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Effects of Thermal Treatments and Storage on Pectin Methylesterase and Peroxidase Activity in Freshly Squeezed Orange Juice

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A specific indicator of freshness, allowing routine distinction between freshly squeezed orange juices (FSOJs) and FSOJ-like products, was to be identified. Using the Actijoule unit of a tubular heater at a flow rate of 60 L/h, FSOJs from *Citrus sinensis* (L.) Osbeck cv. Valencia Late were continuously heated on a pilot plant scale at six different temperatures (42–92 °C), followed by continuous cooling to ambient temperature and subsequent filling into sterilized glass jars. The cloud stability and residual activities of pectin methylesterase (PE) and peroxidase (POD) were monitored over the storage at 4 °C for up to 62 days, thus considering the storage conditions of FSOJs in retail markets. As shown by the viable microbial counts throughout storage, microbial activity was insignificant due to good sanitary practice, thus proving that the enzyme activities detected were of plant origin. The juices processed at temperatures ≥62 °C were characterized by minor residual activities. When exposed to temperatures <62 °C in the genuine acidic matrix of the juices, the heat stability of PE exceeded that of POD. Compared with the aforementioned samples, the juice processed at 52 °C with a residual PE activity of 33.8% was hardly inferior in terms of cloud stability within the first 14 days. After the juice was processed at 42 °C, rapid clarification occurred within the first 8 days, consistent with undetectable PE deactivation. Hence, only the range of ∼50–60 °C is relevant in minimal heat-processing for the retention of cloud stability within the short turnover period of FSOJ-like products, with partial PE and POD deactivation being already sufficient to distinguish those juices from FSOJs. Irrespective of the previous thermal treatment, the total PE activity remained nearly constant during storage, whereas the POD activity rapidly declined to minor levels after 20 days. Consequently, as to the future analysis of samples with unknown processing history, PE was suggested as an indicator enzyme for the freshness of FSOJs, allowing their unambiguous distinction from minimally heat-processed juices.

KEYWORDS: Food quality; freshness; indicator enzyme; orange juice; minimal heat-processing; pasteurization; pectin methylesterase; peroxidase

INTRODUCTION

Citrus juices and soft drinks derived thereof have gained great popularity, which is reflected by firm demand. On the basis of a world production of 59.0×10^6 tons of oranges and 94.8×10^6 tons of total citrus fruits in crop year 2004–2005, the respective percentages of fruits used for processing were 37.0% and 28.1% (*1*). Following the recommendations of modern dietetics, consumers' demands tend toward fresh and minimally processed fruit products. Freshly squeezed orange juices (FSOJs) offer an optimum of fresh taste and vitamin content, thus being considered as premium products with inherent enzyme activity (*2*). In particular, volatile flavor compounds differ between fresh and processed juices (*3*). On the other hand, unpasteurized juices are characterized by a relatively short shelf life, requiring marketing in a cool chain, since thermal preservation resulting in a reduction of microorganisms is not permitted (*4*). The shelf life of FSOJ is usually limited to 10–14 days and can be

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extended to 22 days through continuous chilling and special packaging materials (*5*). While the shelf life is mainly determined by microbial growth, flavor degradation and loss of ascorbic acid concomitantly occur (*2, 5*). The visual appearance is strongly affected by a rapid loss of turbidity, associated with sedimentation, which is crucial for the purchase decision in the case of cloudy and pulp-containing fruit beverages (*6*). Moreover, the cloud particles have a considerable impact on flavor, taste, and color (*7*). Native pectin is chiefly responsible for cloud stability due to its high water-binding capacity, resulting in increased serum viscosity and the formation of hydrate envelopes around the cloud particles (*8*). However, its quality is markedly influenced by the process parameters and the activities of degrading enzymes, primarily pectin methylesterase (PE).

As PE (EC 3.1.1.11) is located in the cell walls of the citrus mesocarp and in the membranes encasing the citrus segments of the endocarp, maximum PE activity was reported for the pulp (*9*). Due to juice extraction procedures that minimize the rupture of the peel and release of the rag, i.e., segment wall tissue enclosing the juice sacs, into the juice, merely approximately 1% of the total PE activity passes over to the juice during processing, still promoting pectin deesterification (*10*). As a consequence, partly deesterified pectin reacts with calcium ions of the fruit, thus resulting in onward flocculation and serum clarification (*11*). This may be enhanced by microbial PE produced by molds (*12*). Therefore, inhibition of PE directly after juice extraction is crucial in the production of storable citrus juice products (*13*).

Peroxidase (POD; EC 1.11.1.7), being responsible for a multitude of oxidative quality and flavor alterations in fruits and vegetables (*14*), is generally considered the most thermostable enzyme in plants (*15*). Consequently, it has widely been used as an indicator of heat treatments in food processing (*15, 16*). However, partial POD regeneration after thermal deactivation was reported (*14*). In oranges, POD activity was mostly detected in the peel and the albedo of the fruits, whereas only minor activity (4–10% of the total) was found in the juice (*16, 17*).

In general, thermal treatments result in deactivation of both enzymes and microorganisms. Although nonthermal alternatives have been developed (*18, 19*), thermal pasteurization is still the most common practice to ensure microbial safety and cloud stability. Although heating at much lower temperatures would impede microbial spoilage, short-time pasteurization at temperatures $\geq$92 °C confers the necessary stability through preventing cloud loss due to the high thermal resistance of citrus PE (*20*). As reported for various citrus juices (*3*), continuous heating at 95 °C for 20 s was required for complete PE deactivation. However, it leads to a remarkable loss of fresh taste (*3*) and to formation of off-flavor as well as a decline in nutritional qualities, notably ascorbic acid (*4*). Among freshly squeezed products offered in retail markets, orange juice that has minimally been heat-processed for shelf life extension and improvement of visual appearance would generate distortion of competition without declaration of this preservation step. Designed to detect marginal residual activities up to 0.5% (*21*), the well-established semiquantitative pasteurization test of IFU method no. 46 (*22*), proving complete PE deactivation through the absence of juice gelling after Ca^{2+} addition, is not applicable to distinguish between fresh and minimally heat-processed juices.

Therefore, the objective of this study was to explore criteria for the routine differentiation of fresh and thermally processed orange juice through a freshness indicator based on the quantification of native enzyme activities. In view of their well-documented roles in citrus and plant foodstuff processing, respectively, the suitability of PE and POD regarding their use in terms of reliable indicators of freshness was investigated. With respect to future evaluation of samples with unknown processing and storage history, the analytical significance of absolute residual enzyme activities, detected at any time after different thermal treatments within a broad range of thermal exposure from mild heating to pasteurization, was to be explored, with storage experiments following thermal processing. In contrast, conventional testing the effectiveness of citrus juice pasteurization (*21–23*) aims at the detection of minor residual activities, often including the comparison of samples before and after thermal processing. Whereas purification and characterization of PE extracted from citrus fruits was the subject of numerous investigations (*10, 11, 13, 24*), information about residual PE and POD activity after thermal treatments in the natural matrix is scarce (*25–27*). The occurrence of phase separation and loss of cloudiness were concomitantly monitored to evaluate to which extent relevant differences in the visual appearance may occur between the juices throughout the turnover period of FSOJs. To exclude a potential impact of microbial enzyme activities, the processing equipment was sanitized and microbial counts were monitored after heating and throughout storage.

MATERIALS AND METHODS

Materials. A 300 kg mass of oranges [*Citrus sinensis* (L.) Osbeck cv. Valencia Late] of Spanish harvest in May 2003 was supplied by a wholesaler in Stuttgart, Germany, and stored at ~4 °C until further use shortly after. Apple pectin with a degree of esterification (DE) of 77% (Pektin Classic, AU-L) was supplied by Herbstreith & Fox (Neuenbürg, Germany). Polyvinylpolypyrrolidone (PVPP), saponin, and hydrogen peroxide (H_2O_2; 30%, v/v) were obtained from Fluka (Buchs, Switzerland). Bovine serum albumin was purchased from Sigma-Aldrich (Steinheim, Germany), while Coomassie Brilliant Blue G 250 tablets were from Roth (Karlsruhe, Germany). All other chemicals were of analytical grade and supplied by VWR (Darmstadt, Germany), including Titrisol ampules for volumetric analyses. Ultrapure water (Milli-Q system, Millipore, Bedford, MA) was used for analytical purposes.

Juice Production and Experimental Design. Prior to juice extraction, the orange fruits (grade 1, size range 6) were divided into six lots of 50 kg for heat treatments at six different temperatures ranging from 42 to 92 °C. The juices were extracted in a semiautomatic Orange X-Press extractor (Brimato Maschinenbau, Hilter, Germany) equipped with two rotating drill heads, each 74 mm in diameter. The extractor was previously sanitized using hot water. The juice of the first lot (thermal treatment variant 42 °C) was finished with a stainless steel strainer with a mesh size of approximately 1 mm. All juices of the other lots were finished using a commercial pulper (PAP 0533, Bertuzzi, Brugherio, Italy) equipped with a finishing screen of 0.8 mm mesh size. For the second lot (thermal treatment variant 52 °C), the rotational speed was adjusted to 700 min^{-1}; the following four lots were finished at 400 min^{-1}. Further improvement of finishing by speed reduction was necessary to facilitate temperature control during heating, because coarse pulp particles of the second juice lot hindered the constant flow in the tubular heat exchanger (6 mm inner diameter). The finished juices were continuously heated at a flow rate of 60 L/h in an Actijoule heating system that was integrated in a tubular heating and cooling device (Ruland Engineering & Consulting, Neustadt, Germany), exposing them to 42, 52, 62, 72, 82, and 92 °C, respectively, for a residence time of 12 s. In the Actijoule unit, heat was directly transferred from the electrically heated tube walls to the product, thus ensuring a constant temperature throughout the tube length. In this way, more precise control of the product temperature was enabled, compared to a conventional tubular heat exchanger. Subsequently, the juices were cooled to 20 °C in the tubular heat exchanger with cold tap water. For the thermal treatment of each juice lot, the pasteurization value ($P^{z=8.9°C}_{T_{ref}=93.3°C}$, min), characterizing the extent of the thermal impact based on the estimated

lethal effect on microorganisms, was determined as previously described (*28*) and computed according to eq 1 for the reference temperature T_{ref} = 93.3 °C and the *z*-value z = 8.9 °C from the product temperatures [$T(t)$] recorded at 11 positions of the heating and cooling sections of the tubular heat exchanger and the flow times (t) until each of those positions. Flow times were calculated from the flow rates and tube dimensions.

$$P^{z=8.9°C}_{T_{ref}=93.3°C} = \int_0^t 10^{[(T(t)-T_{ref})/z]}\,dt \quad (1)$$

The pasteurized juices were filled in sterilized 370 mL glass jars that were manually closed with twist-off caps. Samples were taken for process control and enzyme assays from the juices before and immediately after processing as well as eight times during storage according to a sampling plan. During storage of 62 days at 4 °C in a cooling chamber, the jars were kept without agitation until analysis. Process control included quantification of the pH, contents of titratable acids and total soluble solids, specific weight, relative turbidity, and microbial counts on each day of sampling. Per sampling date and juice variant, two of three jars were used as nonredispersed samples for analyzing the serum phase, i.e., the partially clarified juice above the sediment formed in the jar [serum enzyme activity (A_s) and relative turbidity]. Referred to as a redispersed cloudy juice sample, the third jar was shaken for mixing the sediment and serum until cloud homogeneity [total enzyme activity (A_t) and process control parameters except relative turbidity].

Quality Control of Juices. All process control parameters were determined in duplicate. Determination of the pH was performed according to IFU method no. 11 (*22*) after calibration of the pH meter (Titrino 718 STAT, Metrohm, Herisau, Switzerland) at pH 4.0 and 6.9. The titratable acidity (TA), calculated as citric acid, was determined by titration with 0.25 N sodium hydroxide solution up to pH 8.1, in compliance with IFU method no. 3 (*22*), using an automatic titration system, Titrino 718 STAT. The content of total soluble solids (TSS) was determined at 20 °C with a digital refractometer, RX-5000 (Atago, Tokyo, Japan), and expressed in degrees Brix after correction of the refractometer reading for the impact of TA according to IFU method no. 8 (*22*). The specific weight was recorded at 20 °C by means of a density meter, DMA 48 (Anton Paar Physica, Graz, Austria). From the contents of TSS and TA (g/100 g of juice), the sugar/acid ratios (TSS/TA) of the juices were calculated.

The turbidity of the serum phase ($\tau_{s,i}$), i.e., the partially clarified juice phase in the jar as defined above, was determined nephelometrically with a diffused-light photometer LPT 5 (Dr. Lange, Düsseldorf, Germany), using cuvettes of 50 mm diameter (*8*). For turbidity analysis, the samples were diluted with a thinning agent containing the major constituents of the juices to be analyzed (*8*). It consisted of glucose (28 g L^{-1}), fructose (30 g L^{-1}), sucrose (33 g L^{-1}), citric acid (9.4 g L^{-1}), and malic acid (1.7 g L^{-1}). A stock solution of 1 L was prepared by dissolving the powdery substances in ultrapure water and stored at −20 °C until use. Prior to analysis, the stock solution was thawed, adjusted to the pH of the juice with 5 N potassium hydroxide, and used for appropriate dilution of the sample. The turbidity readings were multiplied with the dilution factor and expressed in formazine turbidity units (TU/F). The turbidities of the juice serum phases were compared with the initial turbidity of the respective heat-treated juices (τ_o), resulting in the relative turbidity, i.e., the percent residual turbidity [$\Delta\tau_{res,i} = (\tau_{s,i}/\tau_0) \times 100\%$], after i days of storage.

The microbial quality of the orange juices was monitored throughout storage to detect potential contributions of microorganisms to measured enzyme activities. For the total viable counts, the samples were serially diluted (1:10) with a sterile solution consisting of 0.85% (w/v) NaCl and 0.1% (w/v) peptone. Aiming at 30−300 colonies per plate for quantification, plating was performed in duplicate per dilution stage, using juice volumes of 1 and 0.1 mL as well as 0.1 mL of diluted sample. The total plate counts were determined on orange serum agar supplied by VWR after incubation at 30 °C for 48 h under aerobic conditions (*29*) and expressed in colony-forming units (CFU) per milliliter of juice. Plates with poor development of colonies were rechecked after a total incubation time of 72 h. Yeasts and molds were microscopically differentiated, using a light-optical microscope (Mikroskop Standard 25, Zeiss, Munich, Germany) with oil immersion at 1000-fold magnification.

Simultaneous Extraction of PE and POD. For enzyme extracts from the cloudy juice, the respective jar was shaken manually before the juice was strained through a screen, DIN 4188 (Retsch, Haan, Germany) of 0.5 mm mesh size to remove coarse particles. Additionally, the serum phases of stored juices from two further jars per variant were extracted. Redispersed juices and serum samples were extracted in duplicate. Samples (100 g), PVPP (3 g), NaCl (0.877 g), and saponin (100 mg) were mixed before adjustment of the pH value to 6.0 with 10 and 1 M NaOH. After being stirred at 4 °C for 120 min, the mixture was centrifuged at 25000*g* and 4 °C for 30 min in a centrifuge, type Suprafuge 22, equipped with a type no. 14290 rotor (Heraeus Sepatech, Osterode, Germany). The centrifugate was decanted through a folded filter (Schleicher & Schuell no. 597½, Dassel, Germany) at 4 °C. The residue was washed twice with ultrapure water (4 °C) and then discarded. The centrifugate and washing water were pooled in a graduated flask and adjusted to 200 mL with ultrapure water (4 °C). Each aqueous extract was divided into portions of 20 mL and stored in plastic containers at −80 °C until further use.

For simultaneous extraction of PE and POD, an aqueous system without the use of buffers was necessary to enable subsequent titrimetric quantification of PE activity. Its applicability was confirmed by a preliminary comparison of extraction methods for POD, with the aqueous extract of orange juice, produced as described above, showing 100.3% of the POD activity that was obtained after application of the reference extraction method (*30*) based on McIlvaine buffer, pH 6.5, to the same sample. The McIlvaine buffer was prepared by mixing 15 mL of citric acid (c = 0.1 mol L^{-1}) with 35 mL of disodium phosphate (c = 0.2 mol L^{-1}) and dilution to a total volume of 100 mL.

PE Activity Assay. Among the various modifications (*31*) suggested for the recorded titration of liberated carboxyl groups (*13*), a standard PE activity assay (*10*), where concurrent alkaline deesterification and β-elimination of the substrate were minimized through a pH value slightly below the pH optima of the citrus PE isoenzymes (*13*), was used with minor adaptations. Titrimetric analysis was performed at 30 °C and pH 7.0 for 30 min with 0.01 N NaOH in a thermostated cell, applying an automatic titrator, Titrino 718 STAT. A 0.5% (w/v) solution of apple pectin (DE 77%), containing 0.15 M NaCl, served as the substrate. After adjustment of 59 mL of substrate to pH 7.0, 1 mL of enzyme extract was added. The pH value was set to 7.0 again, and the titration was performed at constant pH for 30 min. In view of a constant total volume of 60 mL, samples of low activities were analyzed with extract volumes of 2 or 5 mL, reducing the substrate volume accordingly to 58 and 55 mL. Blanks were determined by analogous titration with 0.01 N HCl, using the enzyme extracts after they had been boiled for 3 min. The PE activity (A_{PE}) was calculated as the liberation rate of carboxyl groups according to eq 2 (μkat g^{-1} of sample), i.e., redispersed cloudy juice or juice serum, from the volumes of NaOH and HCl used to titrate the sample extract (V_{NaOH}, mL) and the blank extract ($V_{HCl,B}$, mL), respectively, the corresponding NaOH and HCl concentrations ($c_{NaOH} = c_{HCl}$ = 0.01 mol L^{-1}), the observation time at pH 7.0 (Δt, s), the volumes of sample extract ($V_{extract}$, mL) and blank extract (V_{blank}, mL), and the factor of enzyme extract volume per sample mass (nominal: F = 2 mL g^{-1}). Assays were conducted in duplicate per extract.

$$A_{PE} = \left[\frac{V_{NaOH}\, c_{NaOH}}{\Delta t\, V_{extract}}\right] + \left[\frac{V_{HCl,B}\, c_{HCL}}{\Delta t\, V_{blank}}\right] F \times 1000 \quad (2)$$

Since PE activity was mostly expressed in PE units per gram of sample, with 1 unit corresponding to 1 μmol of liberated carboxyl groups min^{-1} (*10, 13, 32*), PE activity according to eq 2 was transformed into units per gram by multiplying the results obtained (μkat g^{-1}) by 60, corresponding to the observation time in minutes.

POD Activity Assay. POD activity was assayed spectrophotometrically as described previously (*28*), with slight adaptations. In a cuvette, 0.4 mL of enzyme extract was blended with 1.1 mL of the substrate solution that contained tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one; c = 12 mmol L^{-1}) and hydrogen peroxide (c = 3.3 mmol L^{-1})

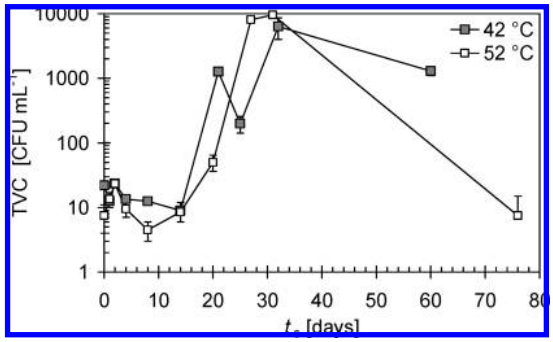

Figure 1. Development of total viable microbial counts (TVC) of orange juice during storage at 4 °C after thermal treatment at 42 and 52 °C, respectively. t_s = storage time.

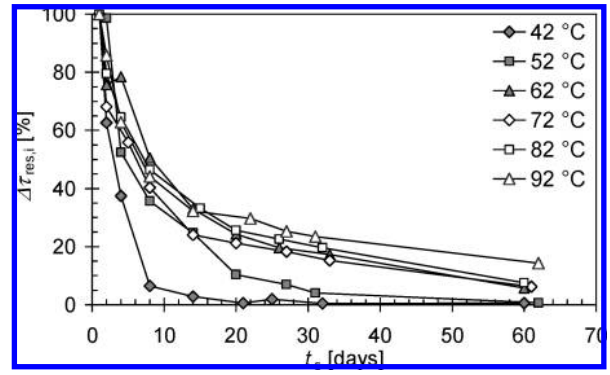

Figure 2. Percent residual turbidity ($\Delta\tau_{res,i}$) in bottled orange juice during storage at 4 °C after continuous thermal juice treatment at 42, 52, 62, 72, 82, and 92 °C. t_s = storage time.

dissolved in McIlvaine buffer (pH 6.5), which consisted of 30% citric acid (c = 0.1 mol L^{-1}) and 70% disodium phosphate (c = 0.2 mol L^{-1}). Tropolone is a potent PPO inhibitor, improving differentiation between POD and PPO (*33*). By means of a Cary 100 spectrophotometer (Varian, Mulgrave, Victoria, Australia), the absorbance at 418 nm was recorded at 25 °C every 15 s over a period of 20 min against a blank, containing the enzyme extract that was previously boiled for 3 min. The increase in absorbance was calculated from the linear part of the absorbance−time curve that partly occurred after a lag phase. The POD activity (A_{POD}) was calculated according to eq 3 (nkat g^{-1} of sample), i.e., redispersed cloudy juice or juice serum, from the increase in absorbance (dE/dt, s^{-1}), the total volume in the cuvette (V_T = 1.5 × 10^{-3} L), the volume of the enzyme extract (V_P = 0.4 mL), the molar extinction coefficient of the reaction product ($\epsilon_{25°C}$ = 2075 L mol^{-1} cm^{-1}), the optical path length (d = 1 cm), and the factor of enzyme extract volume per sample mass (nominal: F = 2 mL g^{-1}). Assays were conducted in triplicate.

$$A_{POD} = \frac{dE}{dt}\frac{V_T}{\epsilon_{25°C}\, d\, V_P} F \times 10^9 \quad (3)$$

Quantification of Protein. The protein contents of the enzyme extracts ($c_{P,extract}$) were determined spectrophotometrically according to the method of Bradford (*34*), using bovine serum albumin as the reference for calibration. Analyses were conducted in triplicate.

Data Analysis. Analysis of variance (one-way ANOVA) was carried out for various process parameters, using Microsoft EXCEL 97.

RESULTS AND DISCUSSION

Juice Characteristics. Analyses of the thermally treated juices of each variant directly after production showed that all juice lots were uniform, displaying specific weights between 1.0442 and 1.0454 kg L^{-1}. Irrespective of heat treatment and storage, the TSS and TA of the bottled juices ranged from 13.6 to 14.0 °Brix and from 12.0 to 13.0 g L^{-1}, respectively, while the pH value was constant at 3.3. Thus, minor differences in the main juice characteristics were negligible.

Microbial Quality of the Orange Juices. Since orange serum agar created environmental conditions similar to those in orange juice, microbial counts detected on this medium represented the number of viable microorganisms able to grow in orange juice.

No growth of microorganisms was detected in juices heated at temperatures exceeding 62 °C. In juices heated at 42 and 52 °C, the initial total viable counts increased during storage up to a maximum of approximately 10^4 CFU mL^{-1} of juice within 30 days. With further storage, a decline to 1300 and 10 CFU mL^{-1}, i.e., to values below the limit of quantification in the second case, was observed for the juices heated at 42 and 52 °C, respectively (**Figure 1**). The flora of the latter mainly consisted of yeasts, whereas molds were not detected. In the juice heated at 42 °C, a change of the microbiological flora was noticed between day 25 and day 32 of storage. At the beginning of the storage, molds were predominant, whereas only a few yeasts were microscopically identified. As from day 32, molds were no longer detectable, but the number of yeasts increased. These findings may explain the two peaks of the total-count curve presented in **Figure 1** for the least heated juice. Compared to juices described earlier (*18, 29*), the microbial quality was excellent, although the fruits had not been washed prior to juice extraction. Since the initial total plate counts were relatively low without a drastic increase, the observed PE activities can be ascribed to genuine orange enzymes, in contrast to the report of Rouse (*35*).

Cloud Stability of the Orange Juices. As expected, clarification during storage strongly depended on the previous heating temperature (**Figure 2**). Compared with the mild treatments (42–52 °C), more intense thermal exposure at 62–92 °C resulted in higher initial turbidity on day 1 after production and in better retention of residual turbidity during storage. The latter was shown by residual turbidities of the serum phases of 5.8–14.4% after 60–62 days of storage and a half-life period [$t_s(\Delta\tau_{res,i}$ = 50%)] of 6.1–8.2 days, which is the storage time (t_s) after the initial turbidity of the juice decreased by 50% in the serum phase in the jar. The respective residual turbidities and half-life periods subsequent to treatments at 42–52 °C were only 0.53–0.70% and 3.0–4.6 days. The total loss in turbidity after 60–62 days steadily increased from 85.6% in the juice heated at 92 °C to 99.5% for the juice heated at 42 °C. Several factors are known to contribute to destabilization of the cloud particles, such as the contents of TA and TSS, storage temperature and cultivar differences, pectin characteristics, particle size distribution, and particle shape (*6*). Hence, the extent of cloud stability after juice pasteurization may vary widely, even after complete PE deactivation. The storage conditions applied closely mimicked the real situation of freshly squeezed juice products displayed for sale in retail markets. Therefore, the results reflect the visual impression of fresh and pasteurized citrus products perceived by the consumers within the time of turnover.

Protein Contents of the Enzyme Extracts. For the juices heated at 42 °C (**Figure 3**) and 52 °C (data not shown), the protein contents of the enzyme extracts from juice serum phases dropped to zero during the first 8–20 days of juice storage. Since this observation is in good accordance with a nearly total loss of turbidity during the same time (**Figure 2**), good correlation between the amount of residual cloud particles of the serum phase and the protein content of the extracts can be assumed. Consistently, protein was extractable from the serum phases of the other variants nearly over the whole period, as exemplarily

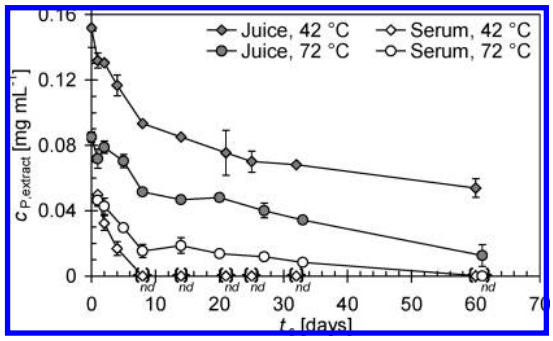

Figure 3. Protein contents ($c_{P,extract}$) of the enzyme extracts obtained from redispersed cloudy juice (filled symbols) and the serum phases of nonredispersed samples (open symbols) during storage of bottled orange juice at 4 °C after thermal treatment at 42 and 72 °C, respectively. t_s = storage time, and $\{\ldots\}_{nd}$ = nondetectable records.

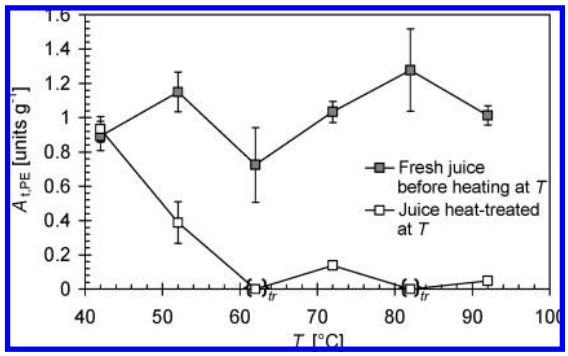

Figure 4. Influence of the nominal temperature (T) on deactivation of native PE by continuous thermal treatments of orange juice: total PE activity ($A_{t,PE}$) of the fresh juice before heating and of bottled juice directly after heating. $\{\ldots\}_{tr}$ = activity in traces.

shown in **Figure 3** for the juice heated at 72 °C. Good protein extractability from the serum phase was achieved even after 62 days of storage for the most cloud-stable juice pasteurized at 92 °C.

The protein contents of the extracts from redispersed cloudy juices gradually decreased toward ~50% of their initial levels during juice storage (**Figure 3**). For the enzyme extracts from the redispersed juices heated at higher temperatures, this reduction of protein extractability was less pronounced, but the amounts of extractable protein directly after heat treatment linearly declined with increasing heating temperature to constant levels following juice treatments at 72–92 °C. This temperature effect on protein extractability was supported by the significantly higher protein contents of extracts obtained from the juices prior to heat treatment (0.163–0.206 mg mL^{-1}). Hence, increasing thermal protein denaturation and improved retention of turbidity had inverse impacts on protein extractability. Due to the differences in protein extractability among the samples, enzyme activities relative to extracted protein were not considered.

Effects of Thermal Treatments on Residual PE Activities of the Orange Juices. The PE activity of the freshly prepared, unheated juices varied from 0.73 to 1.28 units g^{-1} of juice among the six lots (**Figure 4**), with a mean value of 1.02 ± 0.05 units g^{-1} corresponding to 16.9 nkat g^{-1} of juice. Similar PE activities have been described for fresh orange juice by other authors (*24, 32*). In view of homogeneous raw material for all juice variants in this study, the differences observed among the six fresh juice lots reflected the reproducibility of juice extraction, since both minimum and maximum values of the activity range mentioned above were recorded for juices produced with exactly the same nominal extractor and finisher settings. The presented PE activities were recorded for fresh juices from fruits that had obviously been harvested at suboptimal ripeness, even though the TSS of the juices clearly exceeded the minimum of 10 °Brix for early-harvested fruits (*2*). However, with a mean value of 11.44 ± 0.09, the sugar/acid ratios of the juices were slightly below the recommended range of 12.5–19.5, but above the minimum of 10.5 set for early-harvested fruits (*2*).

As PE is a cell-wall-associated enzyme with various isoforms (*10, 11, 32*), the PE activity of fresh orange juice is dependent on the pulp content (*36*), i.e., the quantity of cloud particles. To consider changes in juice concentration during production, the PE activity was partly reported relative to a standardized TSS level in degrees Brix (*18, 36*), which, however, does not necessarily compensate for the former influence. Facing the multiple factors that affect the PE activity of orange juices, it is recommended to express enzyme activities as processing indicators for juices of unknown history on a juice weight basis, rather than on the basis of a standardized juice composition, to cope with the heterogeneity of orange juices offered in fresh-product markets. However, for a potential application of the enzyme assay to unknown samples for verification of citrus juice freshness, the influence of the pulp content on the total PE activity implies standardization of the juice samples prior to enzyme extraction to exclude this effect, in contrast to the application of the assay in enzyme characterization (*27*), where maximum enzyme extraction is important. In the present study, this standardization was achieved by sample filtration, mimicking a typical finishing step through a sieve of 0.5 mm mesh size (*3*). For the same reason, enzyme extracts were analyzed instead of direct juice analysis, the latter being preferred in process control for pasteurization tests (*22*).

After heating at 42 °C, the PE activity did not significantly differ from that of the fresh juice (**Figure 4**), consistent with the observation for an orange juice heated in a water bath of 45 °C for 5 min (*27*). However, when compared with the laboratory-scale exposure to 55, 65, and 70 °C for 5 min, resulting in residual activities >80% and around 30% and 20%, respectively (*27*), enzyme deactivation rose more vigorously with increasing temperature in continuous heating from a residual activity of 33.8% after exposure to 52 °C to an almost total loss of PE activity at temperatures ≥62 °C. Presumably, the functional principle of the Actijoule unit, with its improved heat transfer along the tube, particularly contributed to the more effective deactivation. Similar to the water bath experiments mentioned above (*27*), also continuous heating of various citrus juices at 70 °C for 10 s in a plate heat exchanger resulted in weaker deactivation to residual activities of ~20% (*3*). In contrast to low-temperature blanching for fruit or vegetable firming of plant tissue (*37, 38*), no activation of PE was induced by heating at mild temperatures. This may be ascribed to an increased thermal sensitivity of the isolated enzyme, released from the fruit matrix during juice production. Heating at higher temperatures with corresponding $P^{z=8.9°C}_{T_{ref}=93.3°C}$ values of 0.003 min (72 °C), 0.023 min (82 °C), and 0.107 min (92 °C) resulted in minor residual activities that were difficult to quantify precisely with 13.4% at 72 °C, traces at 82 °C, and 4.8% at 92 °C. Unlike the PE-based pasteurization tests (*22, 23*), the titrimetric PE assay is not designed for the quantification of minor activities (*21*). Nevertheless, those results conformed to the deactivation behavior of purified PE from orange on the laboratory scale (*27*), where the strongest activity decline occurred between ~60 and 65 °C to residual levels of ~10% observed after heating at 65–75 °C. Consistent with **Figure 4**,

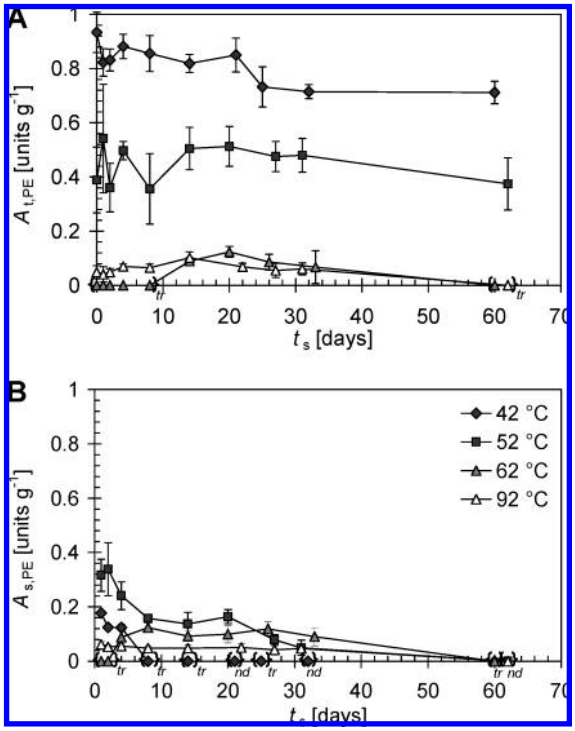

Figure 5. PE activity (A_{PE}) in bottled orange juice during storage at 4 °C after continuous thermal juice treatment at 42, 52, 62, and 92 °C: (**A**) total PE activity ($A_{t,PE}$) of the juices after enzyme extraction from redispersed cloudy juice; (**B**) PE activity of the serum phases ($A_{s,PE}$) after enzyme extraction from the juice serum of nonredispersed samples. t_s = storage time, $\{\ldots\}_{tr}$ = activity in traces, and $\{\ldots\}_{nd}$ = nondetectable records.

minor residual PE activities in the range of 1–4% were reported for various citrus juices after continuous heating at 85 or 90 °C for 10 s (*3*). Furthermore, deactivation of the native PE in the orange juices was largely comparable with that of very early studies (*36, 39*) based on simple continuous heating devices for juice pasteurization. Within the typical pH range of citrus juices, a slightly increasing thermal sensitivity of the total PE activity with a declining pH value (*36*) may additionally contribute to the overall variation in residual activities. As shown for PE from tomato (*40*), purified isoenzymes may be more thermostable than PE of a crude extract from the juice. Among the three PE isoenzymes accounting for 95% of the activity in Navel orange, PE I and II as well as a thermostable high-molecular-weight PE were rapidly inactivated at 70, 60, and 90 °C, respectively, under laboratory conditions, when added to single-strength orange juice of pH 4 (*10*). Minor residual activities of native PE in orange juice, as observed in the present study after continuous heating at ≥62 °C, were ascribed to the thermostable form that represented only ~5% of the total PE activity in Navel orange (*10, 13*). For the use of native enzymes in food analysis as indicators of thermal treatments, requiring a rapid assay without previous steps of enzyme purification, the behavior of crude enzyme extracts from juice processed under conditions of industrial relevance is crucial and was therefore exclusively considered in this study.

On the basis of the analysis of extracts from the redispersed cloudy juices, storage of the heat-treated juices at 4 °C led to an almost constant PE activity for a storage period of 60–62 days, with modest total reduction by 23.7% in the juice treated at 42 °C to a residual activity of 79.7% relative to that of the fresh juice (**Figure 5A**). Since reduction of PE activity during storage was by far lower than the decline in the protein content

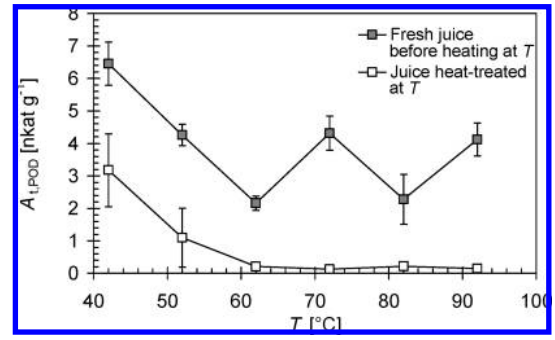

Figure 6. Influence of the nominal temperature (T) on deactivation of native POD by continuous thermal treatments of orange juice: total POD activity ($A_{t,POD}$) of the fresh juice before heating and of bottled juice directly after heating.

of the extracts (**Figure 3**), the extractability of PE was obviously not affected by the lowering of extractable total protein. As shown by addition of three isolated PE isoenzymes to single-strength orange juice preserved with sodium azide, stability during storage at 6 °C markedly varied among the isoenzymes with decimal reduction times from 4.5 to >400 days (*10*), though underlining the high overall stability of PE.

As confirmed by the comparison of parts **A** and **B** of **Figure 5**, the PE activity is closely associated with the cloud particles (*11*). In the increasingly clarifying serum phase of the bottled juices, the PE activity rapidly declined during storage, particularly in the juice treated at 42 °C. This was consistent with a former study (*41*) showing enhancement of cloud loss by the PE activity through simulation of different residual activities by adding increasing levels of isolated citrus PE to fully pasteurized juice. For variants heated at ≥62 °C, the PE activities of the redispersed juices (**Figure 5A**) and those of the cloudy serum phases (**Figure 5B**) were quite similar throughout storage. Hence, minor activities below 0.12 unit g^{-1}, as observed for the more turbid serum phase samples from those juices, were predominantly caused by thermal destruction of the enzyme protein. Furthermore, **Figure 5** indicated missing enzyme reactivation, consistent with reports on PE from other sources (*40, 42*).

Effects of Thermal Treatments on Residual POD Activities of the Orange Juices. The activity of POD in freshly squeezed orange juice varied from 2.16 to 6.46 nkat g^{-1}of juice with a mean value of 3.9 ± 0.64 nkat g^{-1} of juice (**Figure 6**). As most reports only presented percent residual activities after heat treatments, comparison of initial POD activities is difficult. On the basis of the molar reaction rates in nmol s^{-1} g^{-1} expressing the enzyme activities in nkat g^{-1} of juice, the average ratio of PE and POD activity was 4.3/1 in the freshly squeezed, unheated orange juices. Their lower relative POD activity makes PE more suitable for quantitative analysis in terms of a processing indicator, in addition to the key impact of the latter on the citrus juice quality (*13*).

In the range of mild thermal treatments (<62 °C), the overall heat stability of extractable POD (**Figure 6**) was lower than that of PE (**Figure 4**). Residual POD activities after the treatments of the juice at 42 and 52 °C were 49.2% and 25.8%, respectively, whereas heating at ≥62 °C reduced the POD activity to 2.9–9.6% of its initial value. On the other hand, POD is known to be one of the most thermostable enzymes of higher plants and, therefore, is often used as an indicator enzyme in blanching (*16*). Indeed, high thermal resistance of both POD and PE became evident by detectable residual activities after continuous heating at 92 °C ($P^{z=8.9°C}_{T_{ref}=93.3°C}$ = 0.107 min). In

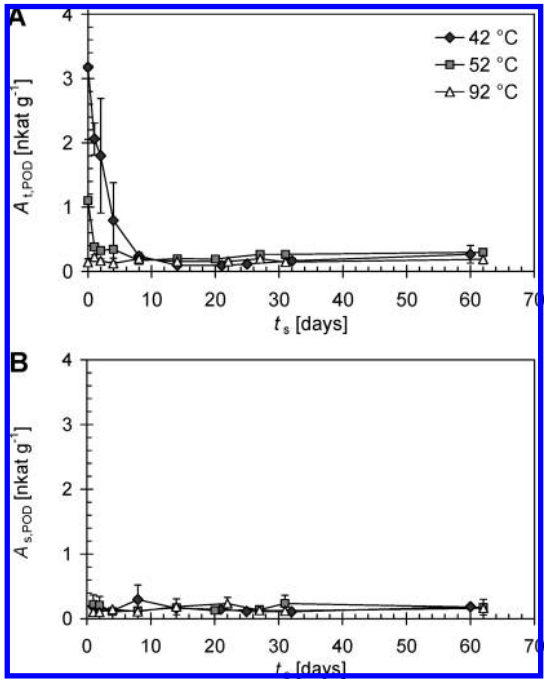

Figure 7. POD activity (A_{POD}) in bottled orange juice during storage at 4 °C after continuous thermal juice treatment at 42, 52, and 92 °C: (**A**) total POD activity ($A_{t,POD}$) of the juices after enzyme extraction from redispersed cloudy juice; (**B**) POD activity of the serum phases ($A_{s,POD}$) after enzyme extraction from the juice serum of nonredispersed samples. t_s = storage time.

terms of PE, this stability was consistent with its use as a pasteurization indicator in conventional citrus juice processing (*13, 22*). In the case of POD, however, the acidic conditions of the orange juices during the heat treatments lowered its overall thermal stability in its natural matrix. Hence, different procedures of minimal heat-processing would be distinguishable more clearly through residual PE activities than through POD. Earlier reports on horseradish (*43, 44*) described a high impact of the pH value on the heat stability of POD, with a total loss of activity occurring at pH 3.5 after the extract was heated at 76 °C for 30 s. In contrast, deactivation studies for the characterization of orange isoperoxidases (*16, 17, 45*) were usually performed by exposing crude and purified POD extracts to heat at nearly neutral pH values, giving evidence of its thermostability. Whereas retention of initial activity exceeded 60%, when a buffered extract containing soluble orange juice POD was heated at 60 °C for 10 min (*17*), continuous heating of orange juice at 62 °C with a dwell time below 1 min caused almost total loss of activity with a retention of 9.6% (**Figure 6**). Such minor residual activities after rigorous heat exposure may be ascribed to the presence of particularly thermostable isoenzymes, since extremely different thermostabilities were reported for the three soluble POD isoenzymes that were isolated from orange juice and purified (*45*). Ionically bound POD isoenzymes also occurring in orange juice were found to be more thermostable than the soluble ones (*17*), even though this difference became negligible in the elevated temperature range when other extraction procedures were applied (*16*). In the present study, both soluble and ionically bound isoperoxidases were assumed to be extracted concurrently from the cloudy juice by using NaCl at a concentration of 0.15 mol kg^{-1} of juice at pH 6.0 in the presence of PVPP and saponin.

Throughout storage, the POD activity of the juices dramatically decreased (**Figure 7A**). Within the first 4 days of storage, a decline by 75.0% (42 °C) and 68.7% (52 °C) was noted relative to the value of the heated juice on the day of production. After 8 days, only trace activities of POD in the range of 0.1–0.2 nkat g^{-1} were detected, irrespective of the previous heating temperature. As the turbidity of the juices decreased at a lesser rate (**Figure 2**), the activity decline of the extractable POD was caused neither by cloud loss nor by limited extractability of the total protein (**Figure 3**), but reflected the poor storage stability of this enzyme. In the juice serum phases, the POD activity was extremely low throughout storage, without significant differences among the six variants (**Figure 7B**). During storage, no regeneration of POD activity was observed. This finding may also be ascribed to the acidic environment of the enzyme. Maximum regeneration of horseradish POD was reported at neutral pH, whereas below pH 5 no regeneration was found (*43, 44*).

Conclusion. Continuous heating of orange juice with an Actijoule unit on a pilot plant scale at temperatures ≥62 °C, i.e., in a time and temperature regime coming close to pasteurization, caused deactivation of both PE and POD to minor residual activities that are ascribed to the heat-resistant isoenzyme fractions. Thus, the juices processed at ≥62 °C were unambiguously distinguished from the fresh product. For the verification of freshness, the observed limited preciseness of the detection of minor PE activities would not be crucial, since even erratic results around the detection limit would indicate thermal processing. In terms of cloud stability, the juice processed at 52 °C was hardly inferior to the samples with minor residual activities within the first 14 days (**Figure 2**). In contrast, processing at 42 °C, causing no detectable PE deactivation (**Figure 4**), was associated with rapid clarification within the first 8 days. Hence, for the production of minimally heat-processed juices with improved retention of cloud stability during the short turnover period, temperatures around 50–60 °C are relevant. In this range, activity reduction was large enough to distinguish those juices from the fresh ones. As shown for Clementine juice (*3*), flavor was insignificantly affected by continuous heating at 50 or 60 °C for 10 s relative to that of the fresh juice. In contrast, processing at 85 °C for 10 s was suggested for the production of high-quality pasteurized citrus juice, resulting in still acceptable flavor deficiencies and minor residual PE activities of ∼3% (*3*). The latter necessitates short-time distribution of such pasteurized juices within a cool chain for improved turbidity retention. Retention of residual activities following processing at temperatures of 42–92 °C was constant throughout storage for PE, making this enzyme a suitable indicator of thermal treatments in orange juices at any time of analysis after processing. In contrast, due to missing storage stability, the POD activity would be an unreliable freshness indicator (**Figure 7**). As confirmed by the monitoring of total viable microbial counts, the observed enzyme activities were clearly ascribable to the juice matrix. For both enzymes, significant reactivation after thermal processing was excluded. The presented results reflected practical conditions, since native enzymes were continuously heat-processed in their genuine matrix and storage conditions mimicked the situation in retail markets. As a result of this study, PE activity is suitable to prove the freshness of orange juices. Elevated production costs for FSOJs are implied by the higher sanitary status required during manufacture, the continuous cool chain needed from production to retail markets, and the highly limited shelf life. As FSOJs gain increasing popularity, higher retail prices may be attained. This fact may provoke

a competition between fresh and minimally heat-processed juices, where differences in flavor, contrasting with an extended expiration date and elevated cloud stability, may create a demand for official analytical procedures for the verification of freshness and the extent of previous thermal processing, respectively. Currently, a comparative PE-based assay for routine analysis of FSOJs has been established and verified. For its broad application, the least detectable difference of PE activities resulting from processing at 45–60 °C relative to that of freshly squeezed juices demands particular attention. From the technological point of view, detailed optimization studies of the temperature range between 45 and 60 °C are required in terms of the contribution of minimal heat-processing on scales of practical relevance to cloud stability, flavor retention, and partial PE deactivation for juices of various citrus species.

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