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TheinfluenceofsterilizationwithEnbioJet® MicrowaveFlowPasteurizeron composition and bioactivity of aronia and blue-berried honeysuckle juices

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A B S T R A C T

In this study, fruit juices that are rich sources of anthocyanins, obtained from aronia (Am. chokeberry, *Aronia melanocarpa*) and blue-berried honeysuckle (*Lonicera caerulea* L. var. *edulis*) were used to examine the preservation of plant phytochemicals and bioactivity upon sterilization—either thermal, or with an EnbioJet® Microwave Flow Pasteurizer. The chemical properties verified included determinations of anthocyanins and other polyphenols by HPLC, total antioxidant activity, and profiles of antioxidants by post-column derivatization. Compared to heat treatment, the higher stability of aronia and blue-berried honeysuckle phytocomplexes during processing with the EnbioJet® device for temperatures ensuring microbial purity (range investigated 90–130 °C) was demonstrated. In the same batches of juices submitted to heating at 100 °C, the rapid decline of anthocyanin content accompanied by lowered antioxidant activity was observed. The changes in chemical composition were reflected in altered biological activity. Both cytotoxicity and protection of DNA against oxidative damage were higher for microwaved juices than for juices processed by the heating method that caused degradation of bioactive phytochemicals.

Keywords: Sterilization Microwaves, Aronia, Blue-berried honeysuckle, Anthocyanins Antioxidant, activity HPLC, Comet assay, Food analysis, Food composition

1. Introduction

Microwave technology is gaining increasing interest in food processing owing to a number of advantages it offers. This way of processing is suggested to surpass conventional processing as regards time, cost effectiveness, ensuring biological safety and preserving sensory properties of fruit and vegetable products (Harrington, 2010). These advantages may be extended to yet another benefit, currently rather less frequently considered. The way of delivering energy and very short exposure to high temperature needed to achieve microbiological safety of plant food products processed with the aid of microwave technology may help also to preserve bioactive phytochemicals (Igual et al., 2010). In the past two decades, these non-nutritive food components have become a focus of interest in health-promoting food production. In particular, the phytochemicals exhibiting antioxidant capacity have quickly become incorporated into a variety of food products following the reports on the etiology of major diseases of civilization resulting from oxidative stress (Halliwell, 2007; de la Monte and Wands, 2008; Calabrese et al., 2010). Special attention has been given to polyphenols belonging to the anthocyanin group. These compounds found mainly in a number of red to dark blue fruits, mostly berries, have been demonstrated to exhibit an array of biological activities important for human health (Howard and Hager, 2007; Szajdek and Borowska, 2008). Those most frequently quoted include: antioxidant, anti-inflammatory and antiplatelet properties, and counteracting cancer and heart diseases (Kong et al., 2003). Importantly, in the case of anthocyanin-rich foods, human intervention studies have also been undertaken. Their results have shown that anthocyanins may be responsible for decreasing LDL cholesterol and the level of triacylglycerols as well as for lowering systolic and diastolic blood pressure (Skoczyńska et al., 2007). Apart from that, clinical studies have shown the hypotensive effect of anthocyanins and their ability to reduce cardiovascular risk markers after myocardial infarction (Naruszewicz et al., 2007).

Unfortunately, anthocyanins are among the most unstable phytochemicals. Factors influencing their stability are diverse and widely discussed in the literature. The metabolic modification of anthocyanins (glycosylation, acylation with aliphatic or aromatic acids), as well as pH, temperature, light, presence of metal ions,

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oxygen and sugar content, all may influence their content in foods (Rodriguez-Saona et al., 1999). In particular, the high temperature of processing affects these phytochemicals, causing their degradation. Therefore, control of temperature conditions and time of heating may benefit the concentration of anthocyanins present in the final product (Seeram et al., 2001).

In this study, the stability of anthocyanins and other antioxidants found in fruit juices was compared for two sterilization methods—conventional thermal and microwave assisted. Microbial purity as well as preservation of phytocomplexes and selected biological activities were assessed for each method, with unprocessed, fresh juices as reference material. As a model sources of anthocyanins, we have chosen two fruits with documented chemopreventive properties and rich sources of anthocyanins: aronia (*Aronia melanocarpa* (Michx.) Elliott) displaying beneficial health effects widely covered in scientific literature (Kulling and Rawel, 2008), and blue-berried honeysuckle (*Lonicera caerulea* L. var. *edulis*), whose biological potential is increasingly recognized (Svarcova et al., 2007).

2. Materials and methods

2.1. Chemicals and biochemicals

HPLC grade methanol and pure p.a. methanol were purchased from Chempur (Poland). Formic acid (98–100%) was obtained from Merck (Germany). Water was purified with a $Q_{PLUS}185$ system from Millipore (USA). The following chemicals and biochemicals were used: 2,2′-azinobis(ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), thiazolyl blue tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), hydrogen peroxide (30%) from Sigma–Aldrich (USA); Folin-Ciocalteu's phenol reagent (FC) from Merck (Germany); phosphate buffered saline (PBS), Triton X-100, sodium pyruvate, L-glutamine, LMP agarose, foetal bovine serum (FBS), penicillin–streptomycin solution, RPMI-1640 and McCoy's media from Sigma (USA); agarose electrophoresis grade (NMP), SYBR® Gold nucleic acid gel stain from Invitrogen™ (USA). The media used for all microbial determinations were purchased from BTL (Poland). The following standard phenolic compounds were used: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, (+)-catechin hydrate, rutin, quercetin from Sigma–Aldrich (USA); caffeic acid, protocatechuic acid, cyanidin-3-glucoside from Fluka (USA); cyanidin-3-galactoside, chlorogenic acid, hyperoside from Extrasynthese (France). All stock solutions of standards were prepared in HPLC grade methanol at a concentration of 1 mg/mL and diluted with this solvent as required. All other reagents used were of analytical grade.

2.2. Plant material

Juices prepared from two fruits: aronia or chokeberry (*A. melanocarpa* (Michx.) Elliott) and blue-berried honeysuckle (*L. caerulea* L. var. *edulis*) were used throughout this study. Aronia fruits collected in September 2009 were purchased from a local fruit processing factory "Fungopol" (Northern Poland), while blue-berried honeysuckle berries collected in June 2009 and 2010 were obtained from a local plantation "Jagódka" (Northern Poland). The fruits were kept at −20 °C before sample preparation. To obtain juices, the fruits were defrosted and squeezed manually through cotton fabric. Then, the liquids were vacuum filtered through glass fiber paper GF/F filters (Whatman, England).

2.3. Microwave sterilization and classic thermal processing

Fruit juices, 9.1 L of aronia juice and 6.5 L of blue-berried honeysuckle juice, were diluted with spring water to a final volume of 25 L. The juices were sterilized using the EnbioJet Microwave Flow Pasteurizer (Enbio Technology Co., Kosakowo, Poland). The power supply of 63 A/20 kW and the temperature range of 90–135 °C were tested. The microwave exposure time amounted to 7 s. Both fruit juices were also submitted to a classic thermal processing, *i.e.* they were kept at 100 °C for 1–5 h. For examination of stability of blue-berried honeysuckle phytocomplex, non-diluted juice prepared with fruits collected in June 2010 was used.

2.4. Microbial determinations

Samples of juices (control and sterilized with EnbioJet device) were serially diluted with saline (Malinowska-Pańczyk et al., 2009). Appropriate serial dilutions of juices were then distributed over plate count agar (PCA) and incubated for 48 h at 30 °C for total bacterial count. Additionally, YM-agar covered plates were used to determine total counts of yeast and moulds in samples of the juices. The plates were incubated at 20 °C for 7 days.

2.5. HPLC determination of phenolic compounds

In the study, an Agilent 1200 Series HPLC-DAD-MS system (Agilent Technologies, USA) was employed for the determination of phenolic compounds. Chromatographic separations were performed on an Agilent Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 μm). The mobile phase consisted of 4.8% (v/v) formic acid in water (solvent A) and HPLC grade methanol (solvent B). The flow rate was set at 0.7 mL/min and the injection volume of all juice samples was 2 μL. The elution was carried out with a linear gradient program with the ratio of solvent B:A going from 10:90 to 55:45 (v/v) over a period of 40 min. MS parameters were as follows: capillary voltage, 3000 V; fragmentor, 120 V; drying gas temperature, 350 °C; gas flow (N_2), 12 L/min; nebulizer pressure, 35 psig. The instrument was operated in a positive ion mode scanning from *m/z* 100 to 800. Individual phenolic compounds were identified by comparing their retention times with those for standards or on the basis of available literature data and mass spectra (Borges et al., 2010). The content of identified phytochemicals (expressed as μg/mL of juice) was calculated on the basis of calibration curves produced by the integration of absorption peaks generated from the analysis of dilution series of corresponding standards.

2.6. Determination of antioxidant activity

Standard methods employing ABTS and DPPH radicals or Folin-Ciocalteu (FC) reagent were used for the colorimetric determination of antioxidant activity as described previously (Kusznierewicz et al., 2011). In each case, a Trolox solution served to generate the standard line (concentration range 0–4.0 mmol/L). All determinations were carried out in 48-well plates at room temperature, and the absorbance was measured with a TECAN Infinite M200 spectrophotometer (Tecan Group Ltd., Switzerland).

2.7. Profiles of antioxidants obtained by post-column derivatization

On-line post-column addition of the ABTS or FC derivatization reagents to HPLC eluent was performed using Pinnacle PCX Derivatization Instrument (Pickering Laboratories, Inc., USA) consisting of pump delivery system and reactor that can be heated. The derivatization reagents were prepared as follows: ABTS was dissolved in aqueous sodium persulphate (2.45 mmol/L) to obtain a concentration of 7 mmol/L, the mixture was stored in the dark at room temperature for 12 h and before use was diluted with methanol to the stock concentration of 30% (v/v). Commercially available FCR was diluted with water to a concentration of

40% (v/v). Derivatization was carried out at the temperature of 130 °C. The flow rate of the individual reagents was set at 0.1 mL/min. In all experiments, the 0.5 mL (PTFE, 0.25 mm, 10 m) coil available as a standard part of the Pinnacle PCX Derivatization Instrument was used. Chromatograms of products formed after derivatization of antioxidant compounds with ABTS or FC reagents were registered at 734 nm and 750 nm, respectively, using a multiple wavelength detector (Agilent 1200 Series MWD, USA).

2.8. Cell culture

TK6 cells (human lymphoblastoid cell line) were grown in RPMI-1640 medium, and HT29 (human colon adenocarcinoma) cells in McCoy's medium, each supplemented with foetal bovine serum (10%, v/v), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), L-glutamine (2 μmol/mL) and sodium pyruvate (200 μg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.9. Determination of cytotoxicity of juices

The cytotoxicity of aronia juices (fresh, heated for 1 h at 100 °C or processed with the EnbioJet device at 130 °C) towards TK6 cell was determined with the proliferation assay (Azqueta et al., 2005). The cells were seeded in 75 cm^2 flasks at a density of 0.2×10^6 cells per 1 mL of medium. After 48 h, samples containing 0.8 mL of cell suspension (1×10^6 cells/mL) were plated in 12-well plates. Cells were incubated for 3 h at 37 °C with 0.2 mL of different concentrations (ranging from 0.0013 to 0.01%, v/v) of aronia juices diluted with PBS. After incubation, the cells were centrifuged ($720 \times g$, 3 min, 4 °C), resuspended in 1 mL of PBS and centrifuged again. Rinsing with PBS was repeated three times. The pellets of TK6 cells were resuspended in fresh medium (0.5×10^6 cells per 1 mL of medium) and were plated in 12-well plates. After 48 h at 37 °C, the cells were counted in a hemocytometer. The influence of processing on the ability of blue-berried honeysuckle juice to inhibit growth of HT-29 cells was assessed with the MTT assay (Carmichael et al., 1987). The exponentially growing cells were seeded in 96-well tissue culture plates (about 20,000 cells/well in 0.15 mL) and allowed to settle for 24 h. Then, to the wells was added 0.05 mL of different concentrations of juices. After treatment for 24 h, the medium was aspirated from the wells, replaced with fresh medium and the cells were incubated at 37 °C for a further 48 h. For the final 4 h of incubation, to each well 0.05 mL of MTT solution (4 mg/mL) was added. To determine the cell growth, the medium was thoroughly removed from wells, farmazan crystals dissolved in 0.05 mL of DMSO added and absorption of the resultant solutions was determined at 540 nm in a TECAN Infinite M200 plate reader (Tecan Group Ltd., Switzerland). For both cellular models, cytotoxicity was expressed as growth inhibition of cells induced by fruit juices in comparison to control non-treated cells.

2.10. Prevention of oxidative DNA damage by aronia juice

Portions of TK6 cells prepared in the same way as for cytotoxicity determination (Section 2.9) amounting to 0.04×10^6 were submitted to the comet assay (Collins, 2004). The cell suspension was mixed with 140 μL LMP agarose (1%, w/v), warmed to 37 °C and two 70 μL aliquots were dropped onto microscope slide precoated with NMP agarose (1%, w/v). Glass coverslips were placed on the drops of agarose, which was allowed to set at 4 °C. One slide was prepared for each concentration of aronia juices. The coverslips were removed and the cells embedded in agarose on the microscope slides were treated with 50 μmol/L of H_2O_2 for 5 min at 4 °C to induce DNA damage. The cells were lysed for 1 h in lysis buffer (0.1 mol/L EDTA, 2.5 mol/L NaCl, 10 mmol/L Tris-base, pH 10 with 1% Triton X-100) freshly added, at 4 °C. After lysis, all slides were placed in a horizontal electrophoresis tank and the DNA was allowed to unwind for 40 min in alkaline electrophoresis buffer (0.3 mol/L NaOH, 1 mmol/L EDTA, pH 13.3) at 4 °C. Electrophoresis was carried out in the same buffer for 30 min at 25 V and 300 mA at 4 °C. The slides were then neutralized with PBS for 10 min and for 10 min with water. The slides were submerged in SYBR® Gold solution (prepared according to producer's recommendations) for 40 min at 4 °C to visualize DNA. After staining, the slides were washed twice with water. Before analysis, the gels were moistened with 20 μL of water and covered with coverslips. Stained nuclear DNA was examined under a Nikon eclipse TS100 fluorescence microscope. The analyses were carried out with the aid of a software scoring system *Comet Assay IV* (Perceptive Instruments) and involved counting of 100 comets selected at random from each slide. The results were expressed as mean % DNA in tail.

3. Results and discussion

3.1. Sterilization methods under comparison

The first method of processing, used here as a reference method of sterilization, was conventional thermal processing. Aronia and blue-berried honeysuckle belong to the fruits that are often traditionally processed at home to produce juices or jams. In fact, in the case of the latter fruit, this is the only way of processing as this berry is rather rare in Poland and can be found mainly in household gardens. This type of processing, being tagged as traditional, is generally perceived by the public as the one producing best quality foods. However, cooking time can be quite lengthy and take even several hours. Accordingly, the aronia and blue-berried honeysuckle juices, diluted with spring water, were heated at 100 °C for up to 5 h in a tightly closed vials.

The other technology under investigation was an innovative sterilization method involving microwave energy for heating the juices. These experiments were carried out with the use of the newly developed high throughput EnbioJet® Microwave Flow Pasteurizer. This device exploits patented technology enabling swift and direct energy transfer (like that in UHT lines) to the flowing medium which reduces the length of the pasteurization/sterilization process to seconds. The higher the temperature (energy supply) the shorter is the holding time. Additionally, due to appropriate design, the undesirable effects of adherance of substances to the walls of the exchanger are eliminated (Patent, 2010). We became interested in this technology because direct supply of energy to water particles, with energy spent only when it is needed to heat up a product, minimises the exposure of labile compounds present in heated medium to high temperature.

3.2. Determination of effectiveness of sterilization

All samples of juices prepared from aronia and blue-berried honeysuckle collected in 2009 were tested for microbiological contamination. In the non-sterilized (control) juices the total bacterial count amounted to 3.7×10^2 and 4.56×10^2 CFU/mL in the case of aronia and blue-berried honeysuckle, respectively. The latter juice additionally contained acidophilic bacteria (1.2×10^3 CFU/mL) and yeast and moulds (1.06×10^2 CFU/mL). The juices submitted to traditional sterilization by heating at 100 °C were devoid of contaminating bacteria and fungi. The microbiological determinations for juice samples sterilized with the EnbioJet device revealed that only the portion of blue-berried honeysuckle juice treated with the lowest temperature (80 °C)

contained some contaminating bacteria. Therefore, samples exposed to such conditions were excluded from further analyses.

3.3. Total antioxidant activity and HPLC profiles of antioxidants

The total antioxidant activities of aronia and blue-berried honeysuckle juices submitted to both sterilization methods, determined by three spectrophotometric tests (employing ABTS and DPPH radicals or FC reagent), are presented in Fig. 1. In aronia juice samples heated at 100 °C, there was a substantial drop of antioxidative potential with increasing length of time of exposure to high temperature. Such a decline was much less pronounced in the case of blue-berried honeysuckle juice treated in the same way. These results are in agreement with other reports. For example, for blood orange juice, it has been shown that antioxidant activity, particularly when measured by the ABTS method, is positively related to the content of anthocyanins and that the reduction of anthocyanin content, typical of commercial long-shelf life juices, leads to a remarkable loss of antioxidant power (Fiore et al., 2005).

In contrast to traditional thermal treatment, sterilization with the aid of the EnbioJet device did not seem to affect strongly the total antioxidant activity of any of the juices investigated (Fig. 1). A rather puzzling observation was the discrepancy between unchanged total antioxidant potentials measured for samples of the both juices sterilized with the EnbioJet device (Fig. 1) and a small, albeit observable, drop in anthocyanin content (Table 1). The explanation of this effect was found when antioxidants present in the juices were profiled by post-column derivatization with either ABTS or FC reagent. Fig. 2 presents a representative set of chromatograms obtained for three samples of aronia juice: control (non-sterilized), and sterilized with the EnbioJet device at the target temperatures of 90 °C and 130 °C. Although peaks corresponding to anthocyanins in chromatograms of phenolic compounds (Fig. 2, Panels A), as well as in chromatographic profiles of antioxidant components (Fig. 2, Panels B and C) were lower in samples treated at the target temperature of 130 °C compared to 90 °C, the drifting baseline in the profiles of antioxidants suggested that these samples contained an array of unresolved degradation products exhibiting antioxidant potential. Such a smear of antioxidative compounds was also detected in thin layer chromatograms prepared for the corresponding samples of juices and visualized with ABTS radical (data not shown). These unidentified degradation products perhaps account for the extra antioxidative activity measured by spectrophotometric tests, despite smaller input from anthocyanins.

3.4. Changes in the composition of phytochemicals during traditional thermal or microwave sterilization

The phytocomplexes of both fruits under study contain compounds representing different groups of polyphenols: anthocyanins, flavonols and phenolic compounds, which are regarded as important chemopreventive dietary ingredients. Therefore, the fate of all these groups of phytochemicals was monitored both during thermal treatment as well as during microwave sterilization with the Enbiojet device. The quantitative data regarding concentration of major polyphenols are collected in Table 1. Although, in the case of both fruits, thermal treatment resulted in a decrease of major phytochemicals, the composition of blue-berried honeysuckle juice seemed to be more thermally resistant. The content of aronia anthocyanins decreased twofold as soon as after 1 h, and essentially they were almost completely destroyed after 3 h of heating at 100 °C, while in the blue-berried honeysuckle juice this decline seemed much smaller. Presumably, on one hand cyanidin-3-glucoside is more thermally stable than its galactoside equivalent, a dominating aronia anthocyanin. On the other hand, as discussed further, the anthocyanins in non-sterilized control blue-berried honeysuckle juice appeared very prone to decomposition as discussed in Section 3.5 in more detail. The profiles of phenolic acids were less affected by the thermal treatment; however, also here aronia phytochemicals were more liable to decomposition. In particular, vitamin C, procyanidin dimer (PC-d) and gallic acid (GA) were detected only in control aronia juice (Table 1). These three compounds as can be seen in Fig. 2 donated a substantial share to the antioxidant potential of this fruit sample. Very noticeable was the conversion of 3- and 5-caffeylquinic acid (3CQA, 5CQA) to non-identified derivative (CQA-d).

The thermal instability of anthocyanins is a well known phenomenon reported widely in the literature (Mok and Hettiarachchy, 1991; Ochoa et al., 1999; Dyrby et al., 2001; Reyes and Cisneros-Zevallos, 2007). The increased content of protocatechuic acid (PCA) observed in the case of heated aronia juice has been reported and is the result of cyanidin degradation (Furtado et al., 1993). Because of health benefits associated with the presence of anthocyanins in foods, their degradation is an undesirable effect of thermal processing. However, the appearance of quercetin, an aglycone not detected in fresh juices, may be regarded as a positive change, that leads to improved bioavailability of aronia and blue-berried honeysuckle flavonoids. A closer look at the results shown in Table 1 suggests that quercetin is probably formed by deglycosylation of rutin, the amount of which decreases in both juices with time of exposure to 100 °C. This transformation takes place very quickly, especially in the case of blue-berried honeysuckle juice, as in samples collected after 1 h of heating quercetin was already present.

The composition and contents of polyphenolic compounds in the samples of aronia and blue-berried honeysuckle juices sterilized with EnbioJet device are shown in Table 1. The general observation made while analysing these data is that the changes in concentrations of phytochemicals are much less than those seen during traditional heat treatment. The amounts of phenolic acids and flavonoids with the exception of anthocyanins were not altered at all; in particular no quercetin was detected. Also, the contents of anthocyanins declined to lesser extents, especially in the case of blue-berried honeysuckle juice where they decreased by no more than 25%.

3.5. Changes in selected biological activities exhibited by aronia juices

In the next set of experiments, we compared the biological activity of three samples of aronia juice processed in ways that

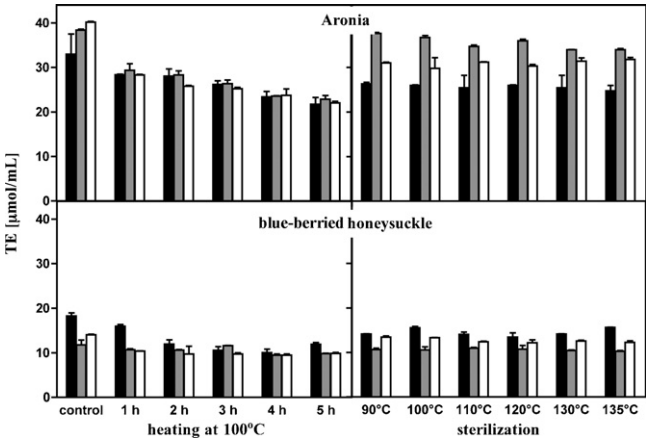

Fig. 1. Total antioxidant activity of aronia and blue-berried honeysuckle juices after classic thermal processing (at 100 °C) or after sterilization with EnbioJet Microwave Flow Pasteurizer (at the indicated target temperature) expressed as Trolox equivalents [μmol/mL] determined by ABTS (■); DPPH (▩) and FC (□) assays. Results represent means ± SD of three independent experiments.

Table 1
Chromatographic, spectroscopic, spectrometric characteristics and concentration of phenolic compounds (μg/mL) in aronia and blue-berried honeysuckle juices submitted to thermal processing at 100 °C (1–5 h) or sterilized with EnbioJet Microwave Flow Pasteurizer at the temperature range of 90–135 °C determined by HPLC-DAD-MS.

Peak	1	2	3	4	5	6	7	8	9	10
Compound	**GA**	**PCA**	**3-CQA**[a]	**PC-d**[b]	**5-CQA**[a]	**CQA-d**[a]	**Cy-3,5-dGlc**[c]	**CA**	**Cy-3-Gal**[c]	**Cy-3-Glc**[c]
t_R [min]	3.8	6.4	6.7	8.2	11.4	12.1	12.9	13.5	16.5	17.9
Major ions [m/z]	153, **171**, 193	137, **155**, 177	163, **355**, 377	291, **579**	163, **355**, 377	163, 355, **480**	287, **611**	163, **181**	287, **449**	287, **449**
λ_{max} [nm]	270	260	325	280	325	325	525	325	525	525
Aronia										
con	197.8 ± 24.3	14.3 ± 0.3	503.2 ± 12.4	423	449.0 ± 19.0	23.6 ± 2.3	nd	tr	616.0 ± 77.0	25.1 ± 3.4
1 h	nd	69.1 ± 0.9	628.1 ± 23.2	nd	431.3 ± 1.0	54.0 ± 1.5	nd	tr	212.0 ± 11.0	11.4 ± 0.7
2 h	nd	84.7 ± 4.3	561.1 ± 13.8	nd	395.0 ± 0.9	74.6 ± 2.1	nd	tr	55.8 ± 4.2	3.8 ± 0.4
3 h	nd	93.7 ± 1.4	536.6 ± 14.5	nd	385.7 ± 5.5	94.1 ± 4.1	nd	tr	20.0 ± 2.2	1.7 ± 0.1
4 h	nd	99.7 ± 0.3	501.5 ± 9.5	nd	363.1 ± 1.9	111.0 ± 7.2	nd	tr	5.3 ± 0.8	0.6 ± 0.1
5 h	nd	103.6 ± 1.6	495.9 ± 16.8	nd	361.3 ± 6.1	128.3 ± 4.3	nd	tr	1.9 ± 0.5	tr
90 °C	nd	18.5 ± 1.0	461.7 ± 12.7	nd	427.3 ± 0.9	19.6 ± 2.3	nd	tr	385.0 ± 30.0	14.6 ± 1.3
100 °C	nd	23.3 ± 1.3	467.1 ± 17.6	nd	438.5 ± 9.3	20.0 ± 1.1	nd	tr	309.0 ± 16.0	11.4 ± 0.5
110 °C	nd	21.0 ± 1.3	458.4 ± 23.2	nd	423.6 ± 0.3	19.3 ± 0.3	nd	tr	317.0 ± 17.0	12.0 ± 0.8
120 °C	nd	22.4 ± 1.3	458.1 ± 21.2	nd	423.8 ± 0.6	20.0 ± 0.8	nd	tr	303.0 ± 22.0	11.4 ± 1.6
130 °C	nd	25.4 ± 0.3	458.1 ± 11.2	nd	421.7 ± 3.5	20.3 ± 1.0	nd	tr	256.0 ± 20.0	9.4 ± 0.8
135 °C	nd	14.8 ± 4.9	453.3 ± 10.1	nd	414.2 ± 0.3	19.7 ± 0.4	nd	tr	303.0 ± 17.5	11.6 ± 0.8
Blue-berried honeysuckle										
con	nd	nd	42.9 ± 0.3	nd	262.9 ± 0.4	nd	53.9 ± 3.4	tr	nd	442.0 ± 20.0
1 h	nd	nd	39.3 ± 0.1	nd	275.8 ± 5.2	5.0 ± 0.7	17.9 ± 1.7	7.2 ± 0.7	nd	387.5 ± 2.1
2 h	nd	nd	38.1 ± 1.4	nd	268.7 ± 7.6	6.8 ± 0.8	13.4 ± 0.5	7.2 ± 0.7	nd	354.9 ± 9.4
3 h	nd	nd	38.1 ± 0.8	nd	276.5 ± 3.5	8.5 ± 1.0	9.0 ± 0.2	6.2 ± 0.7	nd	308.0 ± 12.0
4 h	nd	nd	38.6 ± 0.5	nd	278.0 ± 5.0	9.5 ± 1.1	6.4 ± 0.1	5.4 ± 1.0	nd	254.8 ± 2.7
5 h	nd	nd	36.1 ± 1.1	nd	267.7 ± 3.7	11.9 ± 0.8	4.2 ± 0.1	5.1 ± 0.3	nd	218.1 ± 1.3
90 °C	nd	nd	39.1 ± 1.4	nd	294.8 ± 1.1	nd	50.1 ± 0.4	3.4 ± 0.2	nd	935.0 ± 44.0
100 °C	nd	nd	39.1 ± 0.7	nd	294.5 ± 2.3	nd	46.3 ± 0.2	2.8 ± 0.1	nd	728.0 ± 35.0
110 °C	nd	nd	39.1 ± 0.5	nd	296.1 ± 2.3	nd	49.3 ± 0.5	3.0 ± 0.2	nd	809.0 ± 40.0
120 °C	nd	nd	39.5 ± 0.4	nd	298.0 ± 1.1	nd	47.8 ± 1.1	2.9 ± 0.1	nd	754.0 ± 29.0
130 °C	nd	nd	39.5 ± 1.2	nd	294.0 ± 2.3	nd	48.2 ± 0.3	2.9 ± 0.1	nd	749.0 ± 30.0
135 °C	nd	nd	39.5 ± 0.3	nd	295.0 ± 0.8	nd	48.2 ± 0.4	2.9 ± 0.1	nd	739.0 ± 26.0

Table 1 (*Continued*)

Peak	**11**	**12**	**13**	**14**	**15**	**16**	**17**	Total anthocyanins	Total phenolics
Compound	**Cy-3-Ara**[c]	**C-3-Rut**[c]	**Pn-3-Glc**[c]	**Cy-3-Xyl**[c]	**Q-3-Gal**	**Q-3-Rut**	**Q**		
t_R [min]	19.4	19.5	21.9	24.6	27.1	27.9	32.1		
Major ions [m/z]	287, **419**	287, **595**	301, **463**	287, **419**	303, **465**, 487	303, **611**, 633	**303**		
λ_{max} [nm]	525	525	525	525	360	360	360		
Aronia									
con	190.2 ± 23.2	nd	nd	24.3 ± 3.1	89.6 ± 6.5	133.9 ± 5.6	nd	855.5	3545.6
1 h	59.3 ± 7.4	nd	nd	7.5 ± 1.1	76.7 ± 1.4	141.7 ± 5.9	19.4 ± 1.1	290.2	2000.8
2 h	10.6 ± 2.1	nd	nd	1.5 ± 0.5	77.7 ± 0.7	133.5 ± 0.9	23.9 ± 0.7	71.7	1493.9
3 h	3.1 ± 0.3	nd	nd	tr	94.8 ± 1.4	118.3 ± 1.9	26.5 ± 0.7	24.8	1399.4
4 h	0.9 ± 0.1	nd	nd	tr	86.5 ± 1.5	100.1 ± 1.3	26.9 ± 0.9	6.8	1302.5
5 h	tr	nd	nd	tr	83.4 ± 1.7	93.6 ± 1.9	26.5 ± 1.1	2.0	1296.8
90 °C	102.8 ± 13.3	nd	nd	13.9 ± 0.9	81.8 ± 5.3	124.8 ± 1.7	nd	516.3	2166.4
100 °C	78.8 ± 5.5	nd	nd	11.1 ± 1.0	78.0 ± 16.0	128.8 ± 2.8	nd	410.3	1976.3
110 °C	81.2 ± 7.7	nd	nd	10.9 ± 0.8	76.0 ± 11.0	125.2 ± 1.1	nd	421.1	1965.7
120 °C	78.5 ± 4.2	nd	nd	10.9 ± 0.5	78.7 ± 8.9	122.5 ± 1.7	nd	403.7	1933.0
130 °C	74.1 ± 3.2	nd	nd	10.3 ± 0.6	76.9 ± 8.1	122.5 ± 0.6	nd	349.8	1824.5
135 °C	65.3 ± 6.1	nd	nd	8.9 ± 0.3	78.1 ± 4.9	120.5 ± 1.1	nd	388.9	1878.4
Blue-berried honeysuckle									
con	nd	50.1 ± 0.6	25.2 ± 1.2	nd	nd	181.4 ± 7.2	nd	571.2	1629.6
1 h	nd	26.1 ± 1.4	12.8 ± 1.0	nd	nd	27.4 ± 0.5	87.9 ± 5.9	444.3	1243.4
2 h	nd	25.1 ± 0.8	12.9 ± 0.4	nd	nd	29.0 ± 1.2	73.3 ± 3.7	406.3	1162.4
3 h	nd	16.7 ± 0.5	7.9 ± 0.6	nd	nd	26.6 ± 1.0	77.2 ± 1.1	341.7	1039.2
4 h	nd	13.8 ± 0.7	6.5 ± 0.2	nd	nd	24.3 ± 0.2	81.0 ± 2.4	281.6	918.9
5 h	nd	10.7 ± 0.3	0.3 ± 0.1	nd	nd	25.8 ± 0.6	81.0 ± 1.3	233.3	813.3
90 °C	nd	48.9 ± 0.4	23.6 ± 1.2	nd	nd	160.9 ± 5.8	nd	1057.7	2613.5
100 °C	nd	44.3 ± 1.1	21.3 ± 0.8	nd	nd	167.1 ± 1.4	nd	839.9	2183.3
110 °C	nd	47.8 ± 1.4	21.3 ± 0.5	nd	nd	168.6 ± 2.2	nd	927.4	2361.7
120 °C	nd	45.9 ± 1.0	20.2 ± 0.2	nd	nd	169.1 ± 0.7	nd	867.8	2245.2
130 °C	nd	45.9 ± 0.8	19.8 ± 0.7	nd	nd	166.0 ± 1.4	nd	862.8	2228.1
135 °C	nd	45.1 ± 0.6	19.4 ± 0.5	nd	nd	167.1 ± 2.9	nd	851.7	2207.8

Composition data expressed as μg/mL (mean ± SD of triplicate assays); concentration based upon [a]chlorogenic acid; [b]catechin; [c]cyanidin-3-glucoside as standards (GA, gallic acid; PCA, protocatechuic acid; 3-CQA, 3-caffeoylquinic acid; PC-d, procyanidin dimer; 5-CQA, 5-caffeoylquinic acid; CQA-d, caffeoylquinic acid derivative; Cy-3,5-dGlc, cyanidin-3,5-diglucoside; CA, caffeic acid; Cy-3-Gal, cyanidin-3-galactoside; C-3-Ara, cyanidin-3-arabinoside; C-3-Rut, cyanidin-3-rutinoside; Pn-3-Glc, peonidin-3-glucoside; C-3-Xyl, cyanidin-3-xyloside; Q-3-Gal, quercetin-3-galactoside; Q-3-Rut, quercetin-3-rutinoside; Q, quercetin; nd, not detected; tr, traces). Bolded ions—[M]$^+$.

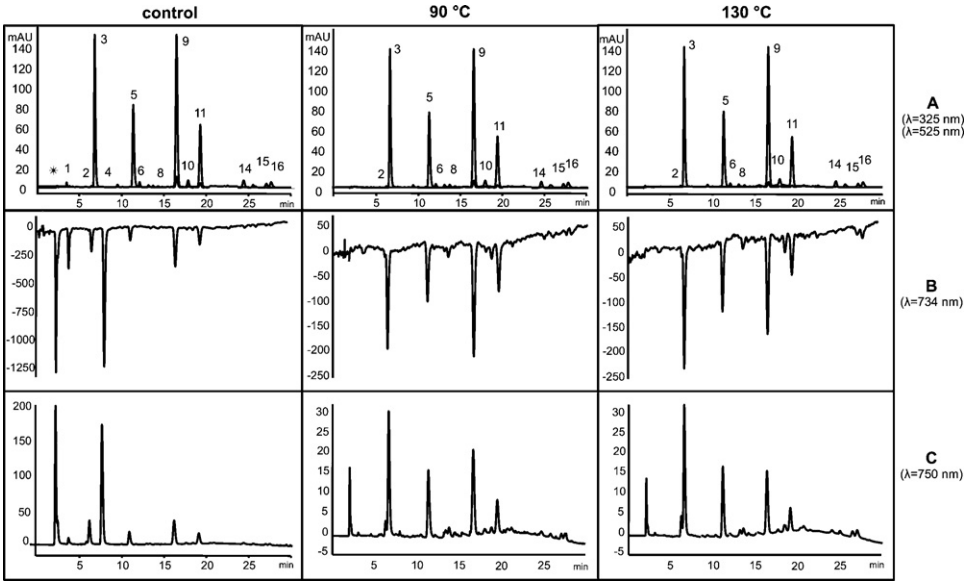

Fig. 2. Combined plots of HPLC profiles of phenolic compounds present in aronia juices sterilized with EnbioJet Microwave Flow Pasteurizer at the target temperatures of 90 °C and 130 °C obtained before (A) and after derivatization with ABTS (B) or FC (C) reagents. The identified peaks include: *—vitamin C, 1—gallic acid, 2—protocatechuic acid, 3—3-caffeoylquinic acid, 4—procyanidin dimer, 5—5-caffeoylquinic acid, 6—caffeoylquinic acid derivative, 8—caffeic acid, 9—cyanidin-3-galactoside, 10—cyanidin-3-glucoside, 11—cyanidin-3-arabinoside, 14—cyanidin-3-xyloside, 15—quercetin-3-galactoside, 16—quercetin-3-rutinoside.

most closely resemble conditions applied in the food industry. The samples chosen included: unprocessed aronia juice, aronia juice heated for 1 h at 100 °C and aronia juice sterilized with the EnbioJet device at the target temperature of 130 °C. The ability of the sample juices to inhibit growth of human lymphoblastoid TK6 cell line was a measure of overall bioactivity. The plots presented in Fig. 3 demonstrate that traditional thermal treatment of aronia juice diminished its growth inhibitory activity in comparison with fresh juice or juice sterilized with the EnbioJet device.

Also, the ability of the same three samples of aronia juices to prevent oxidative DNA damage in TK6 cells challenged with hydrogen peroxide was evaluated by the comet assay. The most efficient, dose-dependent, protection of cellular DNA against oxidative damage was observed for unprocessed juice (Fig. 4). The EnbioJet-sterilized sample for the highest concentrations used, was equally effective. The aronia juice submitted to thermal treatment prevented oxidative DNA damage in TK6 cells in a dose-dependent manner as well, but was the least effective of the three juices under study.

3.6. Instability of blue-berried honeysuckle phytocomplex

The much higher content of anthocyanins in EnbioJet-sterilized blue-berried honeysuckle juice compared with control samples was very puzzling (Table 1). This result suggested that either very efficient endogenous oxidoreductases or very rapid growth of microorganisms caused the degradation of this group of compounds in non-sterilized juice. To get some insight into the processes responsible for this exceptional instability of blue-berried honeysuckle anthocyanins, not seen in the case of aronia juice, we examined HPLC profiles and antioxidant and cytotoxic activity for a new batch of blue-berried honeysuckle juices. The frozen fruits were thawed at ambient temperature. The fruits were pressed to obtain juice, while the still very wet pulp was transferred to −20 °C. The juice was divided into three portions. One was immediately frozen on dry ice and kept at −80 °C (marked as "control"), while another was allowed to undergo spontaneous fermentation for 2 weeks before transferred to −20 °C ("fermented"). The third portion was sterilized in the EnbioJet device at the target temperature of 130 °C as recommended by the producer

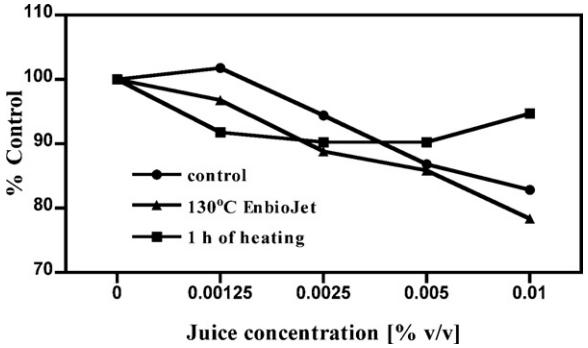

Fig. 3. The inhibition of growth of human lymphoblastoid cell line TK6 by aronia juices: fresh and submitted to either classic thermal processing for 1 h at 100 °C or heated with EnbioJet Microwave Flow Pasteurizer at the target temperature of 130 °C. Cells were incubated with juices for 3 h. Results represent means of three independent experiments, SD values did not exceed 10%.

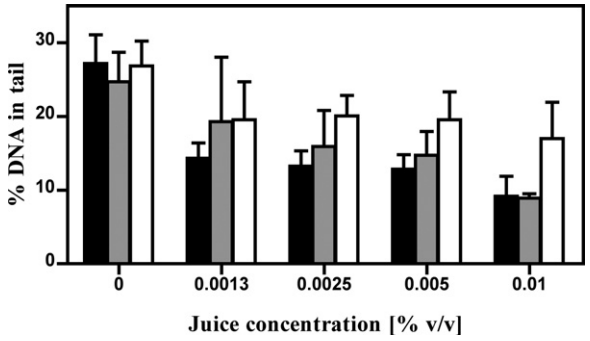

Fig. 4. Protective effect of aronia juices (fresh (■) and submitted to processing with either EnbioJet Microwave Flow Pasteurizer at the target temperature of 130 °C (▩) or heated for 1 h at 100 °C (□) on DNA of human lymphoblastoid cell line (TK6) challenged with oxidative stress induced by H_2O_2 (50 μM). Cells were incubated with juices for 3 h and then submitted to the comet assay. Results represent means ± SD of three independent experiments.

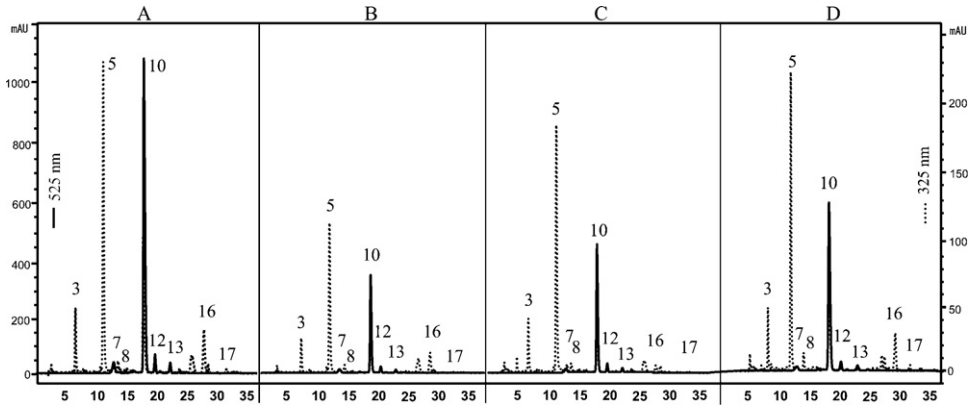

Fig. 5. Combined plots of HPLC profiles of phenolic compounds present in blue-berried honeysuckle juices processed in different ways: fresh stored at −80 °C (A), squeezed from the pulp stored at −20 °C (B), spontaneously fermented (C) and sterilized with EnbioJet Microwave Flow Pasteurizer at the target temperature of 130 °C (D). The identified peaks include: 3—3-caffeoylquinic acid, 5—5-caffeoylquinic acid, 7—cyanidin-3,5-diglucoside, 8—caffeic acid, 10—cyanidin-3-glucoside, 12—cyanidin-3-rutinoside, 13—peonidin-3-glucoside, 16—quercetin-3-rutinoside, 17—quercetin.

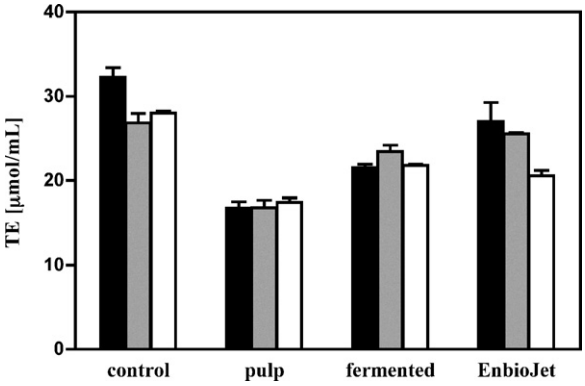

Fig. 6. Total antioxidant activity of blue-berried honeysuckle juices processed in different ways: fresh stored at −80 °C (control), squeezed from the pulp stored at −20 °C (pulp), spontaneously fermented (fermented) or sterilized with EnbioJet Microwave Flow Pasteurizer at the target temperature 130 °C (EnbioJet) expressed as Trolox equivalents [μmol/mL] determined by ABTS (■); DPPH (▩) and FC (□) assays. Results represent means ± SD of three independent experiments.

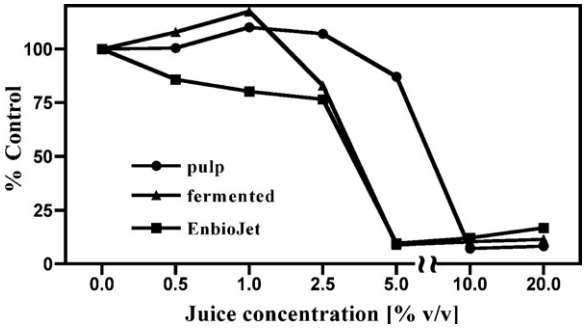

Fig. 7. The inhibition of growth of human colon cancer cell line HT29 treated for 24 h with blue-berried honeysuckle juices processed in different ways: squeezed from the pulp stored at −20 °C (pulp), spontaneously fermented or sterilized with EnbioJet Microwave Flow Pasteurizer at the target temperature 130 °C (EnbioJet). Results represent means of the experiment carried out in triplicate; SD values did not exceed 12%.

("EnbioJet"). After 6 months, all juices and the pulp were thawed, a new portion of juice was squeezed out from the pulp ("pulp") and the samples were analysed. The HPLC profiles of polyphenolic compounds shown in Fig. 5 revealed the profound changes in the content of polyphenols, especially anthocyanins, in all samples compared to the one kept at −80 °C. The smallest decline of anthocyanins as well as of antioxidant potential (Fig. 6) in relation to the "control" sample was observed for the sterilized juice kept at −20 °C ("EnbioJet"). The greatest degradation of polyphenols, paralleled by the most decreased antioxidant activity (Fig. 6) and cytotoxicity (Fig. 7), was found for the juice freshly prepared from frozen stored pulp. This decrease was even stronger than in the fermented sample. All these data suggest that blue-berried honeysuckle may possess very effective oxidoreductases that are inhibited by microwave treatment which therefore slows down degradation of polyphenolic components, anthocyanins in particular.

4. Conclusion

The present results seem to justify the claim that sterilization with EnbioJet® Microwave Flow Pasteurizer is highly conservative as regards bioactive phytochemicals found in aronia and blue-berried honeysuckle and, most probably, this conclusion will hold true for other plant preparations rich in bioactive phytochemicals. One of the mechanisms behind the preservation of labile phytochemicals is the inactivation of enzymatic activities that may catalyse degradation of some valuable components, e.g. antioxidants. Microwave technology has been also applied to vacuum drying of fruits. As in our studies, freeze drying and vacuum microwave drying of cranberries (Leusink et al., 2010) or raspberries (Mejia-Meza et al., 2010) resulted in higher retention of anthocyanins than with other drying methods. In contrast to public beliefs, traditional processing may diminish nutritional value of plant based foods by destroying bioactive components which play an important role in chemoprevention of human diseases.

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