Effects of ultrasound on the enzymatic degradation of pectin

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1. Introduction

Ultrasound (US) technology has gained increasing attention in food processing in the past decade. Particularly in fruit juice production, this emerging technology provides several promising advantages like minimizing processing time, improving sensory quality, and ensuring microbial safety [1–3]. The use of high-intensity US offers a non-thermal alternative to conventional pasteurization techniques that avoids undesired side effects [4]. Benefits have recently been shown also for low-intensity US treatment, e.g., the enhanced yet milder extraction of natural compounds like polysaccharides or polyphenols with higher yields and reduced processing time [3,5]. Additionally, US treatment increases enzyme activities by synergistic effects, also resulting in higher extraction yields of plant materials compared to common enzymatic extractions [6,7].

In juice production, maceration plays a crucial role in defining the quality of the juice. In this process step, the extent of cell wall degradation determines the juice yield and extraction of natural compounds. The addition of pectinolytic enzyme during mashing primarily to increase juice yield is common industrial practice, especially in berry juice production due to the higher pectin content. Commercial enzyme preparations usually contain a mixture of pectinase activities, encompassing mainly polygalacturonase (PG), pectin lyase (PL), and pectin methylesterase (PME) accompanied by a wide range of minor side activities. These mixtures are therefore capable of hydrolyzing pectin being part of the complex polysaccharides of the plant cell wall into ligands containing galacturonic acid units, being part of the complex polysaccharides of the plant cell wall into ligands containing galacturonic acid units.
simpler molecules, thereby enhancing the release of phytochemicals like anthocyanins into the juice. Other polysaccharides like hemicellulose and cellulose remain more or less unchanged depending on the present side activities. [8–10] The quality and extent of cell wall degradation mainly depends on the activity and dosage of the applied enzymes, the enzymes optimal conditions, maceration duration, and the complexity of the fruit’s cell wall matrix [11,12].

Pectin, the most abundant polysaccharide presented in the plant cell wall [13], has been demonstrated to strongly interact with phenolic compounds like anthocyanins. The complexation due to weak bonds like hydrophobic interactions and hydrogen bonds depends on the structure of both pectin and anthocyanins and protects the latter against oxidation and other degradation pathways. The molecular weight (MW) of pectin-derived polysaccharides affects the solubility of the complexes formed, thus determine the nutritional value and color quality of the juice. High MW complexes are very likely removed during pressing, leading to a significant loss of bound phenolic compounds [14–17]. Small oligomers will pass the production process into the juice due to their molecular size and higher solubility enriching the juice in fiber content and stabilizing valuable anthocyanins by complexation [10,15,18]. Because these interactions have the potential to greatly improve nutritional and sensory properties, the control of the extent of cell wall degradation and the size of arising oligosaccharides and polysaccharides during maceration is of pivotal importance for the quality of the juice.

Ultrasound-assisted enzymatic maceration (UAEM) represents a new technological approach to further increase fruit juice quality. To date, only a few studies demonstrated some selected beneficial effects, like higher or similar juice yields at reduced processing temperatures or in less time compared to conventional enzyme treatment. Concomitantly, the extraction of phytochemicals like phenolic acids, flavonoids, and especially anthocyanins was improved, the latter leading to an intensified color. Most of these studies used ultrasonic bath systems and treated samples batch-wise in flasks only at laboratory scale [1,2,19,20]. However, the juice industry strongly prefers continuous systems for juice processing which even fewer studies investigated requiring a flow cell with an integrated US probe. These authors focused on the effect on anthocyanin content, color, and shelf life of several juices in the context of microbial counts reduction [21,22]. To the best of our knowledge, those studies lack a detailed characterization of the arising pool of polysaccharides that strongly interacts with other fruit compounds and determines juice quality.

The US principle of a facilitated extraction primarily by cell wall disruption caused by collapsing cavitation bubbles increases cell membrane permeability and leads to enhanced mass transfer. In comparison with high-intensity US (>100 kHz) treatment, which induces chemical reactions like the formation of hydroxyl radicals, low-intensity US (approx. 20 kHz) recommended for extraction generates mechanical, cavitational, and thermal forces that act on the cell wall [23]. The US-induced forces can also alter the molecular structure of enzymes and their corresponding substrates, affecting significantly their activity [7]. Few studies reported a synergistic effect applying of US and pectinases, in particular PG [24,25]. However, synergistic effect toward the other main pectinolytic enzyme activities PL and PME in fruit juice preparations has not yet been described so far.

All these effects and other general US-induced effects, like cavitation phenomena, pressure oscillation, local shear stress, and the formation of radicals have been demonstrated to be highly variable depending on type US setup, treatment duration, intensity, and food matrix [4,26]. Therefore, it is necessary to evaluate the distinct effects on matrix compounds individually to explain the underlying mechanisms.

The present study focuses on the characterization of pectin degradation by UAEM at a reduced maceration temperature of 30 °C in a continuous circulation system. The results were compared to a benchmark degradation of pectin by the same enzyme preparation at 50 °C for 60 min, employing parameters commonly used in fruit juice processing. To reduce interfering effects of other matrix components that would hamper the investigation in a berry mash, a sugar beet pectin model solution (pH 3.5) was used. Sugar beet pectin has a more complex structure with more similarities to berry pectin compared to industrially extracted apple or citrus pectin. [10,27]. It contains higher amounts of neutral sugars due to higher contents of the rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II) branched subunits, the higher degree of acetylation (DA), and a medium degree of methylation (DM), compared to citrus and apple pectin which mainly consist of linear galacturonic acid chains known as homogalacturonan (HG). To determine the distinct degradation effects of US and enzyme, as well as the combined effects, pectin polysaccharides and oligosaccharides were characterized by the viscosity they induce, high-performance size exclusion chromatography (HPSEC), DM, DA, and the content of three specific monomers in pectin’s complex structure. Galacturonic acid (GalAc), rhamnose, and fucose were quantified because of their location in distinct subunits of pectin. Besides the determination of the specific enzyme activities of the three main pectinolytic enzymes PG, PL, and PME under optimal conditions, the effective enzyme activities were examined regarding the UAEM process conditions to reveal synergistic effects. In this study, synergistic effects for PL and PME of a commercial enzyme preparation by the low-intensity US were evaluated for the first time. The final marker concentration of each enzyme assay was measured and used to assess the total process output of UAEM.

2. Materials and methods

2.1. Materials

Ultrapure water was obtained from a PURELAB flex 2 water purification system (ELGA LabWater, Paris, France). Ethanol (99.7%) and acetic acid were purchased from VWR (Mannheim, Germany). Ethanol (HPLC grade) and citric acid monohydrate (>95.5%) were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Methanol (HPLC grade) and sulphuric acid (95%) were from Th. Geyer (Renningen, Germany). Formic acid (99.9%), malondialdehyde tetraethylammonium salt (≥97%), potassium sodium tartrate tetrahydrate (≥98%), and 3,5-dinitrosalicylic acid (≥98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). n-Propanol, propionic acid, 2-thiobarbituric acid and sodium azide were purchased from Merk (Darmstadt, Germany); sodium hydroxide was from Honeywell (Morris Plains, NJ, USA). D(+)-GalAc monohydrate (99%) was obtained from Fluka (Munich, Germany); sodium nitrate (99%) was from Acros Organics (Geel, Belgium), and trisodium citrate dihydrate (≥98%) was from Alpha Aesar (Ward Hill, MA, USA). Sugar beet pectin Betapac RU 301, apple pectin Classic AU-L 036/18 (low methylated, DM 38%) and apple pectin Classic AU-L 022/17 (high methylated, DM 71%) were kindly provided by Herbstreith & Fox GmbH & Co. KG (Neuenbürg, Germany).

2.2. Ultrasound-assisted enzymatic maceration (UAEM) treatment of pectin model solution in a continuous circulation system (30 °C)

A continuous circulation system (Fig. 1), comparable to that described by Wong et al. [22], was used to sonicate a pectin model solution with added enzyme preparation (Fig. 2). The experimental setup consisted of a thermostatic water bath (1) to maintain the temperature at 30 ± 2 °C during the treatment. The model solution (600 ml) was placed in a beaker (2), from which the solution was continuously circulated at a constant flow rate of 7.3 ml sec⁻¹ (0.44 L min⁻¹) by a peristaltic pump (3); Immatec ecoline from Cole-Parameter GmbH, Wertheim, Germany) through a cylindrical flow cell (4; FCI01LLK-15S from Hirschel Ultrasonics GmbH, Teltow, Germany) equipped with a US probe of 9 cm² (7). The flow cell underneath the probe had a volume of 9 ml. US treatments were performed by a US processor (5; UIP 1000hDT, 1000 W, 20 kHz, Hielscher, Teltow, Germany) fitted with a booster horn (6; 100% amplitude: 53 μm). The amplitude of the US generator (9) was set at 60% (UAEM 60%) or 90% (UAEM 90%), resulting in an energy
input of 190 ± 3 W and 300 ± 6 W, respectively. Specific power density $\delta$ and specific intensity $I$ were calculated by the following Eqs. (1)–(3):

$$\delta = \frac{P}{V_1}$$

$$I = \frac{P}{A}$$

where $\delta$… specific density ($W\cdot ml^{-1}$), $I$… specific intensity ($W\cdot cm^{-2}$), $P$… ultrasonic power (quantified by probe processor), $V_1$… volume of flow cell (9 ml), $V_2$… sample volume in system (600 ml), $A$… surface area of probe ($cm^{-2}$).

The duration of the treatments varied between 60 and 90 min. The sample temperature was measured directly after sonication (8) and was controlled by a cooling unit (10; ProfiCool Novus, PCNO 30.03-NED from NationalLab GmbH, Moelln, Germany).

The applied pectin model solution was prepared by dissolving sugar beet pectin in a 0.05 M sodium citrate buffer (pH 3.5) at a concentration of 1% (w/v) by stirring the suspension overnight. The enzyme preparation (Rohapect Classic, AB Enzymes GmbH, Darmstadt, Germany) is typically applied for fruit juice production during the mashing process. The model solution was tempered to 30 °C ± 2 °C before 100 ppm of the enzyme was added. Effects of US application alone were examined in the same system at highest amplitude (90%) without enzyme addition. Influences of the enzyme preparation alone were determined by running the continuous circulation system without US treatment (0% amplitude). All experiments were carried out in duplicate. Aliquots of samples were heated (100 °C, 3 min) to inactivate the enzymes and subsequently stored at −80 °C until further analyses.

2.3. Batch maceration treatment of pectin at 50 °C

A pectin model solution, prepared as described for UAEM treatment, was incubated with 100 ppm of the enzyme preparation at 50 °C for one hour (Fig. 2) according to the supplier’s recommendation regarding application for fruit juice production, in the following referred to as batch maceration (benchmark). An aliquot of the solution without enzyme preparation was treated in the same way and was used as the control. Inactivation of the enzyme was carried out as described above. The experiments were repeated three times.

2.4. Characterization of the pectin model solution

2.4.1. Determination of the viscosity

The viscosity of pectin model solutions was determined with a rotational viscometer (V-Pad, Fungilab, New York City, NY) equipped with an LCP spindle. The analyses were run at 200 rpm for 1 min at 23 °C.

2.4.2. Determination of the molecular weight (MW) distribution by high-performance size exclusion chromatography (HPSEC)

The distribution of MW was analyzed by HPSEC with refractive index (RI) detection as described previously [15]. Pullulan standards (6.1–708 kDa, ReadyCal-Kit Pullulan, PSS-Polymer Standards, Mainz, Germany) were used to calculate MWs. The chromatograms were divided into three representative fractions and their relative peak areas were calculated: Fraction 1 (117–708 kDa), fraction 2 (30–117 kDa) and fraction 3 (6–30 kDa).

2.4.3. Determination of the degree of methylation (DM) and the degree of acetylation (DA)

DM and DA were determined by quantification of released methanol and acetic acid, respectively, by headspace solid-phase dynamic extraction gas chromatography (HS-SPDE-GC) with flame ionization detection (FID) after saponification as described previously [15]. DM and DA were calculated as mol methyl/acetyl groups per 100 mol GalAc [28].

2.4.4. Quantification of galacturonic acid (GalAc), L-rhamnose and L-fucose in the low molecular weight fraction (<30 kDa)

Three specific monomers, GalAc, L-rhamnose, and L-fucose, were quantified after acid hydrolysis using the respective enzyme-kit from Megazyme (Wicklow, Ireland) [15]. Prior to acid treatment, samples were separated at room temperature by their MW using Vivaspin 15 R (MWCO 30 kDa, Sartorius, Goettingen, Germany) with a Heraeus Megafuge 40R Centrifuge (Thermo Fisher Scientific, Braunschweig, Germany) at 6000g. Monomers were analyzed in the low MW fraction and were compared to the respective amount in an untreated sample representing the total amount of the monomers in the native pectin.

2.5. Determination of enzyme activities

The assays for the determination of the specific enzyme activities (nkat*ml⁻¹) were adjusted to optimal conditions described below based on Li et al. [29]. Incubation time, substrate concentration, enzyme
dilution, and the substrate were evaluated to obtain consistent results. Additionally, these assays were used to examine the effective enzyme activities during batch maceration and treatments in the continuous circulation system under the respective conditions (Fig. 2). Incubation time (60 and 90 min), substrate concentration (1% w/v), and enzyme dilution (100 ppm) were based on the experimental approach and were considered in the corresponding calculations. The final marker concentration, determined in each assay, was analyzed after the whole process of 60 or 90 min to compare the total output of each process. All experiments including the controls without enzyme addition were carried out in triplicate.

2.5.1. Characterization of polygalacturonase (PG) activity

As the marker for PG activity, the amount of reducing sugar released from pectin was determined. Reducing sugars were quantified by the 3,5-dinitrosalicylic acid method [30] with slight modifications. For the determination of the specific enzyme activity, apple pectin (low methylated) was used. The pectin suspension (300 µl; 0.5%, w/v) dissolved in sodium citrate buffer (0.1 M, pH 3.5) and 30 µl of aqueous enzyme solution (30%, v/v) were incubated for 30 min at 45 °C in a shaking water bath. The reaction was terminated by the addition of 1000 µl 3,5-dinitrosalicylic acid reagent. After boiling for 5 min, cooling on ice and centrifugation for 5 min at 9600 g (Heraeus Pico 17, Thermo Fisher Scientific, Braunschweig, Germany), the absorption was measured at 540 nm. PG activity was calculated as GalAc equivalents.

2.5.2. Characterization of pectin lyase (PL) activity

The marker of PL activity was the amount of formylpyruvate formed in alkaline media after enzymatic β-elimination from pectin. The aldehyde was quantified by the thiobarbituric acid method [31] with slight modifications. For the determination of the specific pectin lyase activity, sugar beet pectin was used. The pectin suspension (250 µl; 0.05%, w/v) dissolved in sodium citrate buffer (0.1 M, pH 3.5) and 30 µl of aqueous enzyme solution (30%, v/v) were incubated for 30 min at 45 °C in a shaking water bath. For the formation of formylpyruvate, 300 µl of sodium hydroxide solution (1 M) was added and incubated for 5 min at 80 °C and cooled on ice (1 min). Subsequently, 360 µl of hydrochloric acid (1 M) and 300 µl of thiobarbituric acid (0.04 M) were added and the solution was incubated 30 min at 80 °C to obtain a pink fluorescent dye. After cooling on ice and centrifugation for 5 min at 9600g (Heraeus Pico 17, Thermo Fisher Scientific, Braunschweig, Germany), the absorption was measured at 550 nm. Different concentrations of malondialdehyde, which is of a comparable structure and shows similar absorption characteristics to formylpyruvate, were used for external calibration [32,33].

2.5.3. Characterization of pectin methylesterase (PME) activity

The released methanol was the analyzed marker for PME activity. The concentration of methanol was quantified by headspace solid-phase dynamic extraction gas chromatography (HS SPDE GC) with flame ionization detection (FID) as described previously [15]. For the enzymatic saponification, 1600 µl apple pectin suspension (0.5%, w/v, high methylated) and 30 µl aqueous enzyme solution (30%, v/v) were incubated for 30 min at 45 °C. Enzymatic reactions were stopped by heat treatment (100 °C, 3 min) and samples were cooled on ice. An aliquot of 1300 µl was transferred to a 10 ml GC vial and mixed with 100 µl n-propanol (0.1% w/v) used as an internal standard. External calibration was based on methanol standard solutions (0.125–5% w/v) and was corrected by n-propanol as the internal standard (0.1% w/v).

2.6. Statistical analysis

To determine significant differences, an ANOVA using Bonferroni
post-hoc test was performed by XLSTAT software version 2014.4.06 (Addinsoft, Paris, France). The level of significance was defined as $p \leq 0.05$.

### 3. Results and discussion

#### 3.1. Specific enzyme activities of the preparation

The three specific activities of the commercial enzyme preparation that are PG, PL, and PME were determined (Table 1) under adjusted conditions based on Li et al. [29], who proposed a reproducible assay independent of dilution factors. Based on preliminary tests, the optimal incubation time and the optimal temperature was 30 min at 45 °C for all assays using a specific substrate concentration of 5 g L$^{-1}$ and enzyme preparation amounts of 3% (v/v) for PG assay, 3.6% (v/v) for PL, and 0.56% (v/v) for PME, respectively. The results were in range with other commercial pectinolytic enzyme preparations for fruit juice maceration, where PG and PME activities are usually the most dominant [9]. Pectinases are classified according to the catalyzed reaction during pectin breakdown and grouped into three main categories: lyases, hydrolases, and esterases. PL (E.C. 4.2.2.10) catalyzes the cleavage of the 1,4-glycosidic bond of esterified GaLA by trans elimination leaving unsaturated residues. PG (E.C. 3.2.1.15) catalyzes the hydrolytic cleavage of 1,4-glycosidic bonds between non-esterified GalAc. PME (E.C. 3.1.1.11) catalyzes the de-esterification of the methoxy groups of pectin. [34] All three pectinolytic activities mainly act on the HG subunit consisting of galacturonic acid and are important for cell wall degradation in juice maceration due to the high pectin content of fruits and berries.

#### 3.2. Pectin degradation during batch maceration (50 °C)

A batch maceration using the commercial pectinolytic enzyme preparation was conducted under maceration conditions commonly used in red berry juice production (1 h, 50 °C, pH 3.5). Since most of the above-mentioned positive effects of polysaccharide degradation (viscosity reduction, interactions with anthocyanins) have been shown for such conditions, the characterization of the arising polysaccharides and oligosaccharides of this batch maceration provided the benchmark for the subsequent application of UAEM. Comparing the UAEM-induced effects with the benchmark, significant changes compared to the standard conditions can be revealed. The used pectin contained a medium DM of approx. 50% (Table 2) providing esterified and non-esterified GalAc binding sides for the different specific pectinolytic activities.

The applied enzyme preparation degraded pectin extensively by specific enzymatic cleavage clearly demonstrated by the considerable decrease in viscosity of 70%, indicating a substantial reduction of the average MW of pectin (Table 2). A detailed profile of the resulting MW distribution after batch maceration of the arising oligosaccharides and polysaccharides was determined by HPSEC (Fig. 3a), illustrating a high polydispersity that implies a high diversity of pectin residues. Separated into three MW fractions, the profile predominantly comprises low MW (6–30 kDa) and medium MW (30–117 kDa), while the high MW (117–708 kDa) fraction probably consists of remaining non-degraded pectin (Fig. 4). The medium MW fraction includes resistant fragments of pectin’s hairy region, forming highly branched oligomers of RG I which have previously been structurally characterized by anion-exchange chromatography and SEC analysis [35]. Results are generally consistent compared to these authors, who degraded sugar beet pectin by PG and PME for 24 h. According to Rale et al. [35], it can be suggested that a prolonged maceration time would probably reveal a greater decrease in high MW residues and a greater increase in low MW oligomers due to ongoing degradation of HG by pectinolytic enzymes. However, a certain amount of resistant fragments of medium MW remains after batch maceration since activities of common enzyme preparations cannot degrade the complex subunit of pectin’s hairy region [35].

Based on our previously published findings, oligomers of low MW form soluble complexes with polyphenols like anthocyanins leading to a stabilization of these value adding compounds. It was assumed that HG-derived residues stabilize anthocyanins rich in hydroxy groups, while RG-derived residues probably play a role in the complex formation of anthocyanins rich in methoxy groups [15]. Therefore, pectin model solutions and especially small oligomers in the low MW fraction were further characterized to examine degradation of pectin subunits into a set of corresponding oligomers by the quantification of GalAc, rhamnose, and fucose after MW separation by ultra-centrifugation (MWCO 30 kDa) in case of low MW fraction and acid hydrolysis (Table 2). GalAc is the predominant monosaccharide moiety in pectin and its content can be correlated with HG-derived residues. Rhamnose and fucose, due to their distinct occurrence in pectin substructures, can be related to the amount of RG I-derived and RG II-derived residues, respectively [36].

The absolute amount of these monomers in samples regarding all MW fraction was quantified without MW separation; the native samples contained 306.5 ± 18.1 mg·g$^{-1}$ pectin of GalAc, 3.9 ± 1.3 mg·g$^{-1}$ pectin of rhamnose and 2.3 ± 1.1 mg·g$^{-1}$ pectin of fucose. Since the medium MW polymers did not form soluble complexes with anthocyanins in our previous study [15], they were not further characterized in the present study. Other sugar beet pectin components such as monosaccharic sugars (e.g., arabinose, galactose, glucose, and xylose), proteinaceous matter, ferulic acid, or metal ions were not further examined [37-39].

The low MW fraction composition (<30 kDa, Table 2) show that enzyme activities mainly released GalAc containing residues, indicating a substantial degradation of HG chains to small oligomers. More than half (60.0%) of the absolute amount of GalAc was quantified in the low MW fraction (<30 kDa) after batch maceration, whereas an initial amount of 3.7% was found in the low MW fraction in the native pectin solution. These findings confirm the fact that PG and PL act as main activities by a high extent of a HG chain degradation into large amounts of polygalacturonic acid-residues [25,40]. The amounts of the minor monosaccharides rhamnose and fucose significantly increased in the low MW fraction, indicating a considerable release of RG I- and RG II-derived small oligomers by enzymatic side-activities like rhamnogalacturonase and endo-polymalacturonase (Table 2) [12]. Referred to the absolute amount in native pectin, 23.8% of rhamnose and 34.1% of fucose were detected in the low MW fraction (>30 kDa) after batch maceration, taking into account that the low MW fraction of native pectin solution already contained 15.0% of rhamnose and 11.7% of fucose. The findings demonstrate that pectin’s subunits of RG I and RG II are difficult to degrade or resistant by enzymatic cleavage of the applied preparation. Thus, lower residue amounts of these subunits are degraded to low MW oligomers compared to HG-derived residues. The characterization of the arising oligomers under common batch maceration conditions is in agreement with previously reported findings. The enzymatic degradation has been shown to release polymers and oligomers including polyGalAc residues, alternating rhamnose-GalAc residues from RG I, RG II dimers, whereas complex RG I-derived residues that are resistant to the pectinase activities of common enzyme preparations remain as medium and high MW polymers [10,15,41].

The enzyme preparation contained a comparably high PME activity (Table 1) that reduced the DM of pectin by 23% during batch maceration. In our previous study, DM could be reduced enzymatically by...
Table 2
Characterization of pectin model solution after individual treatments; samples treated in continuous circulation system at 30 °C were compared to batch maceration at 50 °C and untreated native pectin. Different letters indicate significant differences in each column due to treatment.

| Sample         | Time (min) | Power density $\delta_1$ (W*ml$^{-1}$) | Power density $\delta_2$ (W*ml$^{-1}$) | Viscosity (mPa*s$^{-1}$) | DM (%) | DA (%) | Low MW fraction (<30 kDa) |
|----------------|------------|----------------------------------------|----------------------------------------|--------------------------|--------|--------|---------------------------|
| native         | –          | –                                      | –                                      | 7.42 ± 0.27$^a$          | 47.92 ± 17.12$^c$        | 2.92$^c$| 2.3$^c$| 11.25 ± 3.42$^d$          |
| batch maceration| 60         | –                                      | –                                      | 2.26 ± 0.01$^d$          | 36.88 ± 13.88$^c$        | 3.77$^c$| 1.14$^c$| 183.97 ± 0.93$^b$         |
| continuous circulation system | 60 | –                                      | –                                      | 2.38 ± 0.05$^d$          | 39.88 ± 16.39$^c$        | 3.71$^b$| 1.14$^c$| 182.14 ± 0.90$^b$         |
| enzyme only    | 90         | –                                      | –                                      | 2.34 ± 0.03$^d$          | 38.04 ± 17.06$^a$        | 2.17$^b$| 0.94$^c$| 191.18 ± 0.92$^a$         |
| UAEM 60%       | 60         | 21.5                                   | 26.5                                   | 2.37 ± 0.03$^d$          | 40.11 ± 17.72$^a$        | 2.05$^a$| 1.22$^a$| 175.80 ± 1.23$^a$         |
|                 | 90         | 21.2                                   | 26.1                                   | 2.31 ± 0.05$^d$          | 39.95 ± 17.96$^a$        | 2.98$^b$| 1.36$^c$| 205.15 ± 1.37$^a$         |
| UAEM 90%       | 60         | 32.8                                   | 40.4                                   | 2.35 ± 0.02$^d$          | 38.10 ± 17.75$^b$        | 4.9$^b$ | 0.92$^c$| 163.77 ± 1.01$^a$         |
|                 | 90         | 33.0                                   | 40.6                                   | 2.29 ± 0.02$^d$          | 34.46 ± 17.03$^a$        | 2.46$^c$| 1.37$^b$| 24.70 ± 1.46$^a$          |
| US only        | 60         | 33.9                                   | 41.7                                   | 3.28 ± 0.10$^b$          | 47.98 ± 15.39$^b$        | 2.97$^b$| 1.76$^b$| 18.27 ± 18.27$^b$         |
|                 | 90         | 34.0                                   | 41.9                                   | 2.76 ± 0.06$^b$          | 47.03 ± 17.36$^a$        | 1.44$^a$| 0.96$^c$| 22.73 ± 2.38$^a$          |

* monomer sugar content is calculated as mg*g$^{-1}$ pectin, power density $\delta_1$ is calculated with flow cell volume (9 ml), power density $\delta_2$ is calculated with sample volume in continuous circulation system (600 ml).

Fig. 3. HPSEC elution pattern of (a) native pectin, continuously US treated (7.3 mls*c$^{-1}$, 30 °C, A 90%, 300 W, 33 W*cm$^{-2}$, for 60 and 90 min) pectin, and pectin after batch maceration (BM, 50 °C, 100 ppm, 60 min) and (b) pectin after treatment in continuous circulation system (7.3 mls*c$^{-1}$, 30 °C) by enzyme alone (100 ppm for 60 and 90 min), after UAEM 60% (100 ppm, A 60%, 190 W, 21 W*cm$^{-2}$, for 60 and 90 min), and after UAEM 90% (100 ppm, A 90%, 300 W, 33 W*cm$^{-2}$, for 60 and 90 min). Column was calibrated with pullulan standards to estimate the peak MW distribution. A: Amplitude.
This difference can be explained by the different enzyme preparations applied varying in PME activity. Also, PME activity might be limited by substrate concentration due to the lower DM of the pectin used in this study. A high DM of pectin is known to increase gelling properties of pectin in an acidic environment by intermolecular hydrogen bridges between the partly undisassociated carboxy groups and hydrophobic interactions of methylated carboxy groups of the galacturonic acids [42]. High viscosities are impeding juice production and need to be decreased. PME reduces pectin esterification, preparing binding sites for PG, which needs non-esterified galacturonic acid chains as substrate. The cleavage by PG consequently reduces viscosity by reducing the MW of polygalacturonic acid. Thus, a sufficient PME activity is necessary for an efficient pectin degradation by PG activity, especially of high DM pectin.

The DA was reduced by 18%, probably by side activity of an acetyl esterase (Table 2) that has been shown in other enzyme preparations for juice production before [15]. A reduction of DM and DA in pectin structure has been demonstrated to beneficially affect anthocyanin complexation. The reduction provides more binding sites (free galacturonic acid residues) and by lower steric hindrances to form hydrogen bonds or ionic interaction in acidic environments, due to dissociation of carboxylic acid groups (pKₐ 3.5 [43]). Therefore, the characterization of DM and DA of pectin and pectin-derived residues provides important information on their ability to complex anthocyanins and other matrix compounds [15].

3.3. Pectin degradation in a continuous circulation system (30 °C)

To obtain an enhanced pectin degradation into the described set of small oligomers by milder maceration conditions, a continuous circulation flow system with an implemented US probe in a flow cell was applied. Continuous systems have been demonstrated to be most convenient for juice production at an industrial scale. Applying US concurrently increases the temperature of the treated media mainly caused by the temperature of local hot-spots (5000 K) of collapsing cavitation bubbles [44]. By the use of a continuous circulation flow system with an implemented US probe in a cooled flow cell the temperature in the whole system could be controlled accurately at 30 °C ± 2 °C.

The effectiveness of combined enzymatic and US-induced pectin degradation as a milder maceration treatment was compared to the benchmark results of the batch maceration at 50 °C. The application of enzymes and US alone was investigated individually to differentiate between distinct and synergistic effects.

3.3.1. Exclusive enzyme treatment

Pectin degradation by the enzyme preparation in the continuous circulation system at reduced temperature (30 °C) was almost identical to the benchmark degradation during batch maceration at 50 °C for 60 min (Fig. 3). All parameters but DA (Table 2) were equal to those of pectin degraded in batch maceration. The reduction of DA after treatment at 50 °C was more pronounced which can be attributed to a higher temperature optimum of the responsible acetyl esterase [45]. The observed results could be attributed to the fact that the main pectinolytic enzyme activities of the commercial preparation are very effective in pectin degradation even at the lower temperature of 30 °C.

3.3.2. Exclusive ultrasound treatment

In general, low-intensity US-induced effects non-specifically degrade polysaccharides by mechanical effects, in contrast to the enzymatic cleavage occurring specifically at distinct points of action in the substrate. This leads to a fundamentally different MW distribution (Fig. 4) of degraded pectin oligosaccharides and polysaccharides after the exclusive US treatment compared to the enzymatic benchmark.
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The narrow MW distribution of US modified pectin indicates a low polydispersity index, meaning that the homogeneity of MW distribution increased \[46\]. One attribute of US-induced polysaccharide degradation is the high fragmentation rate of large molecules to a narrow and more uniform MW distribution. This phenomenon is explained by a non-random breakage occurring approximately in the midpoint of the oscillating polymers induced by US pressure waves. The final length of the polymers depends on the intensity of the mechanical torque that is necessary to rupture the linkages. The degradation of high MW polymers occurs faster due to the less energy required for their breakage and, thus, the resulted pool of polymers presents a narrow MW distribution \[47\]. This phenomenon is also consistent with the following observations. The MW profile of degradatian products mainly encompasses medium MW polymers (30-117 kDa; Fig. 4), within a prolonged treatment time (90 min) this fraction only slightly increased further (4.3%). The amount of low MW fraction of small oligomers (<30 kDa) increased by only 1% over treatment time of 60 and 90 min. The results indicate that the applied energy in the continuous system was only sufficient for the production of medium MW polymers. The findings confirm the fact that the lower the MW of the polymers, the higher energy is necessary to require further breakage \[48\]. However, it has been claimed that an increase in US intensity only results in higher degradation effects up to a certain level. This is explained by hindrance of the energy transmission in the system as a consequence of an increased number of cavitation bubbles. A limit of up to 50 kDa for dextran and acidic polysaccharides \[47\], and 100 kDa for chitosan \[49,50\] after prolonged treatment times was proposed.

The monosaccharide composition of small oligomers in the low MW fraction (<30 kDa, Table 2) revealed that only the amount of HG-derived and RG I-derived residues increased significantly, resulting in an increase of 5.9% GalAc and 6.5% rhamnose in the low MW fraction, referred to the initial content of native pectin solution. The amount of RG II-derived residues in the low MW fraction was not significantly changed compared to native pectin. These findings indicate that the applied low-intensity US broke the HG chain into only few small polygalacturonic acid residues and released RG I residues from the main chain. This result agrees with previously reported findings, where the authors explained the US-induced reduction MW of pectin was mainly due to the release of RG-I domains \[36,51\]. In accordance with literature, monosaccharides did not emerge during exclusive US treatments \[48,51\]. These findings confirm the fact that US-induced effects only reduce MW of polymers into smaller ones without the liberation of monomers and, consequently, without altering monosaccharide compositions of the produced juice.

The exclusive US treatment did not change DM and DA significantly (Table 2), probably because of the relatively low US intensity applied (max. 34 W/cm^2) and the citric acid medium, which has been shown to inhibit the reduction of DM by US treatments \[51\]. In contrast, higher ultrasonic intensity caused a significant reduction of DM and DA in previous studies \[15,48\]. High US intensities are apparently needed, if a reduction of DM or DA is necessary. In this study, higher US intensities were included in treatment conditions at 30 °C.

Although US treated samples contained polymers of higher MW than that after those after enzymatic treatment, the decrease in viscosity was similar to enzymatic treatment by 62.8% (Table 2). Since viscosity is associated with high MW polymers, the considerable reduction of viscosity by US treatment has to be explained by the reduction of agglomerates and structural changes that lead to more linear polymers \[15,48\]. However, the exclusive US treatment did not produce a similar set of oligosaccharides and polysaccharides like the benchmark enzymatic degradation, which are necessary to enrich juice with soluble fiber and evoke anthocyanin stabilizing properties. Despite the comparable reduction of viscosity, an exclusive US treatment cannot replace the common enzymatic maceration. Additionally, polymers with medium MW created by US-treatment were shown to form insoluble anthocyanin-complexes in our former study, that would deprive valuable anthocyanin compounds in final juice \[15\].

3.3.3. Ultrasound-assisted enzymatic maceration (UAEM)

As indicated above, a combined application of US and enzymes has been demonstrated to enhance plant material extraction and facilitate polymer degradation in certain experimental conditions \[1,6,19\]. Accordingly, the present study was focused on the characterization of the arising polysaccharides and oligosaccharides during UAEM using a continuous circulation system and applying a common enzyme preparation, because certain small HG-derived and RG-derived oligomers have been shown to determine juice quality.

The UAEM application resulted in a quite similar MW distribution pattern (Fig. 3b) compared to that observed after enzymatic degradation alone indicating also a diverse pool of oligosaccharides and polysaccharides. However, the MW distribution obtained after UAEM treatment was shifted to the lower MW fractions (Fig. 3b), meaning that higher amounts of smaller and simpler molecules are formed compared to the benchmark trial (Fig. 3a, Table 2). Compared to enzymatic degradation alone in the continuous system, high MW fraction was decreased significantly for UAEM 60%: −13% and −20% after 60 and 90 min, respectively, and for UAEM 90%: −18% and −31% after 60 and 90 min, respectively. The medium MW fraction was increased for UAEM 60%: +9% and +1% after 60 and 90 min, respectively, and for UAEM 90%: +14% and +10% after 60 min and 90 min, respectively, (Fig. 4). The medium MW fraction increased significantly more after shorter treatment of 60 min compared to the longer treatment of 90 min, due to the ongoing degradation of medium MW oligomers to smaller fragments. Although the MW distribution patterns after UAEM treatment qualitatively correlated with the enzymatic degradation patterns, degradation to polymers of medium MW and oligomers of smaller MW was enhanced with an increase in the applied US intensity. Thus, the higher US intensity (90%) revealed greater degradation effects than the lower (60%) in the UAEM system. It can be concluded that US affects enzymatic degradation, and that higher US intensities provoked more pronounced effects. The latter is explained by higher energy delivered into the system with increasing US intensity, thus increasing US-induced effects like cavitation and micro streaming \[46,48\].

Since the MW distribution of the formed oligosaccharides and polysaccharides is of great importance for stabilizing effects, it is noteworthy that US not only enhanced enzymatic degradation of pectin but also altered the MW distribution pattern, resulting in a profile of degradation products with structures different from those arising from enzymatic treatment alone. This effect can be explained by several mechanisms, like the US-induced alteration of the substrate, the US-induced alteration of the enzyme conformation and action, and the US-induced effects on the surrounding media. [24,36,52] This last effect is a more general effect on the overall reaction speed as it increases turbulence caused by pressure fluctuations that lead to an improved mass transfer accelerating substrate-enzyme exchange. Since the other two mechanisms are more likely to be specific for UAEM, they are discussed in the following.

3.3.3.1. Ultrasound-induced alteration of the substrate pectin.

The findings of the exclusive US treatment demonstrated, that US degraded high MW polymers mainly into high and medium MW polymers. UAEM accordingly produced a significantly larger fraction of medium MW than enzymatic treatment alone. The medium MW fraction after UAEM 90% increased more than after UAEM 60% like in the exclusive US treatment (3.3.2) indicating a prevalent US-induced production of medium MW
polymers. However, it was reported that high cavitation phenomena deteriorate the efficiency of energy transmission into the solvent, reducing US-induced effects when the intensity exceeds a certain level. Zhang et al. [46] reported reduced US degradation effects on apple pectin at intensities >302 W cm$^{-2}$. In the present experiments, the intensity was maintained sufficiently low (max. 34 W cm$^{-2}$) to avoid such effects. As explained in Section 3.3.2, the shear forces of cavitation and micro-jets created by US change the structure of polymers and, thus, alters the substrates; it forms linear oligomers and breaks the complex, voluminous structure of pectin by concomitantly exposing more accessible domains that can be reached more easily by the enzymes. Consequently, enzymes degraded the US-modified polymers further into smaller molecules. [7,36] Thus, samples after UAEM treatment showed a reduced medium MW fraction compared to the medium MW fraction after US treatment alone, due to the additional enzymatic degradation, as can be seen from Fig. 4. Concurrently, all UAEM treated samples presented highest amounts of small oligomers in the low MW fraction that consists of the highest amounts of GalA, rhamnose and fucose monosaccharides (Table 2). After UAEM 90% (90 min) treatment, the content of the small oligomers (>30 kDa) were further elevated. The low MW fraction contained 66.3% of the absolute GalA content, 37.5% of rhamnose and 37.0% of fucose, respectively. This distribution indicates that UAEM released more HG-derived, RG I-derived, and RG II-derived residues into the low MW fraction with increasing US intensity than the benchmark enzyme degradation at 50 °C. These findings are in agreement with Ma et al. [36], who pointed out that sonochemical-enzymatic effects degrade citrus pectin into smaller and simpler oligomers (batch trail, 4.5 W ml$^{-1}$) with strongest synergistic effects at temperatures below 40 °C. The authors reported a higher decomposition of the HG subunits and an increase in contents of RG-I residues of degraded products by sonoenzymolysis reactions compared to enzymatic degradation of pectin. The pectin residues were shown to be less branched proven by atomic force microscopy [36]. In contrast, Muñoz-Almagro et al. [51] reported a decreased degradation of apple and citrus pectin by an US-assisted pectinase treatment compared to simple enzymatic degradation suggesting an inactivation of enzyme activities by ultrasound forces. Different experimental conditions like higher temperature (50 °C), higher US intensities (81.7 W cm$^{-2}$), and different enzyme activities could explain these contrasting results in comparison with the present study.

The above reported mechanisms might suggest that US-induced effects simply prepare pectin for a subsequent enzymatic degradation. Our own preliminary tests and further studies on US pre-treatment before enzyme degradation led to inconsistent results. Lieu et al. [1] noticed enhanced cell wall degradation and juice yield by US-pretreatment of grape mash treatment, whereas Dalagno et al. [24] and our preliminary results did not show any enhanced pectinase activity by US pre-treatment of apple and sugar beet pectin, respectively. Feng et al. [53] described an enhanced papain activity after the US pretreatment of sodium alginate due to favorable substrate-enzyme conformations. This discrepancy in the observed effects corroborates the conclusion that more than one mechanism is responsible, i.e. another mechanism apparently also plays a role for the observed synergistic effects, that is, the US-induced enzyme modification.

### 3.3.3.2. Ultrasound-induced effects on the enzyme.

Mechanisms comparable to US-induced effects cleaving glycosidic bonds of the pectin chains also alter the conformation of enzymes during US treatment. The alterations of amino acid residues, the breakage of hydrogen bonding, and van der Waals interactions in polypeptide chains result in the modification of protein conformation and change the enzymes’ catalytic activity. The tertiary and secondary structure of PG was studied in detail after US treatment (9.9 – 9 W ml$^{-1}$, 30 °C, 40 min) by intrinsic fluorescence and circular dichroism spectra [7]. The authors reported an irreversible structural unfolding and a slight increase in β-sheet conformation that resulted in the formation of more active binding sites. The consequent changes of enzyme conformation thereby increased the activity. In contrast to these positive effects, negative effects of the alteration of proteins in terms of denaturation and inactivation have also been reported [51]. Muñoz-Almagro et al. [51] explained this by molecular unfolding, which was evaluated by intrinsic fluorescence due to tryptophan residues in pectinase. This contrasting results might be explained by harsher experimental conditions (81.7 W cm$^{-2}$, 60 min, 50 °C) leading to enzyme denaturation.

Another theory attributes the improved enzyme activity to US induced hot-spots causing locally increased temperature, and, thereby shortly reaching the enzymes’ temperature optimum [52]. This is substantiated by the observation that pectinase activity was enhanced by US mostly at temperatures below 40 °C which was indeed observed in preliminary experiments as well as in several studies [24,36]. At higher temperatures close to the optimum temperature of the enzymes (45–50 °C), US effects became negligible. A further increase in temperature (>60 °C) during US-treatment might even affect pectinases’ conformation adversely and lower cavitation effects. The latter is explained by an increase of vapor pressure in the solution due to the increasing temperature leading to a decrease of viscosity and surface tension reducing collapse temperature [19,36,51,54]. The temperature in our experiments was thoroughly controlled by a cooling system and kept at 30 °C ± 2 °C to avoid such negative effects.

DM was only slightly reduced by UAEM (Table 2), which might be related to an impaired PME activity as discussed in detail in 3.4. However, longer treatments (90 min) decrease DM values more (25% by UAEM 60% and 28% by UAEM 90%) than the enzymatic treatment of the benchmark trail (60 min, 23% by batch maceration). This means that PME is continuously active releasing more methoxy groups at prolonged reaction time. DA values of pectin were not affected by any UAEM treatment (Table 2), which demonstrates that US application has no synergistic effects on the acetyl esterase activity. The findings are in agreement with those previously reported, where the combined enzyme and US treatment of citrus pectin had no influence on the DA [36]. Regarding the exclusively enzymatic treatments in the benchmark trail, DA was only reduced by acetyl esterase at 50 °C. Therefore, it can be assumed that the hot-spot theory (momentarily reaching temperature optimum) might be less important in case of acetyl esterase than conducive conformational modifications of the enzymes. While DA was significantly decreased solely by acetyl esterase at 50 °C, local hot-spots did not stimulate enzyme activity at 30 °C. The exact mechanisms of US-induced effects on the conformation of acetyl esterase still needs to be investigated.

Regarding the quality of degradation, the present results demonstrate that UAEM in a continuous flow system can be an effective method to produce a set of smaller polymers or oligomers with a greater diversity, which could be beneficial for fruit juice processing. The applied US treatment alone did not reveal a comparable degradation, but US holds synergistic effects by modification of both, substrate and enzyme, leading to a pronounced pectin degradation. Smaller oligomers that are not removed during juice filtration increase fiber content in juices and are assumed to possess higher anti-oxidant activities compared to bigger molecules [48]. Furthermore, low MW oligomers stabilize anthocyanin contents in soluble complexes during storage. A set of HG-derived and RG-derived oligomers has been shown to beneficially complex most anthocyanins that differ in their number of hydroxy groups and methoxy groups. Additionally, a decrease in DM beneficially affects these interactions due to an increased number of free binding sites on the galacturonic acid residues and less steric hindrances [15].

### 3.4. Influence of ultrasound on effective enzyme activities during maceration

The observed results support the theory that low-intensity US treatment synergistically affects enzyme activities for enhanced pectin

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**Table 2**

| Treatment | MW Fraction (%) | HG | RG I | RG II |
|-----------|----------------|-----|------|-------|
| Batch     |                | 66.3| 37.5 | 37.0  |
| US 90%   |                | 66.3| 37.5 | 37.0  |
| DA        |                | 66.3| 37.5 | 37.0  |

**Figure 4**

Intrinsic fluorescence and circular dichroism spectra [7]. The authors reported an irreversible structural unfolding and a slight increase in β-sheet conformation that resulted in the formation of more active binding sites.
degradation, which has also been reported previously for pectinases in general, but only examined for PG activity in particular [2,24,25]. To evaluate these effects for each main pectinolytic activity, the effective enzyme activity was determined individually for PG, PL and PME in the continuous circulation system under the process conditions applied (Fig. 5).

The effective enzyme activities were generally higher during batch maceration at 50 °C (benchmark) compared to the continuous circulation system at 30 °C (Fig. 5). These results are in agreement with the optimum temperature of the specific enzyme activity given by the manufacturer and previous studies [24,25]. The results obtained by the specific assays are somehow contradicting the results discussed for the UAEM effects, which clearly demonstrated a sufficient pectin degradation. The applied assays are used to characterize individual pectinolytic enzyme activities, but the degradation of pectin is a combination of all reactions. The observed differences between the lower distinct enzyme activities and the comparable pectin degradation can be explained by the marker compounds that are used for the assays, which do not necessarily reflect the effective degradation. However, the individual activities are observed to reveal the different synergistic effects of US on the three enzymes PG, PL and PME. The common assays only examine individual pectinolytic enzyme activity.

Regarding the individual enzyme effective activities, UAEM treatment significantly enhanced the PG and PL activities at 30 °C compared to enzymatic treatment alone within comparable treatment time (Fig. 5, I-II). The results prove a synergistic effect of PL and US treatment that has not been published so far. Thus, the recently proposed synergistic effects of US on the “so-called” pectinase activity [2,24,25], that has misleadingly only been proved for PG activity, also applies to PL activities. For the enzyme preparation used here, synergistic effects for PL were even higher than for PG. The effective enzyme activity of PL during UAEM 60% treatment was almost equal to the results of the benchmark trial at 50 °C (Fig. 5, II). It has already been reported that similar US parameters have various effects on different enzymes. Yu et al. [55] reported the inactivation of α-amylase and papain under US irradiation, whereas the activity of pepsin was activated under the same experimental conditions. The authors attributed these deviant effects to the different secondary and tertiary structures of the enzymes. This could also apply to the individual conformations of PG and PL that are apparently influenced differently by US-induced effects. Both enzymes fold into a righthanded parallel β-helixes, but they differ in the number of β-sheets and the localization of disulfide bridges determining their conformation. In contrast to the three β-sheets in PL, the PG β-helix is comprised of four β-sheets [56-58]. Such β-sheets have been shown to be more prone to US irradiation, and might convert into α-helix, β-turn and random coil structures under higher US intensities (6 W·ml⁻¹) [53,55,59]. In contrast, at lower US intensities (2 W·ml⁻¹, 15–30 min) an increase in β-sheets and a decreases of α-helix and random coil in soy protein isolate was observed under US treatment. [59] Also, Ma et al. [7] reported an increase of β-sheet and α-helix conformation after US treatment (US probe, 900 W, 9 W·ml⁻¹, 40 min, 40 °C). The substrate binding site of PG is located on the exterior of the β-helix consisting of β-sheets indicating its essential conformation for catalytic cleavage [7,60]. The findings highlight the thin line between increasing and decreasing effects on enzyme activity by US treatment by either exposing or destruction of active sites, depending on the nature of enzymes conformation, US intensity, and other properties related with the ultrasound setup. Thus, within the US intensities applied in this study, PG and PL activity might be enhanced by conformational changes exposing of favorable β-sheet conformation. Although PG contains more β-sheets, PL activity was more enhanced suggesting other important conformational changes that have not been described so far. One reason might be that US-induced effects have been described to break disulfide bridges, decreasing intramolecular bindings of the enzymes [53,59]. Consequently, different amounts and localization of disulfide bridges, that are four in PG and two in PL, might affect conformation differently.

Fig. 5. Effective enzyme activity (nkat/ml) of PG (I), PL (II) and PME (III) of the commercial preparation Rohapect®Classic (100 ppm) in pectin model solution (1%, pH 3.5) referred to the actual process conditions. Batch maceration (BM, grey bar) was carried out at 50 °C. US only (waved bar), enzyme only (white bar) and UAEM (dotted bar) were carried out in a continuous circulation system at 30 °C. Different letters indicate significant differences due to treatment. US + : A 90%, 300 W, 33 W·cm⁻² US + : A 60%, 190 W, 21 W·cm⁻², enzyme + : 100 ppm, enzyme − : no enzyme.
and change the enzymes’ activity \[56,58\]. Subsequently, the more di-sulfide bridges are destroyed the more conformational changes might occur and thus adversely affect enzyme activity. A detailed investigation of conformational changes of the enzymes by different US intensity treatments is necessary to eventually prove this hypothesis.

The effective enzyme activities of PG and PL during the exclusive enzymatic treatment were lower after 90 min than after 60 min treatments (Fig. 5, I-II), suggesting a partial inactivation of the enzymes over time. This loss of activity was compensated by UAEM treatments, which retained the effective enzyme activity over the longer periods of treatment for PG and PL. Similar effects were described by Ma et al. [7], who observed a prolonged lifetime and thermostability of PG activity by US treatment. For effective PL activity, UAEM 60% revealed slightly higher results than UAEM 90%, suggesting a greater synergistic effect at the lower US intensity (Fig. 5, II). This fact could be attributed to a partial deactivation of PL by higher US intensities, since higher intensities may alter enzyme conformation detrimentally by more free radicals and stronger shear forces resulting in a denaturation and inactivation [36,61].

In contrast to PL and PG, the effective activity of PME was not enhanced by UAEM treatment. It was even slightly reduced by UAEM 90% application over 90 min (Fig. 5III). These findings suggest a possible specific sensitivity of PME activity toward US treatment, while other pectinase activities are improved. Tiwari et al. [62] reported the inactivation by 62% of PME even by low intensity-US treatments (1.05 W*ml\(^{-1}\)) in orange juice. Fungal-PME folds into distinctive parallel \(\beta\)-helix structures containing even more \(\beta\)-sheets than the other two pectinases (PG and PL) encompassing several active-site residues. Additionally, it contains a cysteine ladder facing internally into the core of the \(\beta\)-helix [63]. Besides the above discussed vulnerability of \(\beta\)-sheets toward US irradiation, which can be adversely altered, US-induced oxygen radicals are assumed to oxidize free sulphydryl groups forming sulfinic and sulfonic acids, destroying susceptible functional groups of the enzyme [59,64]. However, the applied US intensity in this study rather decelerated PME activity than inactivated it, since DM values of pectin was still significantly reduced after UAEM treatments compared to the native pectin (Table 2).

Enzymatic degradation of pectin is depended on the ensemble acting of all applied enzymatic activities and US. The observed results indicate a US-sensitivity of PME, consequently DM is only slightly reduced after incubation. However, PG activity requires de-esterified galacturonic acid residues as substrates and therefore depends on PME activity, if only methylated pectin is available. Consequently, PME inactivation entails a reduced PG activity. In contrast to PG, PL needs esterified galacturonic acid residues as substrates, which are more available, if PME activity is reduced by US application. This effect could also explain the higher PL activity rates during UAEM. To examine the US-induced effects on individual pectinolytic activities, specific tailor-made enzymes with only one activity should be examined. However, in juice production enzyme preparations mainly produced by Aspergillus Nigerial encompass several activities that has to be considered for the complex pectin degradation.

The observed results demonstrate that the different enzyme activities of PL, PG and PME are affected individually by similar US parameters and experimental conditions. This dependency might be explained by the differently susceptible enzyme conformation leading to activation, deceleration or inactivation. Positive and negatives effects are acting at the same time summing up to a net-effect that can be observed and quantified.

3.5. Determination of total process output

Assessed only by the effective enzyme activities in the continuous circulation system at 30 °C compared to the benchmark in the batch maceration at 50 °C, it might be assumed that pectin degradation was less effective by UAEM. However, by considering the total output of the whole process, characterized by the corresponding final marker concentration determined in each assay (reducing sugars for PG [30], methanol for PME [28], and formylpyruvate for PL [31]), it can be seen that the UAEM treatments at 30 °C lead to similar results as the benchmark at 50 °C (Fig. 6). UAEM 90% treatments resulted in final marker concentrations for PG and PL equal to the benchmark, while quantities after UAEM 60% showed only a slightly lower level (Fig. 6, I-II). Final marker concentration of PME was also higher after UAEM 90% treatments compared to UAEM 60%, but slightly lower compared to benchmark (Fig. 6, III). This is explained by considering the final marker concentration over the whole process revealing comparable outputs at the end of each process, while the effective enzyme activities are expressed by rate of conversion per sec.

The results demonstrate that US affects enzyme activities at reduced temperature, rendering them at least as effective by revealing similar outputs in the applied process as at the given temperature optimum of the specific enzyme activity (50 °C, benchmark). Therefore, UAEM shows potential as an alternative processing technique for milder maceration conditions for juice production. A temperature reduction provides not only favorable conditions for heat-sensitive compounds, but also an energy-saving potential. Besides the similar total process output of enzymatic marker compounds compared to the benchmark, UAEM degraded pectin into a set of smaller oligomers derived from HG and RG subunits with reduced DM. The increased degradation of pectin may be promising for an increased cell wall degradation and juice yield in juice production. This primarily results in a higher extraction of plant compounds like polyphenols, but is also beneficial due to higher fiber contents in juice and the complexation of anthocyanins by UAEM-induced small oligomers to more stable complexes improving juice color and quality.

4. Conclusions

The applied enzyme preparation revealed effective pectin degradation in a continuous circulation system at reduced temperature (30 °C) regarding MW distribution, reduction of viscosity, and decrease in DM comparable to the batch maceration at 50 °C. UAEM in the continuous system resulted in an altered MW distribution by the formation of higher amounts of oligomers with medium MW (Mp approx. 150 kDa) and an enhanced release of HG-derived, RG I-derived, and RG II-derived smaller oligomers into the lower MW fraction (>30 kDa). The results suggest that the application of US affected enzymatic degradation by two mechanisms. On the one hand, it produced less complex oligomers mainly of medium MW with a high homogeneity of MW distribution, which could be subsequently degraded more easily by the enzymes. On the other hand, US affected enzyme’s conformation resulting in syner-gistic effects that enhance the effective enzyme activities during process conditions for PG and PL by even prolonging their activity over time. The effective activity of PME was not enhanced by low-intensity treatment of UAEM, whereas UAEM 90% (33 W*cm\(^{-2}\)) slightly reduced the effective PME activity indicating a US sensitivity. The individual effects on enzyme activities might be explained by the different susceptibility of their conformation. Finally, the total process output of UAEM 90% treatments demonstrated for PG, PL and also nearly for PME that UAEM at reduced temperature was as efficient as the conventional batch maceration at 50 °C.

The potential of UAEM for degradation of pectin at reduced temperature offers several prospects for fruit juice production. Milder process conditions preserve heat-sensitive compounds, in particular vitamins and phenolic compounds including anthocyanins. Soluble small oligomers arising from sufficient pectin degradation add nutritional fiber to the juices. Modified polysaccharides like RG I residues have been described to act as health-promoting ingredients, showing antimicrobial and anti-cancer activity potential [21,22]. Low MW oligosaccharides provide an additional beneficial effect by complexing polyphenols like anthocyanins [15]; since these complexes prevent
Fig. 6. Final marker concentration (nmol/ml) of the specific enzyme assay of PG (I), PL (II) and PME (III) of the commercial preparation Rohapect Classic (100 ppm) in pectin model solution (1%, pH 3.5) referred to the actual process conditions. Batch maceration (BM, grey bar) was carried out at 50 °C. US only (waved bar), enzyme only (white bar) and UAEM (dotted bar) were carried out in a continuous circulation system at 30 °C. Different letters indicate significant differences due to treatment. US +/+: A 90%, 300 W, 33 W cm⁻² US +/+: A 60%, 190 W, 21 W cm⁻², enzyme +/+: 100 ppm, enzyme -: no enzyme.

anthocyanins from oxidation and other degradation pathways, this may improve juice quality and nutritional value [15,18]. Ongoing research will need to verify our observed effects in actual juices, and might also be applied in other food matrices.

CRediT authorship contribution statement

Lena Rebecca Larsen: Conceptualization, Methodology, Investigation, Writing - original draft, Conceptualization, Methodology, Investigation, Writing - original draft. Judith van der Weem: Investigation, Formal analysis. Rita Caspers-Weißenbach: Investigation, Formal analysis. Andreas Schieber: Resources, Funding acquisition. Fabian Weber: Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing financial interest or personal relationships that could influence the reported work in this paper.

Acknowledgments

The authors gratefully thank AB Enzymes GmbH (Darmstadt, Germany) for providing the enzyme preparation and Herbstreith & Fox GmbH & Co. KG (Neuenbürg, Germany) for supplying pectin material.

Funding sources

The project is supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program (281A102716).

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