of ultrasonic power (W) and extraction time (min) on rutin yields, F) Surface plot for the effect of extraction temperature (°C) and ultrasonic power (W) on rutin yields.

Fig. 4. Scanning electron microscopy (SEM) images of A) freeze dried lemon by-product without extraction, B) freeze dried lemon by-product after hot water extraction, C) freeze dried lemon by-product after organic solvent conventional extraction, and D) freeze dried lemon by-product after aqueous ultrasound-assisted extraction.

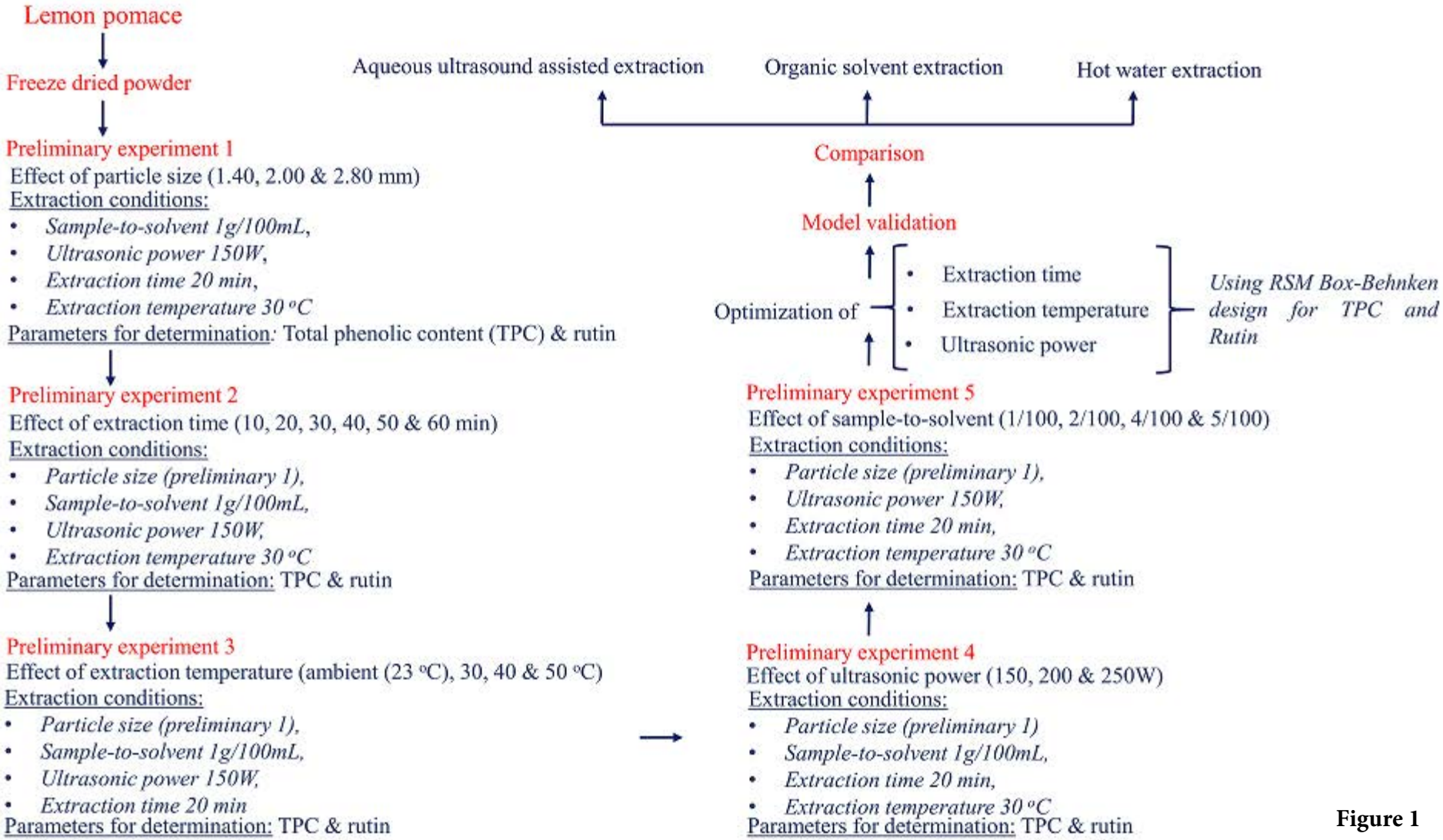
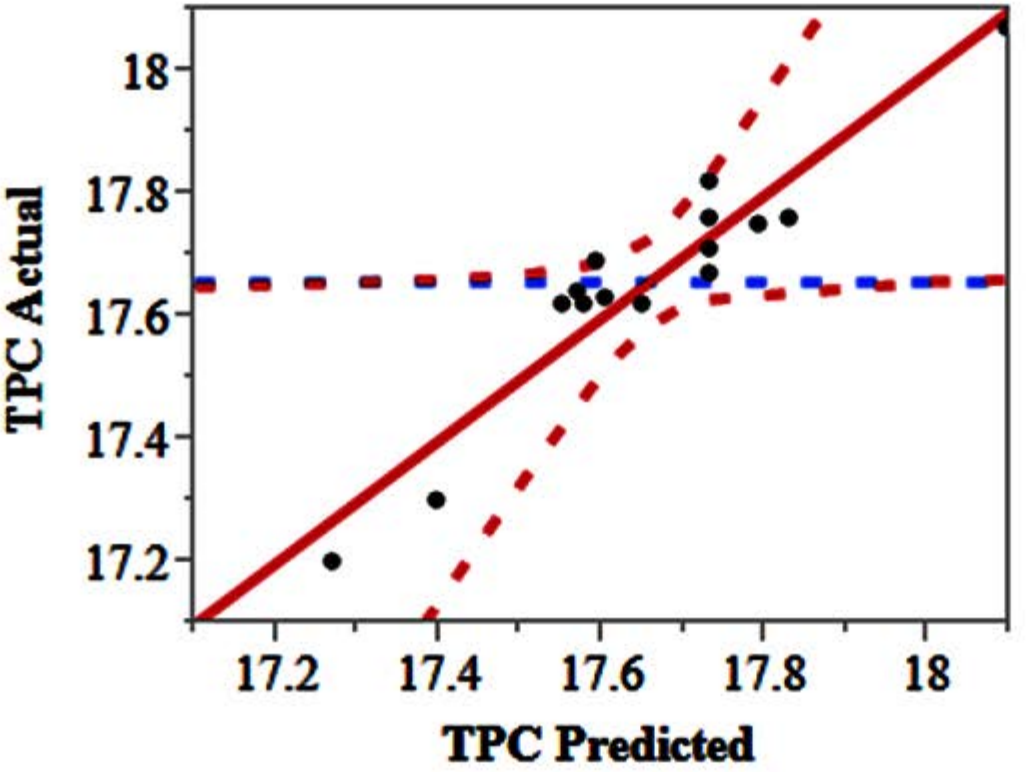
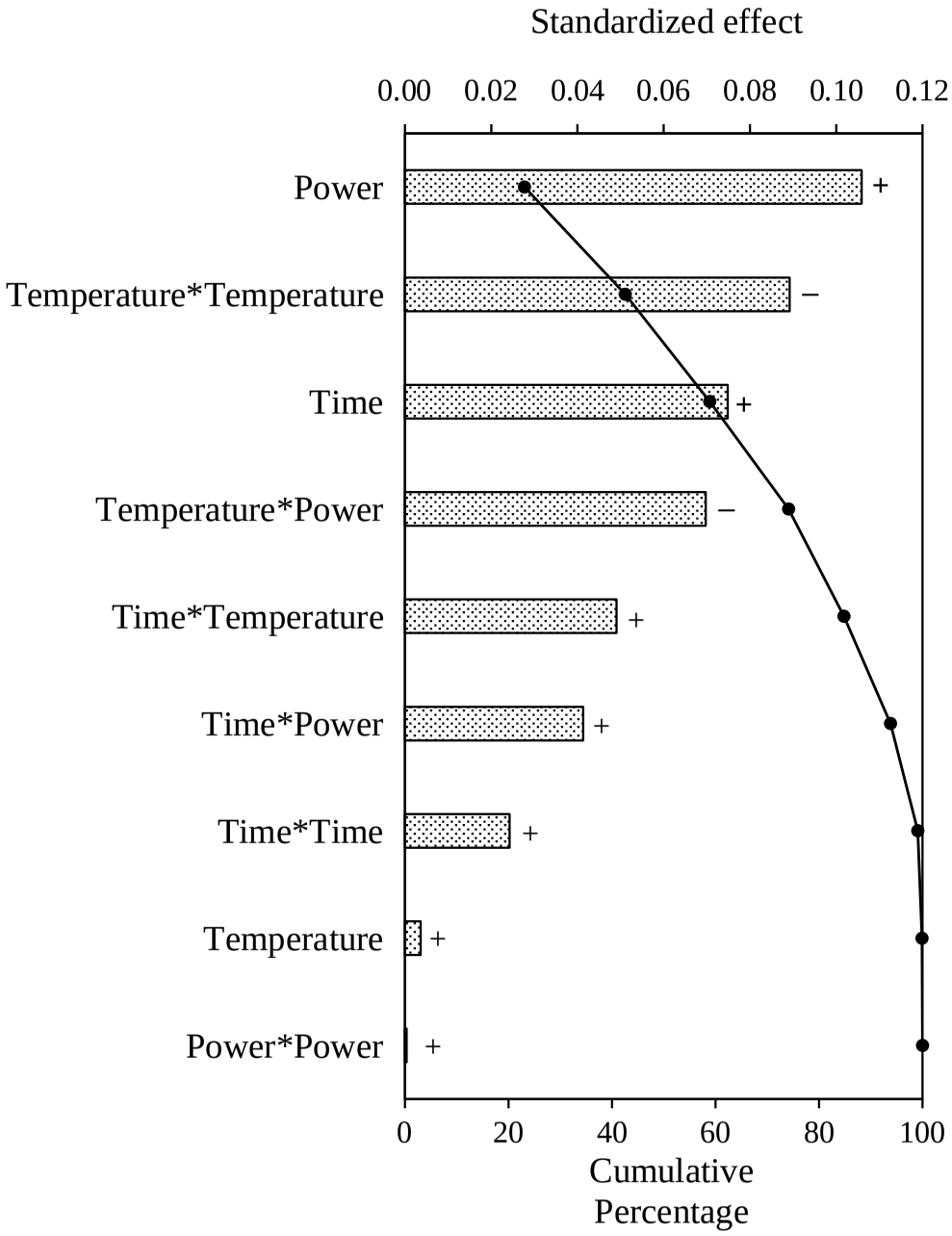

Lemon pomace
Freeze dried powder
Preliminary experiment 1
Effect of particle size (1.40, 2.00 & 2.80 mm)
Extraction conditions:
• Sample-to-solvent 1g/100mL,
• Ultrasonic power 150W,
• Extraction time 20 min,
• Extraction temperature 30 °C
Parameters for determination: Total phenolic content (TPC) & rutin
Preliminary experiment 2
Effect of extraction time (10, 20, 30, 40, 50 & 60 min)
Extraction conditions:
• Particle size (preliminary 1),
• Sample-to-solvent 1g/100mL,
• Ultrasonic power 150W,
• Extraction temperature 30 °C
Parameters for determination: TPC & rutin
Preliminary experiment 3
Effect of extraction temperature (ambient (23 °C), 30, 40 & 50 °C)
Extraction conditions:
• Particle size (preliminary 1),
• Sample-to-solvent 1g/100mL,
• Ultrasonic power 150W,
• Extraction time 20 min
Parameters for determination: TPC & rutin
Preliminary experiment 4
Effect of ultrasonic power (150, 200 & 250W)
Extraction conditions:
• Particle size (preliminary 1)
• Sample-to-solvent 1g/100mL,
• Extraction time 20 min,
• Extraction temperature 30 °C
Parameters for determination: TPC & rutin
Preliminary experiment 5
Effect of sample-to-solvent (1/100, 2/100, 4/100 & 5/100)
Extraction conditions:
• Particle size (preliminary 1),
• Ultrasonic power 150W,
• Extraction time 20 min,
• Extraction temperature 30 °C
Parameters for determination: TPC & rutin
Optimization of
• Extraction time
• Extraction temperature
• Ultrasonic power
Using RSM Box-Behnken design for TPC and Rutin
Model validation
Comparison
Aqueous ultrasound assisted extraction
Organic solvent extraction
Hot water extraction

Figure 1



R^2=0.90; R^2 adjusted=0.72; *P*-value of model=0.0457; Lack of fit=0.2985; RMSE=0.11

Figure 2A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0.02
0.04
0.06
0.08
0.10
0.12
Power
+
Temperature*Temperature
−
Time
+
Temperature*Power
−
Time*Temperature
+
Time*Power
+
Time*Time
+
Temperature
+
Power*Power
+
0
20
40
60
80
100
Cumulative
Percentage

Figure 2B

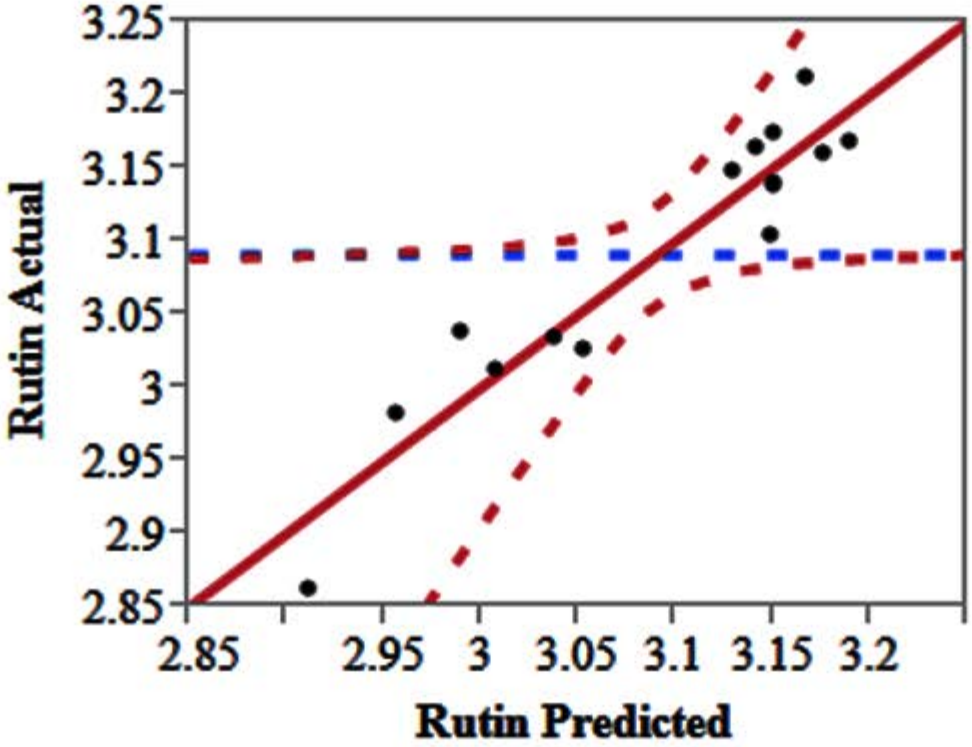

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Figure 2C

Standardized effect
0.00
0.02
0.04
0.06
0.08
Cumulative Percentage
0
20
40
60
80
100
Temperature*Temperature
Temperature*Power
Temperature
Time
Power*Power
Power
Time*Temperature
Time*Time
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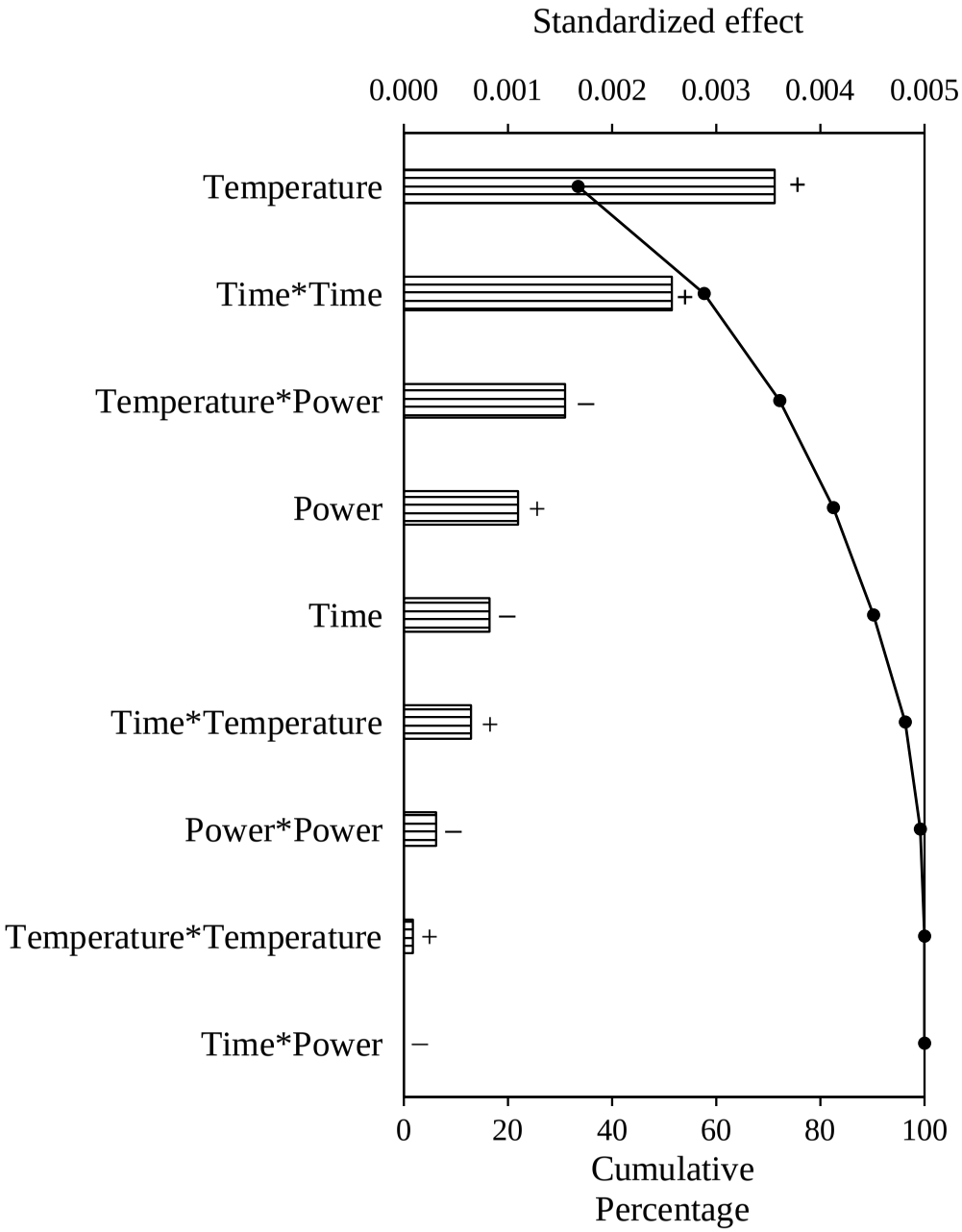
–
+
–
+
–
+
–
+

Figure 2D


Standardized effect
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0.001
0.002
0.003
0.004
0.005
Temperature
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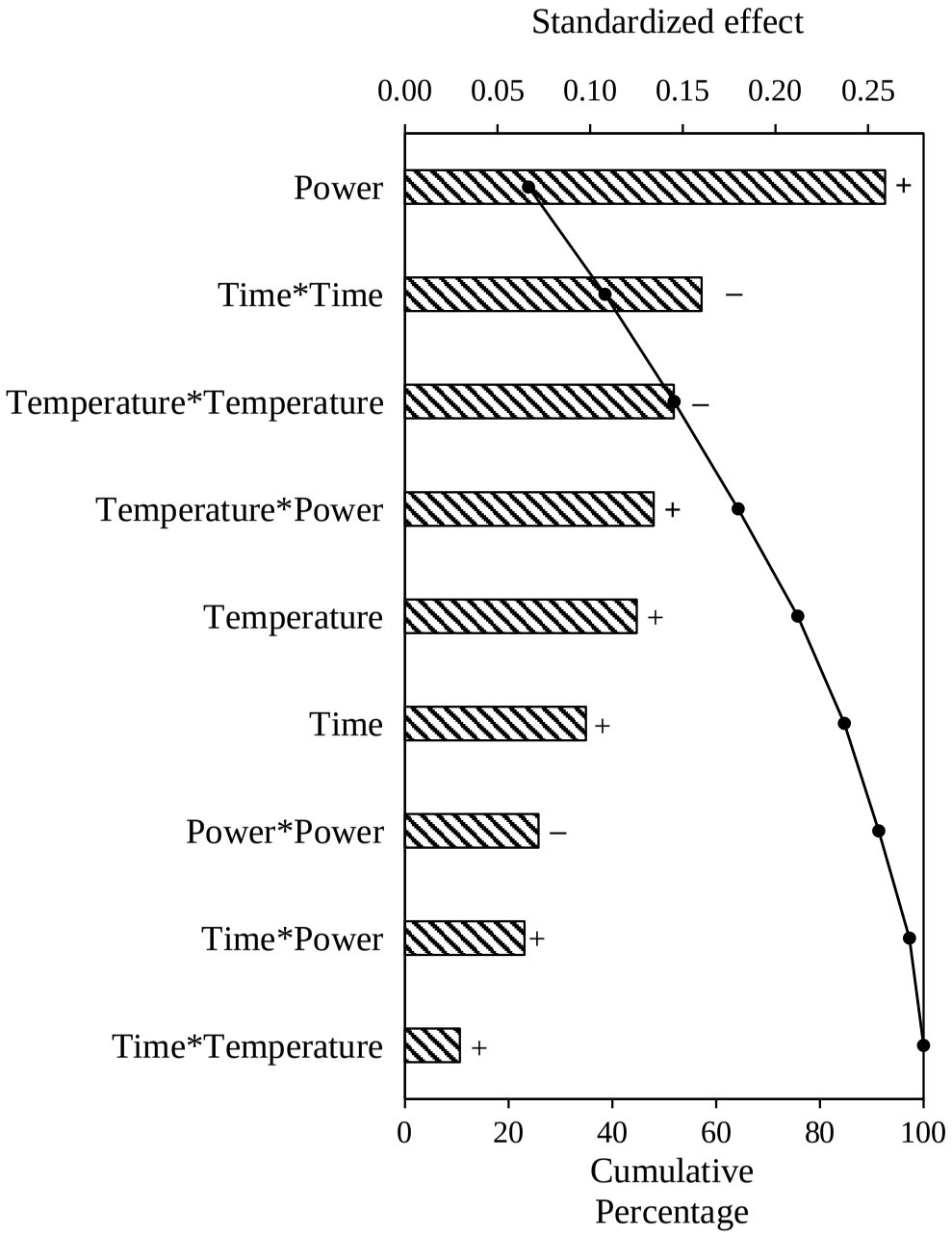
Time*Time
+
Temperature*Power
−
Power
+
Time
−
Time*Temperature
+
Power*Power
−
Temperature*Temperature
+
Time*Power
−
0
20
40
60
80
100
Cumulative
Percentage

Figure 2E


Standardized effect
0.00
0.05
0.10
0.15
0.20
0.25
Power
+
Time*Time
−
Temperature*Temperature
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Temperature*Power
+
Temperature
+
Time
+
Power*Power
−
Time*Power
+
Time*Temperature
+
0
20
40
60
80
100
Cumulative
Percentage

Figure 2F

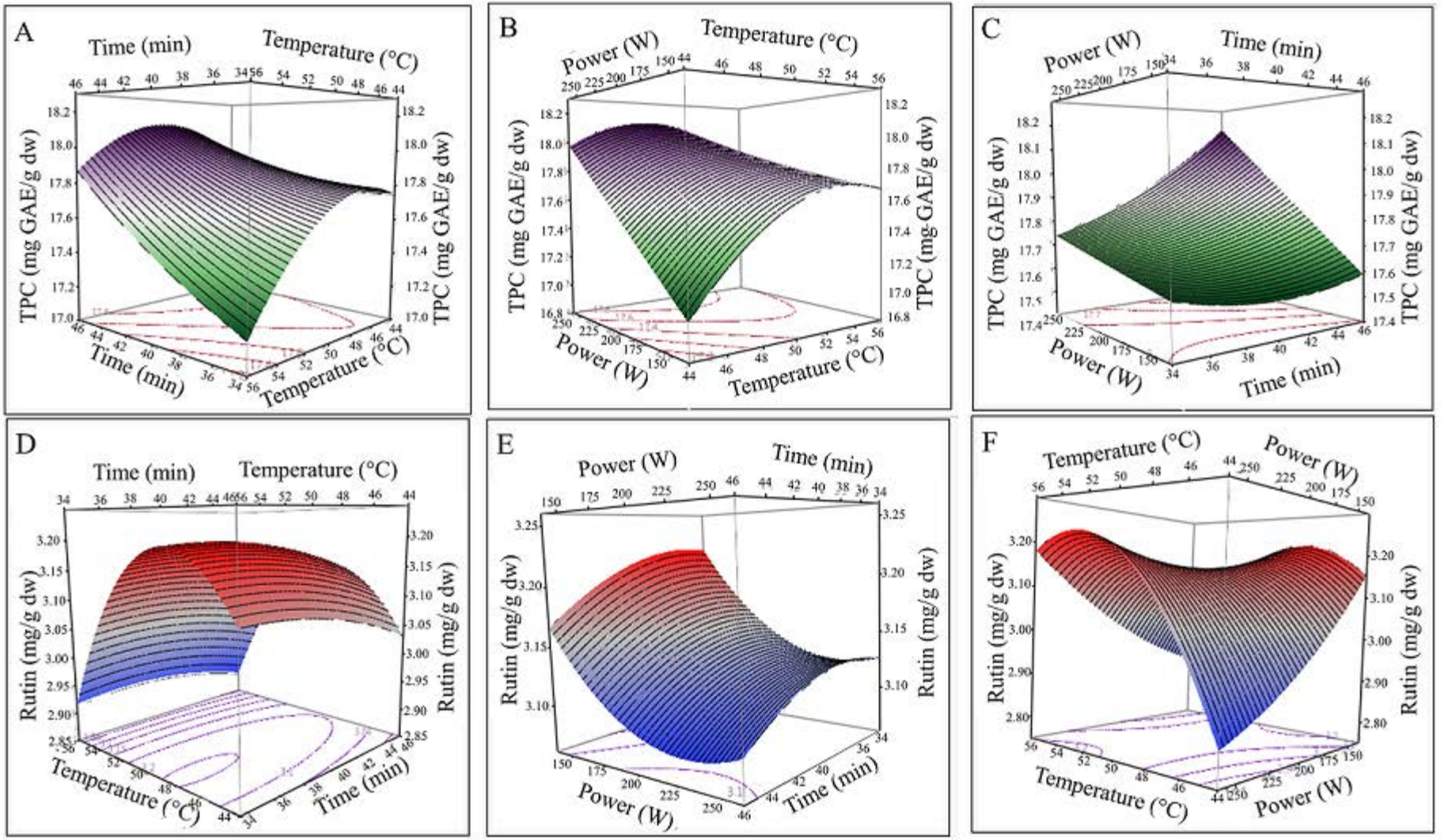
A
Time (min)
Temperature (°C)
TPC (mg GAE/g dw)
B
Power (W)
Temperature (°C)
TPC (mg GAE/g dw)
C
Power (W)
Time (min)
TPC (mg GAE/g dw)
D
Time (min)
Temperature (°C)
Rutin (mg/g dw)
E
Power (W)
Time (min)
Rutin (mg/g dw)
F
Temperature (°C)
Power (W)
Rutin (mg/g dw)

Figure 3

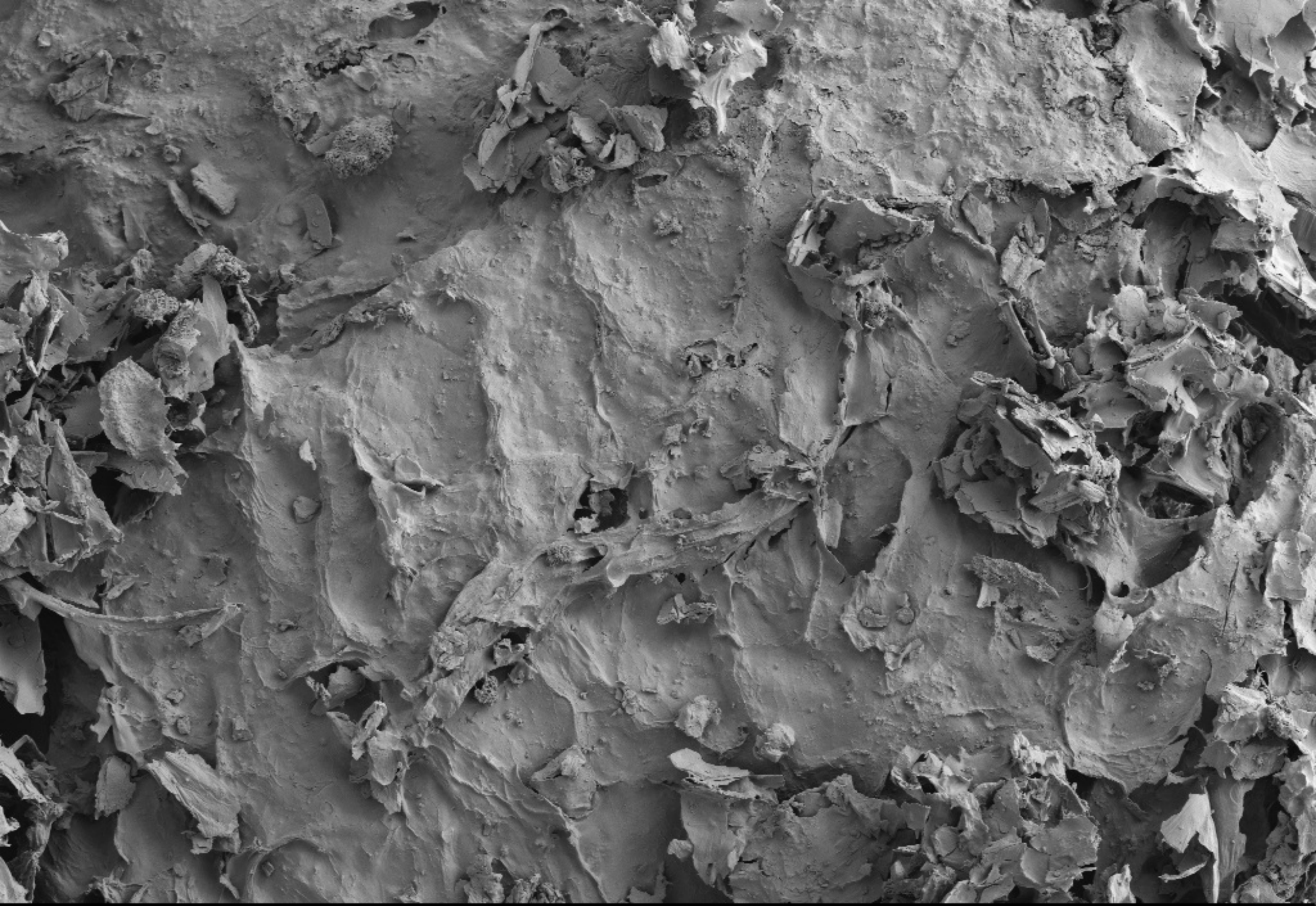
100 µm
Mag = 500 X
Width = 603.9 µm
WD = 8.4 mm
Signal A = SE2
Signal B = NTS BSD
Signal = 1.000
Gun= 3.46e-010 Torr
Chm=2.92e-006 Torr
Sys= 2.92e-006 Torr
File Name = Freeze dried - 08.tif
31 Jan 2017 14:56:50
5.00 kV
x = 67.749 mm
y = 80.511 mm

Figure 4A

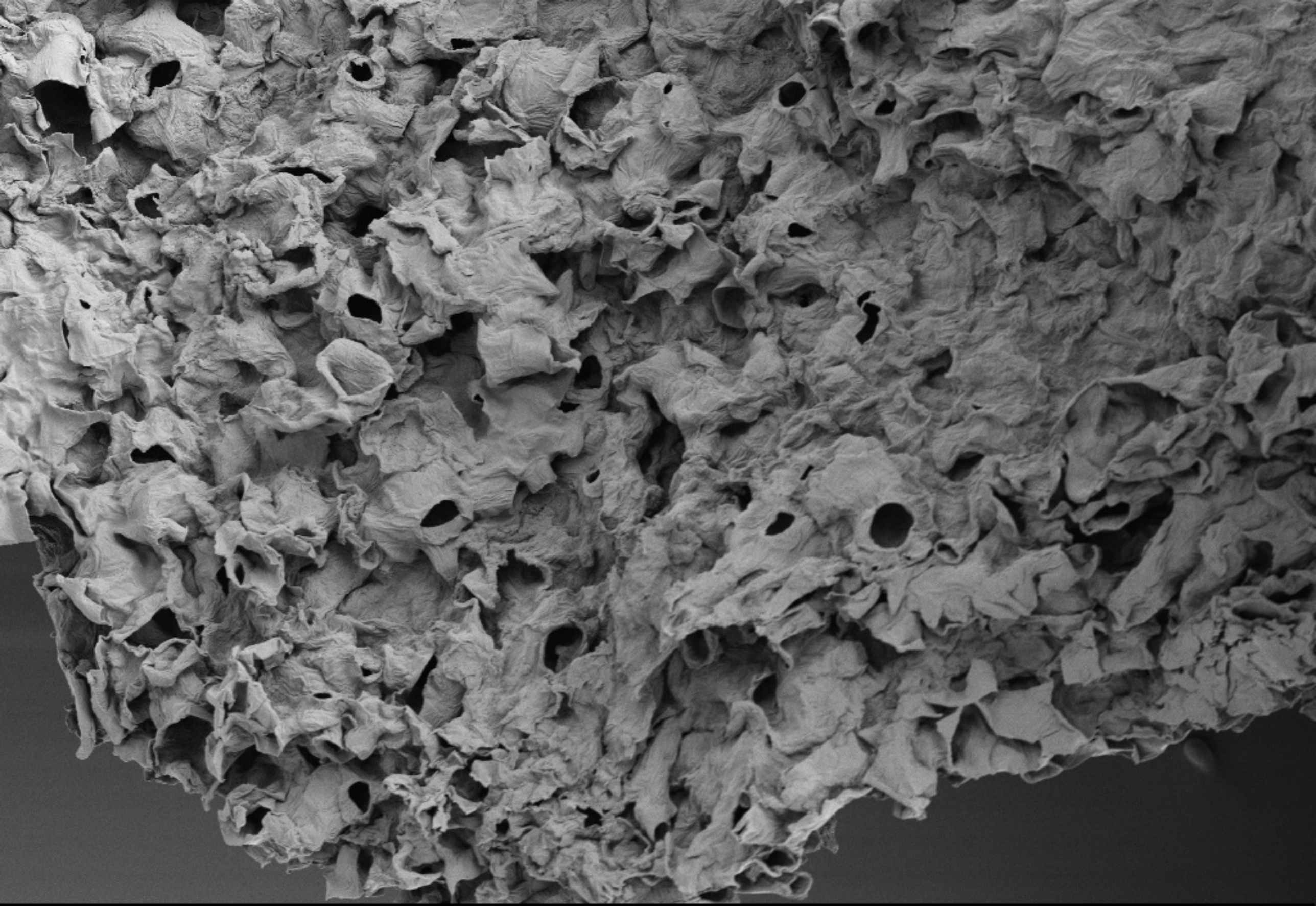
100 µm
Mag = 500 X
Width = 603.9 µm
WD = 8.8 mm
Signal A = SE2
Signal B = NTS BSD
Signal = 1.000
Gun= 3.53e-010 Torr
Chm=2.44e-006 Torr
Sys= 2.44e-006 Torr
File Name = Hot water extraction - 06.tif
31 Jan 2017 15:12:54
5.00 kV
x = 79.334 mm
y = 68.164 mm

Figure 4B

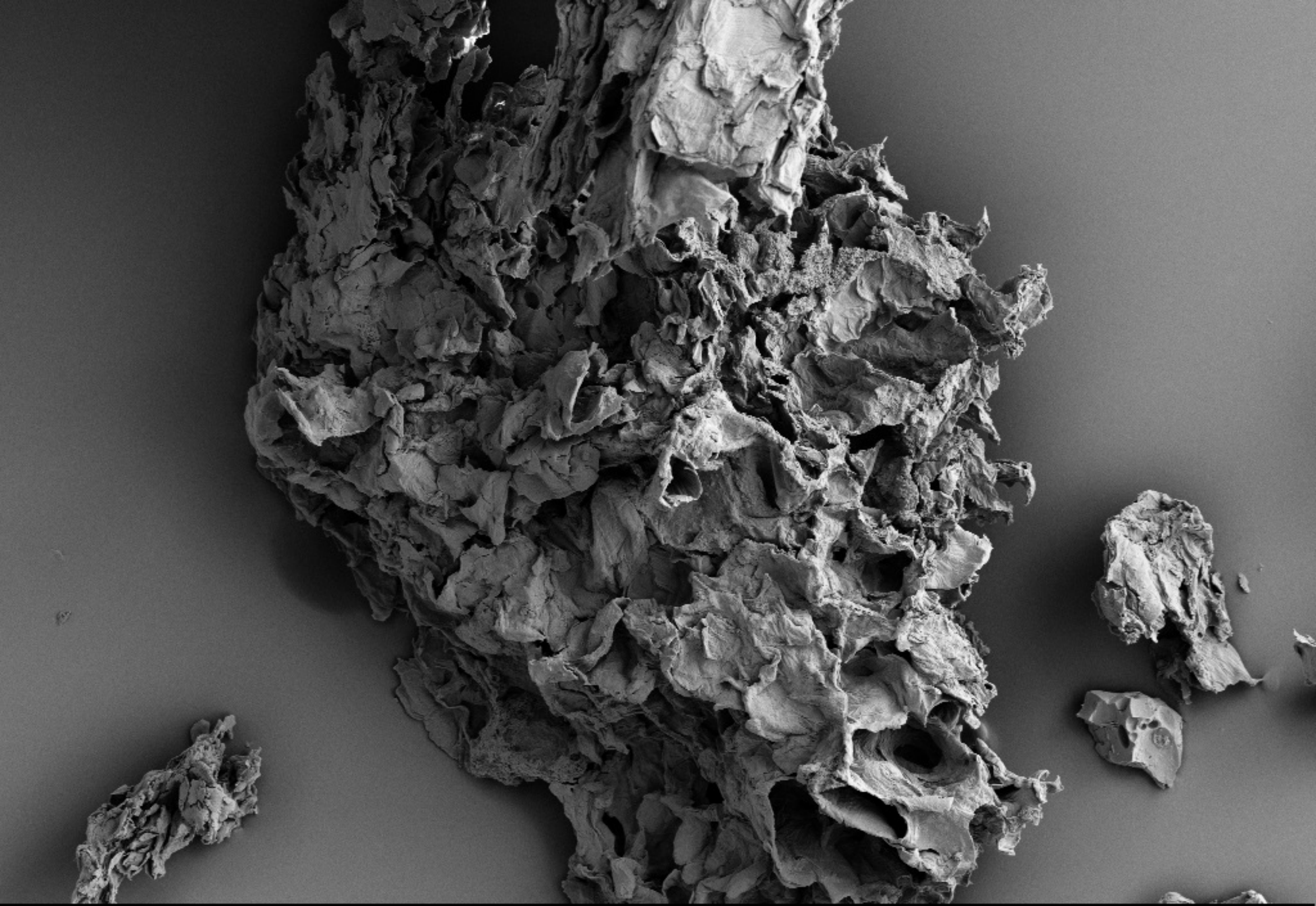
100 µm
Mag = 500 X
Width = 603.9 µm
WD = 9.2 mm
Signal A = SE2
Signal B = NTS BSD
Signal = 1.000
Gun= 3.56e-010 Torr
Chm=1.68e-006 Torr
Sys= 1.68e-006 Torr
File Name = OSCE- 03.tif
31 Jan 2017 16:24:30
5.00 kV
x = 68.867 mm
y = 42.703 mm

Figure 4C

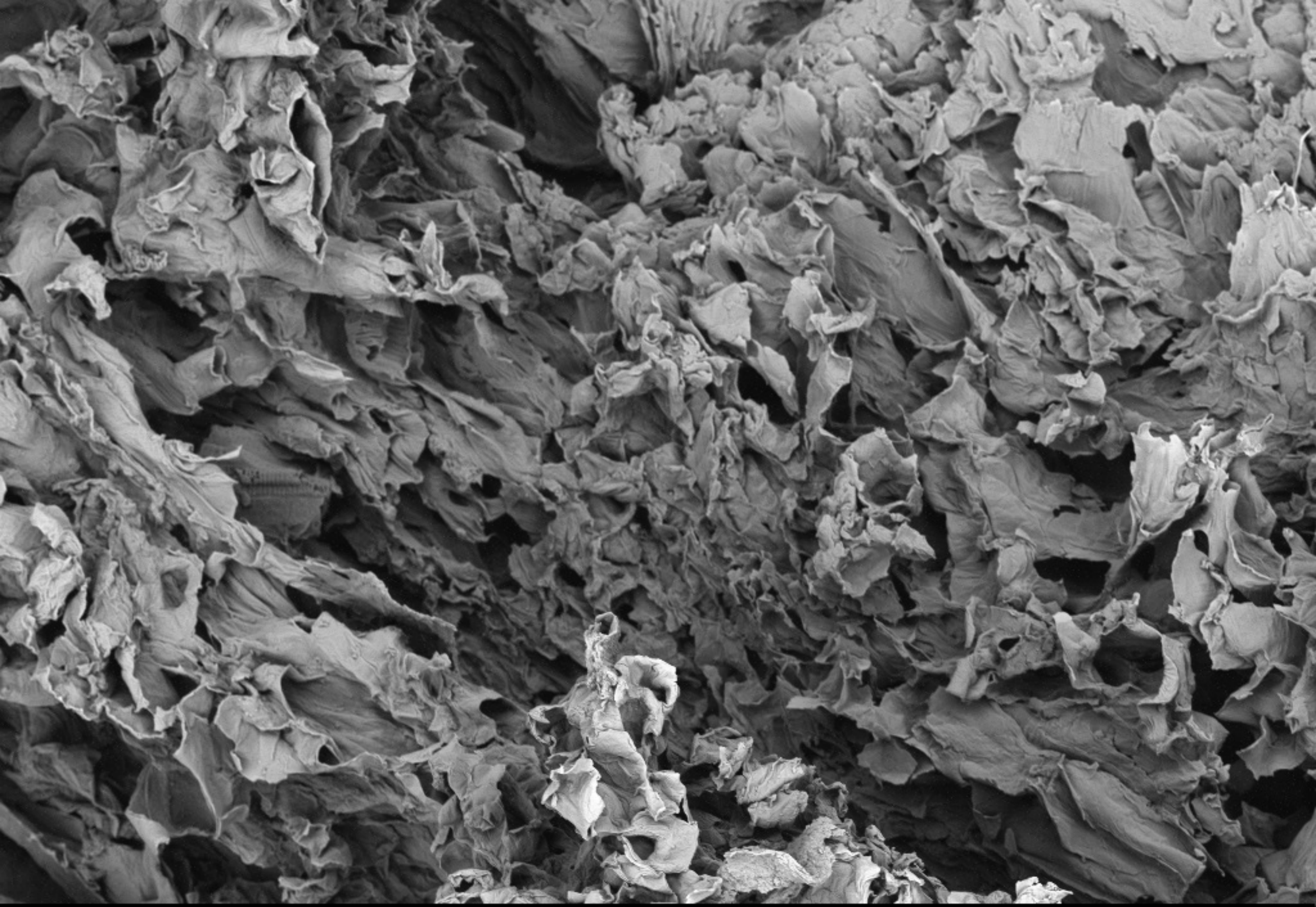
100 µm
Mag = 500 X
Width = 603.9 µm
WD = 8.5 mm
Signal A = NTS BSD
Signal B = NTS BSD
Signal = 1.000
Gun= 3.46e-010 Torr
Chm=1.85e-006 Torr
Sys= 1.85e-006 Torr
File Name = AUAE- 02.tif
31 Jan 2017 15:58:29
10.00 kV
x = 80.080 mm
y = 50.935 mm

Figure 4D