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Validation and applicability of a standardized procedure for evaluating freshness of *Citrus* juices based on pectin methylesterase activity quantitation

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Summary

A method developed for freshness authentication of freshly squeezed *Citrus* juices (FSCJ) was evaluated for routine application. It involved titrimetric assessment of pectin methylesterase (PE) activity after enzyme extraction from pulp-standardized juice samples. Standard test conditions enabled reliable discrimination between FSCJ and chilled *Citrus* juices that had comparative advantages due to extended shelf life. Unlike the latter, FSCJ always displayed PE activities in the linear range between the limit of identification (LOI, 0.42 units g^{-1} of juice) and the maximum activity found for FSCJ (1.94 units g^{-1}), equivalent to 0.0035-0.016 units during titration. However, for model samples having activities < LOI due to production by respective dilution of FSCJ, the responses abruptly fell to unspecific levels below the limit of detection (LOD, 0.21 units g^{-1}). Accuracy was substantiated by 100-106 % recovery for model juices with PE activities of 0.87-1.22 units g^{-1} resulting from FSCJ dilution or PE standard addition, but it was lower (76-80 %) near LOI. The average of the mean activities, which were detected by 3 analysts with intra-assay precision ≤ 8.4 %, varied with relative standard deviations of 8.2 % for FSCJ and 3.9 % for a sample of the same juice diluted to 60 % (w/w), thus proving reproducibility. FSCJ batches were unambiguously distinguished from four commercial chilled juices, because the activities detected for the latter were by far ≤ LOD and thus confirmed labeled mild preservation.

1. Introduction

Orange fruit accounts for the most popular flavor of fruit juices and nectars in Europe (~35 %) with a portion of even 55-62 % in some EU countries (AIJN, 2010). The total of ~7.5 billion L of fruit juice, which was consumed in Europe in year 2009 regardless of the flavor, mostly comprised ambient products, either from concentrate (FC) or not from concentrate (NFC), with a shelf life of up to 18 months. Chilled commodities though added up to notable 18 % (AIJN, 2010). The latter are uniformly perceived by consumers to be premium (Crupi and Rispoldi, 2002), but their role in the juice market greatly varied among countries (e.g., 6-10 % of fruit juice in Germany and France, 55-69 % in the UK and Sweden; AIJN, 2010). Their distribution involves a cold chain, which is either needed for the retention of food quality and safety or chiefly associated with premium market positioning. Since freshly squeezed orange juices (FSOJ), i.e. freshly squeezed *Citrus* juices (FSCJ) in general, are offered without previous pasteurization, their shelf life is limited to a few days despite chilled supply chain (Crupi and Rispoldi, 2002), as expressed by a labeled best-before date within two weeks after juice extraction (FSA, 2008). However, the diverse group of chilled *Citrus* juices also includes NFC with a shelf life of a few weeks owing to mild pasteurization (Collet et al., 2005; Sentandreu et al., 2005). While they most resemble FSCJ with respect to fresh taste (Ruiz Perez-Cacho and Rouseff, 2008), microbial load and pectin methylesterase (PE; EC 3.1.1.11) activity causing decay (Kopelman and Rauchwerger, 1984) and rapid cloud loss (Hirsch et al., 2008), respectively, have been reduced by minimal preservation (Crupi and Rispoldi, 2002). The same shelf life-extending effects may be attained by various non-thermal preservation techniques (Crupi and Rispoldi, 2002; Guiavarc'h et al., 2005), as recently discussed by Hirsch et al. (2011) as to PE deactivation. By contrast, due to complete PE deactivation during their production, chilled FC constituting the third subgroup would only require cold supply, if the pasteurization of the juice that was reconstituted from concentrate was merely mild (Crupi and Rispoldi, 2002) or if FC was blended with FSCJ or gently pasteurized NFC. FC even prevailed among the chilled juices in individual EU countries (AIJN, 2010). Hence, freshness is an outstanding feature of the first subgroup, demanding strictest sanitation standards during processing and distribution (Crupi and Rispoldi, 2002; Hirsch et al., 2008). Analytical distinction of FSCJ from other chilled *Citrus* products via authentication of juice freshness by food inspection boards is thus crucial when labeled specification of product types comes under scrutiny.

In view of the great impact of processing on *Citrus* juice aroma, analysis of aroma compounds has been used for discrimination between different juice types of this highly diverse product group (Tønder et al., 1998). By classifying juices via multivariate analysis of aroma profiles, FSOJ were found among various kinds of ambient orange juices (FC and NFC) (Shaw et al., 1993). Being basically characteristic of FSCJ, activities of endogenous enzymes, e.g., acid phosphatase (Gel Moretó, 2000), have generally been regarded as the criterion of choice to distinguish this juice type from ambient products, provided that the test enzyme is stable throughout juice distribution. Enzyme stability of peroxidase during cold juice storage was unacceptable for a freshness indicator of FSCJ, unlike that of PE (Hirsch et al., 2008). The latter is also the technologically most relevant enzyme for the design of *Citrus* juice preservation (Duvetter et al., 2009). Suitability of PE as a freshness indicator (Hirsch et al., 2008) was substantiated by its universal applicability across *Citrus* cultivars and species, as deduced from the activity range and the overall uniform thermal resistance observed for PE of numerous FSCJ of known production history (Hirsch et al., 2011). However, discrimination between different types of chilled juices, especially FSCJ and chilled NFC, has appeared by far less trivial, because partial PE deactivation by mild preservation may lead to activities in a broad range (Hirsch et al., 2011). The analytical method suggested for freshness evaluation (Hirsch et al., 2008, 2011) was though deemed suitable for the distinction of FSCJ from those chilled NFC that have comparative advantages in terms of significantly enhanced shelf life, when mild pasteurization almost completely inactivated their thermo-labile PE fraction.

Freshness authentication implied a standardized procedure aiming at routine assessment of PE activity in unknown samples. Unlike other *Citrus* juice types, specifically FSCJ may contain notable amounts of entire juice sac particles, following the organoleptic characteristics of the fresh fruit (Crupi and Rispoldi, 2002). Consequently, the analytical method involved initial juice standardization by manual finishing via filtration to exclude interference by varying contents

of pulp with adherent PE (Hirsch et al., 2008). Solubilization of tightly bound thermo-stable PE fractions was reported to be always incomplete (Wicker et al., 1988). Enzyme extraction was thus adjusted to the release of the cell wall-bound enzyme under standard conditions (Hirsch et al., 2011) that were chiefly selective for the heat-sensitive PE isoenzymes (Wicker et al., 1988), because the latter were considered crucial for discrimination between FSCJ and chilled NFC (Hirsch et al., 2011). For the analysis of the crude extract based on recorded titration of liberated carboxyl groups (Schols and Voragen, 2003), assay temperature and pH were chosen with respect to reasonable rates of enzymatic pectin deesterification (Wicker et al., 1987), but minimal concurrent alkaline de-esterification and β-eliminative degradation of the substrate (Hirsch et al., 2008). Unlike the well-established titrimetric PE assay for monitoring pasteurization via relative de-activation percentages (Collet et al., 2005), routine evaluation of *Citrus* juice freshness relies on precise and accurate PE activities that are to be quantitated in extracts of standardized juices.

This study aimed at validation of the complete analytical procedure of Hirsch et al. (2008, 2011) for freshness evaluation of *Citrus* juices as regards precision, accuracy, linearity, and sensitivity prior to its application to the analysis of commercial chilled products. In this way, the possibilities to distinguish between various types of chilled *Citrus* juices were to be further substantiated. Concurrently, the limits for freshness authentication were to be identified in order to refine the procedure for routine application.

2. Materials and methods

2.1 Chemicals and reagents

A commercial preparation of pure PE from *Aspergillus niger* (Rapidase® FP 15000) was kindly provided by DSM Food Specialties (Seclin, France). Apple pectin with a degree of esterification (DE) of 77 % (Pektin Classic AU-L) was supplied by Herbstreith & Fox (Neuenbürg, Germany). Polyvinylpolypyrrolidone (PVPP) and saponin were obtained from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and supplied by VWR International (Darmstadt, Germany), including Titrisol® ampoules for volumetric analyses. Ultra-pure water (Milli-Q system, Millipore, Bedford, USA) was used for analytical purposes. To dissolve the pectin for the preparation of the substrate solution for the enzyme assay (cf. 2.4.3), the pectin (0.5 % w/v) and NaCl (0.15 M) were slowly strewed into water (55 ± 3 °C) under stirring in a beaker until complete dispersion of the polysaccharide prior to adjustment of the volume in a volumetric flask (20 °C). The final substrate solution was used after a swelling time of ~16 h in the refrigerator.

2.2 Plant material and juice samples

Fresh orange fruit (*Citrus sinensis* (L.) Osbeck cvs. 'Navelina' and 'Salustiana') of class I from Spain (harvests 2004 and 2005, respectively) was obtained from retailers in Stuttgart, Germany, as raw material for the production of freshly squeezed orange juices on the laboratory scale (cf. 2.3) directly after purchase. The size codes were 6-7 for 'Navelina' fruit and 6 for 'Salustiana'.

Commercial *Citrus* juices, all of which were distributed in a cold chain, were purchased from various retailers in France and Germany in year 2004 as sample material for an application study (cf. 3.4) in order to apply the analytical method for freshness evaluation (cf. 2.4) to chilled *Citrus* juices of unknown production history. Sample collection was at random, based on the availability of respective chilled products in retail markets. As specified on the packages by the producers, three juices had been produced from oranges and one from mandarin fruit and they had been subjected either to (gentle) pasteurization or, in the case of one orange juice, to high-pressure treatment. Storage in the refrigerator or at maximally 8 °C was indicated by the producers in each case. After purchase (approx. 2-4 weeks prior to the specified best-before date), the samples were immediately deep-frozen until analysis of the defrosted product according to 2.4.

2.3 Production of freshly squeezed orange juices and model juices

For the manufacture of freshly squeezed orange juices (FSOJ), fruit (cf. 2.2) was de-juiced in the semi-automatic Orange X-Press extractor (Brimato Maschinenbau, Hilter, Germany), which had previously been used for *Citrus* juice production on the pilot plant scale (Hirsch et al., 2008, 2011). On the small production scale of this study, 'Navelina' FSOJ was obtained by extracting 4.5 kg of fruit of the respective cultivar, 'Salustiana' FSOJ by the use of 21 kg. Since the finisher used in the past (Hirsch et al., 2008, 2011) was inapplicable on this scale, the juices were finished by passing them through a stainless steel strainer of approx. 1 mm mesh size for removal of coarse pulp particles and manual disruption of remaining juice sacs.

Besides an aliquot used for the analysis of FSOJ according to 2.4, further aliquots of the 'Navelina' FSOJ were immediately processed after finishing into a series of freshly squeezed model orange juices (FSMOJ) by dilution for the analysis of products, which clearly differed in their PE activities owing to different contents of original FSOJ. After finishing, 'Salustiana' FSOJ was completely filled into 1 L plastic bottles and deep-frozen at -20 °C until analysis of the FSOJ or preparation of FSMOJ from defrosted juice for different types of validation experiments (cf. 2.5).

For the production of these model juices, an aliquot of the respective FSOJ was subjected to the same standardization procedure based on juice filtration (cf. 2.4.1), which was otherwise applied as sample preparation to those FSOJ aliquots that were directly used for freshness evaluation (cf. 2.4). Proportional differences in pulp contents and PE activity could thus be assumed among the individual samples of each dilution series. For each FSMOJ, the exactly weighed amount of pulp-standardized FSOJ was made up to a total mass of 100 g of model juice with an aqueous solution, which consisted of glucose (28 g/L), fructose (30 g/L), sucrose (33 g/L), citric acid (9.4 g/L), and malic acid (1.7 g/L) and had been adjusted to the pH value of the FSOJ with 5 N potassium hydroxide (Hirsch et al., 2008). FSMOJ from 'Navelina' FSOJ comprised a series of nine model juices with FSOJ contents of 0.1-75 % (w/w). The 'Salustiana' FSMOJ series covered model juices containing the respective original FSOJ at ten levels from 1 to 75 % (w/w). The model juices were directly subjected to the enzyme extraction step (cf. 2.4.2) of the freshness evaluation method.

2.4 Analytical method for evaluating *Citrus* juice freshness

2.4.1 Sample preparation

For standardization of the juice sample to uniform pulp content prior to enzyme extraction, it is shaken up and an aliquot of ~110 g is strained through a screen DIN 4188 (Retsch, Haan, Germany) of 0.5 mm mesh size and 10 mm diameter (Hirsch et al., 2008, 2011). In this study, this procedure was accordingly applied to the FSOJ (cf. 2.3) and the commercial products (cf. 2.2), respectively. For FSMOJ, this step was omitted, because it had already been applied to the respective FSOJ prior to its conversion into FSMOJ by dilution (cf. 2.3).

2.4.2 Enzyme extraction

The enzyme extract is prepared following the standard procedure described by Hirsch et al. (2008, 2011). Accordingly, the exactly weighed mass of ~100 g of pulp-standardized juice sample (cf. 2.4.1) or FSMOJ (cf. 2.3) was added to 3 g of PVPP, 877 mg of NaCl, and 100 mg of saponin. After immediate adjustment to pH 6.0 with 10 N and 1 N NaOH, the mixture was stirred at 4 °C for 120 min and subsequently centrifuged at 25,000×*g* for 30 min at 4 °C in a Suprafuge 22 with a rotor of the type 14290 (Heraeus Sepatech, Osterode, Germany). The centrifugate was decanted through a folded filter (Schleicher & Schuell no. 597 ½, Dassel, Germany) at 4 °C into a volumetric flask (200 mL). The residue was washed twice with ultra-pure water (4 °C) and was subsequently discarded. The combined volume of centrifugate and washing water was made up to 200 mL with ultra-pure water (4 °C). This extract was divided into portions of 20 mL, filled into plastic vials, and stored at -80 °C until analysis of PE activity (cf. 2.4.3).

As concluded from a preliminary comparison of this one-time extraction method according to Hirsch et al. (2008, 2011) with a procedure involving repeated extraction, the former was clearly acceptable for the analytical method of freshness evaluation. In the other procedure, extraction was repeated by resuspension of the aforementioned residue in 20 mL of ultra-pure water (4 °C), centrifugation (25,000×*g*, 30 min, 4 °C), resuspension of the second residue in 20 mL of ultra-pure water (4 °C), further centrifugation (25,000×*g*, 30 min, 4 °C), and pooling of all three centrifugates and the washing water in the volumetric flask (200 mL). PE activities after one-time extraction and this repeated extraction procedure, respectively, were insignificantly different (data not shown).

2.4.3 Pectin methylesterase assay

PE activity (A_{PE}), expressed in units g^{-1} of pulp-standardized juice sample (cf. 2.4.1) according to **Eq. 1** as the liberation rate of carboxyl groups during enzymatic pectin de-esterification, is quantitated as detailed by Hirsch et al. (2008, 2011). Hence, titrimetric analysis was carried out in a thermostated cell at 30 °C and pH 7.0 for 30 min with 0.01 N NaOH using an automatic titrator Titrino 718 STAT (Metrohm, Herisau, Switzerland). The 0.5 % (w/v) solution of apple pectin (DE 77 %), containing 0.15 M NaCl, served as the substrate. After adjusting 59 mL of substrate solution to pH 7.0, 1 mL of enzyme extract (cf. 2.4.2) was added. The pH value was set to 7.0 again, and the titration was performed at constant pH within 30 min. Another aliquot of the enzyme extract was boiled for 3 min and analyzed by analogous titration with 0.01 N HCl (blank). As calculated from the NaOH equivalents required for pH retention (**Eq. 1**), A_{PE} (units g^{-1} of sample, i.e., juice or FSMOJ) resulted from the volumes of NaOH and HCl used to titrate the extracts of sample (V_{NaOH}, mL) and blank ($V_{HCl,B}$, mL), 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 extract volumes of sample ($V_{Extract}$, mL) and blank (V_{Blank}, mL), and the factor of enzyme extract volume per sample mass (nominal: F = 200 mL/100 g of sample). One unit of PE activity denoted a liberation rate of carboxyl groups of 1 µmol min^{-1} (i.e., 60 µkat). Each extract was analyzed in triplicate, if not otherwise stated.

$$A_{PE} = \left(\frac{V_{NaOH} \cdot c_{NaOH}}{\Delta t \cdot V_{Extract}} + \frac{V_{HCl,B} \cdot c_{HCL}}{\Delta t \cdot V_{Blank}} \right) \cdot F \cdot 60{,}000 \qquad (1)$$

Unless otherwise declared, the standard assay with an extract volume of 1 mL was used. However, when no NaOH consumption was induced because of too low PE activity, titration of sample and blank was additionally performed with 5 mL of active and boiled extract, respectively, while the substrate volume was reduced to 55 mL for a constant total volume of 60 mL.

2.5 Methods used for validating the procedure of freshness evaluation

2.5.1 Evaluation of repeatability

Intra-assay precision and time-dependent intermediate precision (Green, 1996) of the analytical method described in 2.4 were explored by analyzing defrosted 'Salustiana' FSOJ 2-3 times per day by the same person and with the same equipment on 3 different days within a period of 4 months. Each of the 2-3 enzyme extracts (cf. 2.4.2) obtained per day was titrated 3-4 times according to 2.4.3 for evaluating the precision of the titration step in comparison with that of the whole method.

Intra-assay precision of the analytical method detailed in 2.4 (i.e., precision under repeatability conditions; Thompson et al., 2002) was concluded from the standard deviations (SD) and the relative standard deviations (RSD) observed on each day i ($SD_{r,i}$, $RSD_{r,i}$, n = 2-3). Time-dependent intermediate precision (i.e., precision under day-to-day conditions) was evaluated based on the SD and RSD of the mean calculated from the average results of 3 different days (SD_{day}, RSD_{day}, n = 3). Total precision was expressed by SD_{run} and RSD_{run} of all individual runs that were performed on these three days (n = 7) (ICH, 1996; Thompson et al., 2002).

2.5.2 Evaluation of reproducibility

For standardization of an analytical method that is designated for official application, reproducibility is usually deduced from the application of this method to aliquots of homogeneous samples by different analysts in multiple laboratories (ICH, 1996). This concurrently provides information on the ruggedness of the method (Thompson et al., 2002). Since an inter-laboratory trial was beyond the scope of this study, reproducibility was estimated as the analyst-dependent intermediate precision. For this purpose, defrosted 'Salustiana' FSOJ and a FSMOJ prepared by dilution of the former to a juice content of 60 % (w/w) (cf. 2.3) (in this experiment referred to as FSMOJ 1 and FSMOJ 2, respectively) were analyzed by three analysts (P_A, P_B, P_C) on different days in the same laboratory. Each analyst investigated both samples in triplicate, following the analysis specification as detailed in 2.4. However, since sample preparation (juice standardization; cf. 2.4.1) had to be applied to the FSOJ before its conversion into FSMOJ (cf. 2.3), each analyst began the analysis of FSMOJ 2 by standardizing the respective FSOJ aliquot prior to dilution of the pulp-standardized juice to 60 % (w/w) and subsequent enzyme extraction. The activity resulting from each individual FSMOJ 2 run was thus corrected to a juice content of exactly 60 % (w/w) by multiplying the reading by the quotient of nominal to real FSOJ content before any averaging calculation for FSMOJ 2.

In addition to the intra-assay precision of each analyst ($SD_{r,p}$, $RSD_{r,p}$, n = 3 per analyst and sample), reproducibility was expressed by SD_R and RSD_R of the average of the three analyst-specific mean PE activities. Moreover, the grand mean characterized by $SD_{R,run}$ and $RSD_{R,run}$ was computed from the total of 9 complete runs for each sample, regardless of the analyst. Additionally, the relative deviation of the means determined by analyst P_B and P_C, respectively, from the mean obtained by analyst P_A was calculated for each sample (Green, 1996).

2.5.3 Evaluation of recovery

Accuracy of the analytical method specified in 2.4 was assessed by two different approaches. The first one was based on the comparison of the measured value with the true value, the other one involved standard addition at different levels to the sample material (Green, 1996).

For the first purpose, the data set produced for evaluating reproducibility (cf. 2.5.2) was explored accordingly. The grand mean obtained for the PE activity of the FSMOJ with a juice content of 60 % (w/w) (FSMOJ 2), representing the measured value, was compared with the value predicted as the A_{PE} mean of the corresponding FSOJ (FSMOJ 1), multiplied by the juice dilution factor of FSMOJ 2. Recovery was calculated as the percentage of found A_{PE} relative to the predicted value.

The enzyme preparation Rapidase® FP 15000 (microbial PE) served as the standard for the second approach. As specified by the producer based on titration at pH 4.5 and 30 °C with citrus pectin (0.5 %) as the substrate, its PE activity was ≥ 5000 units g^{-1}. Its activity at pH 7.0 was assessed according to 2.4.3 by examination of six enzyme solutions obtained by diluting Rapidase® FP 15000 with ultra-pure water in the range of 1:20-1:60 (2-4 titrimetric runs per enzyme solution). Respective mean PE activity of Rapidase® FP 15000 was 55.6 ± 2.7 units g^{-1} of commercial enzyme preparation with a 95 % confidence interval of ± 5.7 units g^{-1}. The latter activity was used for the calculation of the spiking doses in terms of added A_{PE}. FSMOJ prepared from defrosted 'Salustiana' juice by dilution to three different juice contents (30, 60, and 75 % w/w; cf. 2.3) served as different types of authentic test material and were analyzed as described in 2.4, both before and after addition of known doses of Rapidase® FP 15000. Each FSMOJ was used for standard addition at 1-2 different concentrations to assess the recovery as the percentage of found A_{PE} relative to the predicted PE activity of the spiked FSMOJ for five different samples. The exact mass of ~100 g of FSMOJ was spiked by adding a defined volume of the enzyme standard (either without or after aqueous dilution of Rapidase® FP 15000) by weight. The predicted activity of the spiked FSMOJ was calculated as the sum of the activity, which was added via the standard, and the activity, which was found for the unspiked FSMOJ after correcting the latter by the ratio of the juice contents of spiked and unspiked sample. This correction factor was necessary, because standard addition slightly lowered the juice content of the spiked FSMOJ. To FSMOJ 3 (juice content 60 % w/w), the exact mass of 1 mL of pre-diluted enzyme solution containing 2.5 and 1.67 % (v/v) of Rapidase® FP 15000, respectively, was added. For FSMOJ 4 (juice content 75 % w/w), the mass of 0.25 mL of Rapidase® FP 15000 was used without previous dilution. FSMOJ 5 (juice content 30 % w/w) was spiked by weight with 0.5 and 1 mL of undiluted Rapidase® FP 15000, respectively. From each FSMOJ, one enzyme extract was prepared (cf. 2.4.2) before and after spiking, respectively. The titrimetric assay (cf. 2.4.3) was carried out 3-4 times per enzyme extract.

2.5.4 Evaluation of linearity and the limits of detection, identification, and quantification

To verify the linearity of the analytical method specified in 2.4, authentic sample material differing in known PE activity over a wide range was provided by the FSMOJ series produced from 'Navelina' and 'Salustiana' FSOJ by dilution to juice contents in the range of 0.1-75 % (w/w) (cf. 2.3). At the top, the range was thus naturally limited by the maximum PE activity observed for FSOJ. Linearity was verified by plotting the predicted value (x) versus the measured one (y) for each FSMOJ, irrespective of the cultivar. Predicted A_{PE} was computed as the product of the mean PE activity of the FSOJ, which had been diluted to produce this FSMOJ, and the respective juice dilution factor. From each FSMOJ, one enzyme extract was obtained (cf. 2.4.1-2.4.2) and subjected to the titrimetric enzyme assay (cf. 2.4.3) 3 times, sporadically up to 6 times (m = 1 complete analysis according to 2.4 per FSMOJ).

Concurrently, information regarding the limits of detection (LOD), identification (LOI), and quantification (LOQ) was deduced according to DIN 32645 (1994) from the cultivar-independent calibration line (ICH, 1996) that was generated from the combined data sets of the 'Navelina' and 'Salustiana' FSMOJ series for the linearity check. The critical minimum A_{PE} level that was unequivocally measurable (y_c) under the conditions specified for the method in 2.4 was calculated as the sum of the ordinate intercept (a) of the calibration line and the width of its one-sided prediction interval (Δa), with the latter originating from the residual standard deviation of the measured values used for calibration ($SD_{y,x}$), the quantile of the Student's t-distribution (P = 0.05) for $f = n$-2 degrees of freedom ($t_{0.05,n-2}$), the mean ($\bar{x}$) and the deviance (Q_x) of all specified values (x) of the calibration line, the number of calibration measurements (n), and the number of analyses per sample (m = 1) (DIN 32645, 1994). The LOD, being the lowest analyte concentration producing a detectable response above the noise level in analytical chemistry (Green, 1996), consequently resulted as the A_{PE} level expected (x_{LOD}) for y_c from the linear calibration function ($y = a + bx$) with the slope b. In this context, the LOD represented the decision limit for the presence of PE activity ($P \leq 0.05$) (DIN 32645, 1994). The LOI, defined as the minimum analyte concentration that can be detected with a specified probability, was calculated by multiplication of the LOD by the factor 2 for a probability of 95 %, assuming the same rates of type I and II errors ($\alpha = \beta = 0.05$) (DIN 32645, 1994). The LOQ, being the lowest analyte level that can be measured accurately and precisely (Green, 1996), was estimated on the basis of P = 0.05 and the standard deviation of the procedure ($SD_{xo} = SD_{y,x}/b$) for a relative uncertainty of 33.3 % (k = 3) by solving **Eq. 2** (DIN 32645, 1994) iteratively. The LOQ was thus the specified PE activity (x_{LOQ}), when the relative uncertainty, defined as the quotient of the half two-sided prediction interval and x_{LOQ}, equaled k^{-1} = 1/3. Iteration of **Eq. 2** was started by using $(k \cdot LOD - \bar{x})^2$ as approximation of $(LOQ - \bar{x})^2$ (DIN 32645, 1994).

$$LOQ = k \cdot SD_{xo} \cdot t_{0.05,n-2} \sqrt{m^{-1} + n^{-1} + (LOQ - \bar{x})^2 / Q_x} \qquad (2)$$

2.6 Statistical analyses

Besides SD, RSD, and the standard error (SE), the half two-sided confidence intervals (CI) of the quantitated A_{PE} means were calculated based on the Student's t-distribution (P = 0.05) with estimation of the quantiles $t_{0.05;n-1}$ via the TINV function of Microsoft Office Excel 2003. If indicated by deviations between arithmetic and trimmed means, the arithmetic means were checked for outliers by means of Dean-Dixon tests and Grubbs tests. Significant differences (P = 0.05) between variants were identified by multiple pairwise comparisons of means (Duncan tests) with the GLM procedure of SAS 9.1 (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1 Precision of the analytical procedure under study

The same analyst determined the PE activity of the pulp-standardized 'Salustiana' FSOJ after previous enzyme extraction on 3 individual days (**Tab. 1**). The relative standard deviations of the daily means of 2-3 runs only amounted to 3.1-4.3 % ($RSD_{r,i}$) on two days, but greater dispersion (13.0 %) occurred as well. Intra-assay precision of the whole three-step method specified in 2.4 was thus ≤ 13 %. By contrast, the RSD of the titrimetric enzyme assays (2.4.3) performed for each enzyme extract only varied between 0.9 and 7.5 % around a median of 2.9 % among all 7 enzyme extracts of this juice sample (**Tab. 1**). Greater dispersion of the whole analytical procedure of 2.4 on analysis day 2 thus indicated that the performance of sample

Tab. 1: Repeatability of the method for pectin methylesterase activity quantification after enzyme extraction from pulp-standardized juice: PE activity (A_{PE}) of freshly squeezed 'Salustiana' orange juice, which was immediately frozen after juice production and defrosted for analysis on different days.

Enzyme extraction no.	A_{PE} [units g^{-1} of juice]						
	Mean	SD	RSD [%]	SE	n_{titr} [ ]	n_{total} [ ]	CI (P=0.05)
Analysis day 1 after 6 days of frozen juice storage							
Run 1	1.28 c	0.01	0.9	0.007	3		0.028
Run 2	1.23 c	0.01	1.2	0.008	3		0.036
Subsample $mean_{r,i}$ (i = day 1)	1.25	0.04	3.1	0.027		2	0.345
Analysis day 2 after 35 days of frozen juice storage							
Run 3	1.23 c	0.03	2.2	0.016	3		0.068
Run 4	1.02 d	0.08	7.5	0.038	4		0.122
Subsample $mean_{r,i}$ (i = day 2)	1.13	0.15	13.0	0.104		2	1.317
Analysis day 3 after 128 days of frozen juice storage							
Run 5	1.41 b	0.09	6.1	0.050	3		0.215
Run 6	1.48 ab	0.05	3.2	0.027	3		0.117
Run 7	1.54 a	0.04	2.6	0.023	3		0.098
Subsample $mean_{r,i}$ (i = day 3)	1.48	0.06	4.3	0.036		3	0.157
Analyses of days 1-3							
Grand $mean_{run}$	1.31	0.18	13.5	0.067		7	0.164

Mean values with different letters (a-d) are significantly different ($P \leq 0.05$). SD, standard deviation; RSD, relative standard deviation; SE standard error; n_{titr}, number of individual titrations performed per enzyme extract; n_{total}, number of complete runs consisting of enzyme extraction from the juice and subsequent titration (i.e., number of extractions); CI, confidence interval ($t_{0.05;n-1} \cdot SD/\sqrt{n}$).

standardization regarding the pulp content (cf. 2.4.1) and enzyme extraction (cf. 2.4.2) could sporadically be suboptimal.

Analysis of the 'Salustiana' FSOJ after frozen storage for 128 days resulted in significantly increased PE activity (1.48 units g^{-1}; **Tab. 1**). It was 1.2-1.3 times higher than the daily mean activities found by the same analyst after shorter periods up to ~1 month (1.25 and 1.13 units g^{-1}). The average of the mean activities detected on 3 individual days was 1.29 ± 0.10 units g^{-1} (RSD_{day} = 13.8 %). Its 95 % confidence interval (1.3 ± 0.44 units g^{-1}) reflected the greater variation on analysis day 2 and the significantly higher PE activity at the end. The time-dependent intermediate precision of 13.8 % (RSD_{day}) though came quite close to the intra-assay precision (maximum $RSD_{r,i}$). The grand mean of all 7 runs performed by the same analyst regardless of the day of analysis implied a total precision of 13.5 % (RSD_{run}) for the method and a PE activity of 1.31 ± 0.07 units g^{-1} for the 'Salustiana' FSOJ (**Tab. 1**). Minimum and maximum activities suggested by runs 4 and 7, respectively, were beyond its 95 % confidence interval (1.3 ± 0.16 units g^{-1}).

Analysis of the same sample in triplicate by each of 3 people on different days (FSMOJ 1 in **Tab. 2**) overall confirmed the PE activity of 'Salustiana' FSOJ presented in **Tab. 1**. Identical results were obtained by the analysts P_A and P_C with individual intra-assay precision of 4.3-4.9 % ($RSD_{r,p}$). The PE activity found by the analyst P_B (1.27 ± 0.004 units g^{-1}) with an $RSD_{r,p}$ of 0.6 % was significantly lower (**Tab. 2**), but consistent with the values reported in **Tab. 1** for short frozen storage. The results of the analysts P_C and P_B deviated from that achieved by analyst P_A by 1.5 % (insignificant) and 14.1 %, respectively. To mimic an inter-laboratory trial for best possible estimation of reproducibility, reagents were prepared by each analyst individually. The average of the mean activities that were found by the 3 analysts was 1.40 ± 0.07 units g^{-1} with a 95 % confidence interval of ± 0.29 units g^{-1}, and the reproducibility of the method was hence 8.2 % (RSD_R). The grand mean of all 9 runs irrespective of the analyst ranged at 1.40 ± 0.04 units g^{-1} with a 95 % CI of ± 0.085 units g^{-1} (**Tab. 2**), thus suggesting a total precision of the method of 7.9 % ($RSD_{R,run}$) for the involvement of different analysts. PE activity quantification after enzyme extraction from pulp-standardized FSOJ was thus reproducible and overall robust. Investigation of the defrosted sample by various analysts (**Tab. 2**) or at different times (**Tab. 1**) did not further increase the dispersion that had to be expected from intra-assay precision in the worst case.

Examination of a sample, which ranged at a lower PE activity level because of adjustment to 60 % juice content during FSMOJ production, even provided more uniform results among the 3 analysts (FSMOJ 2 in **Tab. 2**). However, one run of analyst P_B was probably an outlier (**Tab. 2**). The variance of the titrimetric assays performed for the enzyme extract of this run was extreme (RSD = 21.4 %). Without consideration of this outlier, analyst-specific intra-assay precision ($RSD_{r,p}$ = 5.3-8.4 %) was slightly inferior to that usually observed at the higher activity levels of FSOJ, but $RSD_{r,p}$ though remained clearly below the maximum $RSD_{r,i}$ listed in **Tab. 1** for the intra-assay precision. The average calculated from the respective mean results of three analysts was 0.87 ± 0.02 units g^{-1} with a 95 % CI of ± 0.085 units g^{-1}. Its RSD_R (3.9 %) even indicated enhanced reproducibility at the lower activity level. Accordingly, the average results of the analysts P_B and P_C deviated from that of P_A only by 4.7 and 7.4 %, respectively. These deviations were comparable

Tab. 2: Reproducibility of the method for pectin methylesterase activity quantification after enzyme extraction from pulp-standardized juice: PE activity (A_{PE}) of freshly squeezed model orange juices (FSMOJ)[a] as determined by three different analysts (P_A, P_B, P_C).

Analyst	Enzyme extraction no.	A_{PE} [units g^{-1} of model juice]						
		Mean	SD	RSD [%]	SE	n_{titr} [ ]	n_{total} [ ]	CI (P=0.05)
FSMOJ 1								
Analyst P_A	Run P_A 1	1.41 bc	0.09	6.1	0.050	3		0.215
	Run P_A 2	1.48 ab	0.05	3.2	0.027	3		0.117
	Run P_A 3	1.54 a	0.04	2.6	0.023	3		0.098
Subsample $mean_{r,p}$ P_A		1.48	0.06	4.3	0.036		3	0.157
Analyst P_B	Run P_B 1	1.26 d	0.02	1.9	0.014	3		0.060
	Run P_B 2	1.26 d	0.04	3.4	0.021	4		0.067
	Run P_B 3	1.28 d	0.08	6.0	0.044	3		0.191
Subsample $mean_{r,p}$ P_B		1.27	0.007	0.6	0.004		3	0.018
Analyst P_C	Run P_C 1	1.49 ab	0.06	3.8	0.033	3		0.142
	Run P_C 2	1.50 ab	0.007	0.5	0.005	2		0.061
	Run P_C 3	1.37 c	0.01	1.0	0.010	2		0.128
Subsample $mean_{r,p}$ P_C		1.46	0.07	4.9	0.041		3	0.177
Grand $mean_{R,run}$ FSMOJ 1		1.40	0.11	7.9	0.037		9	0.085
FSMOJ 2								
Analyst P_A	Run P_A 1	0.97 A	0.02	1.6	0.009	3		0.038
	Run P_A 2	0.88 ABC	0.04	4.4	0.022	3		0.096
	Run P_A 3	0.88 ABC	0.05	6.0	0.026	4		0.084
Subsample $mean_{r,p}$ P_A		0.91	0.05	5.3	0.028		3	0.119
Analyst P_B	Run P_B 1	0.82 BC	0.02	2.1	0.010	3		0.042
	Run P_B 2	0.57[b] D	0.12	21.4	0.055	5		0.152
	Run P_B 3	0.92 AB	0.01	1.6	0.008	3		0.036
Subsample $mean_{r,p}$ P_B		0.77[c] (0.87)[d]	0.18[c] (0.07)[d]	23.4[c] (8.4)[d]	0.104[c] (0.05)[d]		3[c] (2)[d]	0.446[c] (0.66)[d]
Analyst P_C	Run P_C 1	0.91 AB	0.02	2.5	0.016	2		0.200
	Run P_C 2	0.84 BC	0.04	4.8	0.023	3		0.100
	Run P_C 3	0.78 C	0.0004	0.05	0.0003	2		0.004
Subsample $mean_{r,p}$ P_C		0.84	0.06	7.6	0.037		3	0.159
Grand $mean_{R,run}$ FSMOJ 2		0.84[c] (0.87)[d]	0.12[c] (0.06)[d]	13.8[c] (6.9)[d]	0.039[c] (0.02)[d]		9[c] (8)[d]	0.089[c] (0.05)[d]

Mean values with different letters (a-d and A-D, respectively) are significantly different ($P \leq 0.05$). SD, standard deviation; RSD, relative standard deviation; SE standard error; n_{titr}, number of individual titrations performed per enzyme extract; n_{total}, number of complete runs consisting of enzyme extraction from the juice and subsequent titration (i.e., number of extractions); CI, confidence interval ($t_{0.05;n-1} \cdot SD/\sqrt{n}$). [a] 'Salustiana' FSOJ was immediately frozen after juice production and defrosted for analysis by each analyst on the individual day, either without (FSMOJ 1) or after (FSMOJ 2) previous dilution of defrosted FSOJ to a juice content of 60 % (w/w). [b] Outlier according to the Dean-Dixon test ($P \geq 0.02$) and Grubbs test ($P \geq 0.05$). [c] Calculated irrespective of potential outliers. [d] Calculated after removal of the probable outlier.

to the dispersion indicated by the range of the analyst-specific $RSD_{r,p}$. Consequently, the grand mean of all 8 runs, irrespective of the analysts, was justified and amounted to 0.87 ± 0.04 units g^{-1} ($RSD_{R,run}$ = 6.9 %), with the 95 % CI being ± 0.05 units g^{-1} (**Tab. 2**). However, even if the debatable run of analyst P_B was no outlier (n = 9), $RSD_{R,run}$ would be 13.8 % (**Tab. 2**), thus meeting the worst intra-assay precision found for FSOJ (**Tab. 1**).

3.2 Accuracy of the analytical procedure under study

Since FSMOJ 2 in **Tab. 2** was prepared from 'Salustiana' FSOJ (FSMOJ 1 in **Tab. 2**) by dilution to a juice content of 60 % (w/w), the PE activity of the former could be predicted by multiplication of the mean PE activity of the latter (1.40 ± 0.04 units g^{-1}; **Tab. 2**) by the juice dilution factor (0.6). The PE activity expected for FSMOJ 2 (0.84 units g^{-1}) was compared with the measured value of this sample (0.87 ± 0.02 units g^{-1}; n = 8; **Tab. 2**). A recovery percentage of 104.1 % was derived from the ratio of measured to predicted activity. Its standard error of ± 3.7 % resulted from error propagation of the standard errors, which were reported in **Tab. 2** for the A_{PE} grand means of FSMOJ 1 ('Salustiana' FSOJ) and FSMOJ 2 and consequently specified the errors of the predicted and the measured value (± 0.04 and 0.02 units g^{-1}, respectively). High accuracy of the analytical method was thus indicated.

FSMOJ 3-5, which were used for the standard addition experiment (**Tab. 3**), were also produced from 'Salustiana' FSOJ. Analysis of a single extract per sample by means of 3-4 titrimetric assays led to the measured PE activities reported in **Tab. 3** for these FSMOJ before spiking with the commercial enzyme preparation. When those activities were likewise related to the A_{PE} grand mean of 'Salustiana' FSOJ (1.40 ± 0.04 units g^{-1}; FSMOJ 1 in **Tab. 2**), high accuracy was confirmed by the recovery of 100.8 ± 3.9 % that was estimated for unspiked FSMOJ 4 (75 % juice content). After standard addition to FSMOJ 4, comparison of measured and predicted PE activity of the spiked sample equally showed

Tab. 3: Recovery of pectin methylesterase activity (A_{PE}) after addition of a respective enzyme preparation (diluted or undiluted Rapidase FP 15000) to freshly squeezed model orange juices (FSMOJ)[a] prior to enzyme extraction.

Model juice sample	A_{PE} [units g^{-1} of model juice]					Recovery[b] [%]
	added	predicted	measured[c]	RSD [%]	CI (P=0.05)	
FSMOJ 3	0	---	0.61 ± 0.013 e	4.4	0.04	---
	0.010	0.613	0.68 ± 0.015 d	3.9	0.07	111.2 ± 3.5
	0.015	0.619	0.75 ± 0.023 cd	5.4	0.10	120.4 ± 4.6
FSMOJ 4	0	---	1.06 ± 0.030 b	4.8	0.13	---
	0.150	1.22	1.30 ± 0.043 a	6.6	0.14	106.0 ± 4.4
FSMOJ 5	0	---	0.34 ± 0.007 f	3.6	0.03	---
	0.307	0.64	0.59 ± 0.007 e	2.1	0.03	92.1 ± 2.6
	0.650	0.99	0.82 ± 0.006 c	1.3	0.03	82.4 ± 2.7

Mean values with different letters (a-f) are significantly different ($P \leq 0.05$). RSD, relative standard deviation of the measured A_{PE} value; CI, confidence interval of the measured A_{PE} value ($t_{0.05;n-1} \cdot SD/\sqrt{n}$). [a] 'Salustiana' FSOJ was immediately frozen after juice production and defrosted for analysis after previous dilution of defrosted FSOJ to a juice content of 60 % (w/w) (FSMOJ 3), 75 % (w/w) (FSMOJ 4), or 30 % (w/w) (FSMOJ 5) and addition of differently diluted Rapidase FP 15000. [b] Recovery calculated as the ratio of measured to predicted A_{PE} of the spiked FSMOJ (mean ± standard error based on error propagation). [c] Mean ± standard error of 3-4 titrimetric assays per extract.

good recovery (106 ± 4.4 %; **Tab. 3**). Accuracy of the analytical method was thus also proven by adding microbial PE to model juice as the second approach (GREEN, 1996) applied, although the additional analytical error of the PE activity detected at pH 7.0 for the commercial enzyme preparation (cf. 2.5.3) further raised the variance of the recovery percentage.

Since the doses of microbial PE added to FSMOJ 3, which was the first model juice batch prepared for the standard addition experiment, turned out to be in the range of the standard errors of the PE activities detected before and after PE admixture (**Tab. 3**), spiking of FSMOJ 3 was not expected to cause demonstrable changes. The PE activities of the spiked FSMOJ 3 batches though significantly exceeded that of the unspiked model juice. Apparently excessive recovery percentages of 111 ± 3.5 % and 120 ± 4.6 % after standard addition (**Tab. 3**) were predominantly ascribed to a too low result for the PE activity of the unspiked FSMOJ 3 batch (0.61 units g^{-1}). The latter was 30.6 % below the A_{PE} level that was uniformly confirmed by different analysts for FSMOJ 2 (0.87 ± 0.02 units g^{-1}; **Tab. 2**), although both FSMOJ batches contained 60 % juice. A possible outlier ranging in the same order as A_{PE} of unspiked FSMOJ 3 was among the results of another analyst for FSMOJ with this juice content (**Tab. 2**). Hence, the findings for FSMOJ 3 in **Tab. 3** pointed out to a weakness of the analytical method that rather concerned its robustness than limited accuracy.

Two notable doses of microbial PE added to FSMOJ 5 with a juice content of only 30 % (w/w) were interrelated by a ratio of 1 : 2.1. Despite significant increments in A_{PE} relative to the unspiked sample, the PE activities of both spiked juices were only related to each other by a ratio of 1 : 1.4. Consistently, merely 92 ± 2.6 and 82 ± 2.7 % of the A_{PE} predicted after spiking, respectively, were found (**Tab. 3**). For FSMOJ 5 without admixture of microbial PE, detection of the A_{PE} level, which was expected from the content and the PE activity of the parent FSOJ of FSMOJ 5, was limited to 80 ± 2.7 %. Hence, the risk of underestimation seemed to be enhanced, when only ~30 % of the PE activity of FSOJ could be expected (FSMOJ 5).

Accuracy was though overall acceptable (recovery ≥ 80 %) and, at least for the activity range of FSOJ (HIRSCH et al., 2011), even good (~100 % recovery). Samples analyzed with high accuracy among those listed in **Tab. 2** and **3** (FSMOJ 2, 4, and 1) displayed PE activities of 0.87, 1.06, and 1.40 units g^{-1} of model juice. This equated to activity levels of ~0.0073, 0.0088, and 0.0117 units, respectively, in the test system of the titrimetric assay. Activity analyses for standard solutions of commercial orange peel PE by means of a spectrophotometric assay, which was adapted to a kinetic microplate reader for quantitating the liberation rate of free carboxyl groups, confirmed high accuracy (100 % recovery) for the same range (0.009-0.015 units; CAMERON et al., 1992). However, for a sample with 0.006 units, these authors only reported ~50 % recovery. A respective FSMOJ analyzed with the method of 2.4 would have a PE activity of 0.72 units g^{-1}, corresponding to ~51 % of the activity of FSMOJ 1 ('Salustiana' FSOJ; **Tab. 2**). Even for FSMOJ 5 with a juice content of only 30 %, recovery was ≥ 80 % (**Tab. 3**). Hence, accuracy of the titrimetric assay according to 2.4.3 was superior in the lower activity range. Moreover, high accuracy in the upper range was obviously not affected by the procedures of juice standardization and enzyme extraction, being part of the quantitation method detailed in 2.4.

3.3 Range, linearity, and limits of the analytical procedure under study

The range of the analytical method was estimated by comparing the measured PE activities of FSMOJ (y) with their expected levels (x) for a series of 10 'Navelina' and 12 'Salustiana' samples that contained 0.51-100 and 1.02-100 % of the respective FSOJ (**Fig. 1**). After different times of frozen storage, two 'Salustiana' FSOJ batches were defrosted for the determination of their PE activity and the use as parent juices for FSMOJ production for this experiment. Since their mean PE activities significantly differed from each other (**Tab. 1**), the batch-specific mean (1.25 ± 0.03 and 1.48 ± 0.04 units g^{-1}, respectively) served as the reference value for predicting the A_{PE} levels of model juice batches that were prepared from the respective parent FSOJ batch. However, PE activity of the 'Navelina' FSOJ batch (1.94 ± 0.05 units g^{-1}) notably exceeded the A_{PE} records obtained for the 'Salustiana' FSOJ at different times (**Tab. 1**) and by different analysts (**Tab. 2**) and thus represented the experimental upper limit of the range (**Fig. 1**). The PE activity of a FSMOJ with 29.86 % (w/w) of juice (expected A_{PE}: 0.44 ± 0.01 units g^{-1}) marked the bottom of the linear range (white rhombs in **Fig. 1**). A slope closely approximating 1.0 suggested that the measured values confirmed the predicted ones rather accurately,

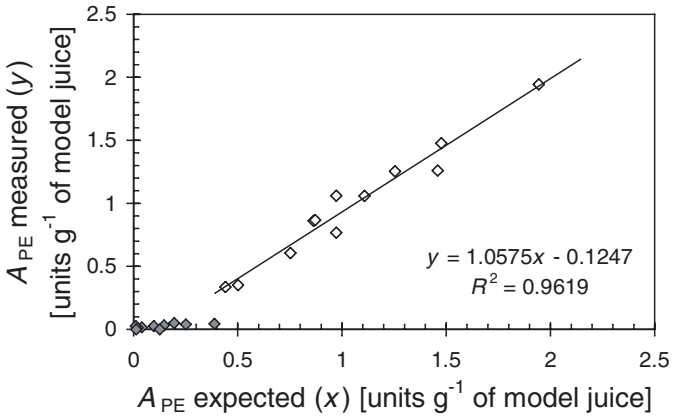

Fig. 1: Range and linearity of the procedure validated for pectin methylesterase activity quantification after enzyme extraction from standardized model juices: PE activity (A_{PE}; rhombs) of 22 freshly squeezed model orange juices (FSMOJ), which comprised 'Navelina' and 'Salustiana' FSOJ and various dilutions thereof with juice contents of 0.5-75.1 % w/w. The expected A_{PE} levels of the model juices (x) were calculated as the mean A_{PE} of the respective FSOJ, multiplied by the juice fraction of the model juice prepared thereof by dilution. Open rhombs represent values included in the linear regression model (n = 12 model juices with juice contents of 30-100 % w/w).

although the coefficient of determination (R^2 = 0.962) indicated deviations of individual records. Accordingly, the residual standard deviation of the measured values ($SD_{y,x}$) was 0.0949 units g^{-1}. As expected from the A_{PE} result of the unspiked FSMOJ 5 (**Tab. 3**; cf. 3.2), the negative ordinate intercept (a = -0.1247 units g^{-1}) confirmed slight underestimation of PE activity near the bottom of the range also for other model juices.

Irrespective of juice content and cultivar, FSMOJ with expected PE activities ≤ 0.39 ± 0.01 units g^{-1} due to juice contents ≤ 20 % (w/w) could not be distinguished analytically (gray rhombs in **Fig. 1**). For those samples, the enzyme assay was even performed with an extract volume of 5 mL, whereas the standard assay with 1 mL was applicable to all samples in the linear range (cf. 2.4.3). Hence, the selectivity of the method was abruptly lost, when the expected PE activity (x) declined from 0.44 to 0.39 units g^{-1} of model juice. This corresponded to a drop from 0.0037 to 0.0032 units in the test system of the standard titrimetric assay. The expected PE activity at the experimental upper limit of the linear range caused 0.0162 units in this test system, i.e. the 4.4-fold value of the expected level at the bottom of the linear range. However, if even the 5-fold extract volume was insufficient for proper analysis of both FSMOJ samples with 20 % (w/w) of 'Navelina' and 'Salustiana' FSOJ, respectively, the crucial factor limiting sensitivity was not the enzyme assay (cf. 2.4.3), but obviously the enzyme extraction (cf. 2.4.2) from the diluted sample.

The regression line of **Fig. 1** was analyzed according to the calibration line method of DIN 32645 (1994) in order to specify the sensitivity of the analytical procedure of 2.4. Presence of PE activity was indicated by a record of at least 0.095 units g^{-1} of model juice (y_c), being the critical minimum A_{PE} value that could unambiguously be measured under the conditions applied (P = 0.05). The minimum activity (x_{LOD}) producing this lowest detectable response (y_c) was an expected A_{PE} level of 0.207 units g^{-1} of model juice, which was thus defined as the limit of detection (LOD). It equated to 0.00173 units in the titrimetric standard test system with 1 mL of enzyme extract (cf. 2.4.3).

As deduced from the LOD, the expected PE activity that represented the minimum level for A_{PE} detection with 95 % probability (x_{LOI}) accounted for 0.415 units g^{-1} of model juice (limit of identification, LOI). Such a sample would yield 0.00346 units in the test system during titration. The LOI ranged between the predicted A_{PE} levels of the two FSMOJ batches, which marked the zone of abrupt selectivity loss in **Fig. 1**. The value recorded for a sample with an activity equal to the LOI would be 0.314 units g^{-1} of model juice (y_{LOI}) according to the regression equation. The recovery at the LOI would thus be 76 %, similar to the recovery found for the unspiked FSMOJ 5 of **Tab. 3** with an expected PE activity coming close to the LOI (cf. 3.2).

As iteratively estimated from **Eq. 2** based on a standard deviation of the procedure (SD_{xo}) of 0.0897 units g^{-1} of FSMOJ, precise and accurate A_{PE} detection with a relative uncertainty ≤ 33.3 % was assumed for model juices with expected PE activities (x_{LOQ}) ≥ 0.537 units g^{-1} of FSMOJ (≥ 0.00448 units in the titrimetric test system). This was considered as the limit of quantification (LOQ). For a sample with a PE activity ranging at the LOQ, the regression equation of **Fig. 1** suggested a measured value of 0.444 units g^{-1} (y_{LOQ}) and thus a recovery of 82.5 %.

If all titrimetric records within the linear range were treated as individual runs, a regression line based on 52 values could be computed, with the slope (1.0546) and the ordinate intercept (-0.1222 units g^{-1}) deviating somewhat less from 1.0 and 0, respectively. Whereas the regression based on mean activities (**Fig. 1**) reflected linearity and sensitivity of the whole analytical method, this approach rather addressed the quality of the titrimetric assay itself. The dispersion of the titrimetric records reduced R^2 to 0.942 and enhanced $SD_{y,x}$ to 0.1100 units g^{-1} and SD_{xo} to 0.1043 units g^{-1}. Estimation of y_c, LOD, and LOI from that regression line suggested slightly lower values of 0.08, 0.19, and 0.38 units g^{-1}, respectively. However, that LOI would already be located in the noise range found for FSMOJ (**Fig. 1**).

On the whole, good linearity could be assumed for the method of 2.4 above an A_{PE} bottom limit represented by the LOI (x_{LOI} = 0.415 units g^{-1} of model juice; cf. **Fig. 1**). Activities > ~0.0035 units should finally be ensured in the test system for the enzyme assay. Consequently, the method was more sensitive than the microplate reader method of Cameron et al. (1992), requiring ≥ 0.009 units (cf. 3.2). Sampedro et al. (2008) reported a detection limit of 0.057 units for a titrimetric assay that was likewise performed at pH 7.0, but at 22 °C under other concentration ratios and for shorter reaction times. Although estimates of LOD, LOI, and LOQ greatly depend on the approach used (ICH, 1996), **Fig. 1** and the comparison of our LOI with that detection limit suggested that the facilitated reaction at the higher temperature of 30 °C (cf. 2.4.3) probably contributed most to the increase in sensitivity by 16 times relative to the assay of Sampedro et al. (2008). For their macro and semi-micro enzyme assays performed at 30 °C, but only for 3 min at pH 7.5, Bartolome and Hoff (1972) estimated a sensitivity of 3 ppm of methanol in the test system prior to conversion of methanol to methyl nitrite for gas chromatographic quantitation. According to the description of those test systems, this indicated a minimum release of methanol of ~0.2-0.3 µmol min^{-1}. The minimum PE activities required were thus ~85 times greater than our LOI (~0.0035 units), but were though acceptable for the analysis of PE activity in plant tissue. Sensitivity of the analytical method described in 2.4 overall appeared superior. However, because of the reduced accuracy near LOI and LOQ with anticipated recoveries of 76 and 82.5 %, respectively, analysis should be repeated with the 3-fold enzyme extract volume in case of records ranging in the order of the LOI or LOQ. As suggested by the results obtained for

FSMOJ 4 (**Tab. 3**) and 'Navelina' FSOJ (**Fig. 1**), best performance is expected, when the enzyme assay is run in the presence of 0.0088-0.0162 units.

3.4 Applicability to *Citrus* juices of unknown production history for freshness evaluation

The PE activities of the FSOJ batches, which were prepared on the small scale for this study from 'Navelina' (1.94 ± 0.05 units g^{-1}; **Fig. 1**) and 'Salustiana' fruit (**Tab. 1**-**2**), represented the ~1.3-fold values relative to those of the FSOJ, which had previously been produced from the respective cultivars on the pilot plant scale (Hirsch et al., 2011). Hence, this earlier report was overall confirmed. Slightly higher activities in the present case could be caused by the less efficient manual finishing process, but the differences were within the wide range documented by Snir et al. (1996) for the natural PE activity variation among fruit based on an assay at 30 °C and pH 7.5. According to the authors, the ratio of maximum to minimum activity varied from 1.4 to 3.5 among cultivar-specific FSCJ batches and overall amounted to 4.5 for the total of 17 FSCJ batches from 8 *Citrus* cultivars. When different titrimetric assays involving the same temperature (30 °C) were used for quantitation at pH 7.0, three 'Valencia' FSOJ batches produced throughout the season and six ones of 'Valencia Late', which were consecutively manufactured from the same fruit lot, displayed PE activities of 2.22 ± 0.86 units mL^{-1} (Wicker et al., 1987) and 1.02 ± 0.05 units g^{-1} (Hirsch et al., 2008), respectively. Since the PE activities of the cultivar-specific *Citrus* juices produced on the pilot plant scale by Hirsch et al. (2008, 2011) originated from exactly the same analytical method, they were directly compared with the results of this study in **Fig. 2**. The former comprised both freshly squeezed and thermally treated juices from oranges [*C. sinensis* cvs. 'Valencia Late', 'Salustiana', 'Navelina', and 'Navelate'], lemons [*C. limon* (L.) Burm. f. cvs. 'Verna' and 'Primofiori'], Clementine mandarins (*C. reticulata* Blanco cvs. 'Clemenules' and 'Marisol'), and the mandarin hybrid cultivars 'Clemenvilla' and 'Ortanique'. The records for all FSCJ batches accumulated between 0.6 and 1.4 units g^{-1} within the full range of 0.44-1.94 units g^{-1} (**Fig. 2**), clearly exceeding the LOD of the method. 'Navelina' samples ranged on the top, followed by those of 'Salustiana'. The PE activity was within the linear range of the method (**Fig. 1**) for all FSCJ samples, with the maximum equating to the 4.4-fold level of the minimum activity (**Fig. 2**) by analogy with the range observed by Snir et al. (1996). However, the minimum (Hirsch et al., 2011) was hardly above the LOI (0.42 units g^{-1}; **Fig. 2**). If the recovery was assumed to gradually decline to 76-80 % near the LOI, as suggested by **Fig. 1** and the unspiked FSOJ 5 of **Tab. 3**, the difference in the true activities between the FSOJ, which displayed the minimum

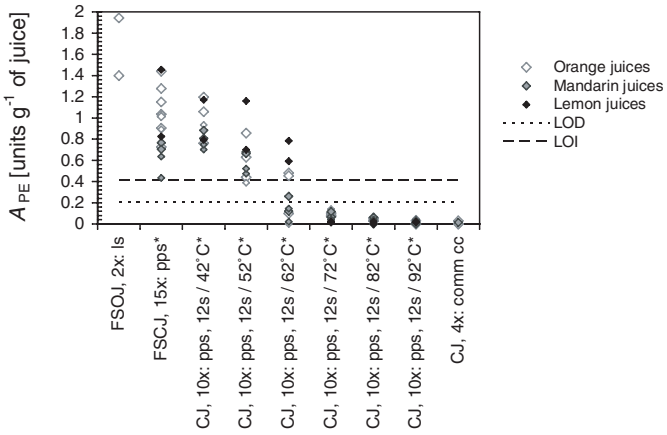

Fig. 2: Discrimination between different types of *Citrus* juices by means of the evaluated analytical method: PE activities (A_{PE}; rhombs) detected in the freshly squeezed 'Navelina' and 'Salustiana' orange juices produced on the laboratory scale for this study (FSOJ: ls), the 4 chilled commercial *Citrus* juices of **Tab. 4** (CJ: comm cc), as well as the pilot plant scale products of Hirsch et al. (2008, 2011), comprising 15 freshly squeezed *Citrus* juices (FSCJ: pps) and *Citrus* juices that were continuously heat-treated at 42-92 °C, respectively, for 12 s (CJ: pps, 12s / 42…92 °C). The juice samples of Hirsch et al. (2008, 2011) are marked by asterisks. Dotted and dashed lines indicate the limits of detection (LOD) and identification (LOI), respectively, as deduced from the linear range (**Fig. 1**) by means of the calibration method according to DIN 32645.

A_{PE}, and the majority of the other samples might have been lower than that indicated by **Fig. 2**.

Whereas chilled FC products accounted for 57 and 44 % of the chilled *Citrus* juices on the German market in years 2005 and 2009, respectively, their portion on the French market was almost negligible (3.0-2.5 %) (AIJN, 2010). As far as quantitative results were attainable at all, the PE activities found for the four commercial chilled *Citrus* juices (**Tab. 4**) were far below the LOD of 0.21 units g^{-1} (**Fig. 2**), despite the use of the 5-fold extract volumes in the enzyme assays of all four samples (cf. 2.4.3). In view of the LOD definition (cf. 3.3), the labeled treatments (**Tab. 4**) thus apparently inactivated the PE isoenzymes (**Fig. 2**). However, confirmation of complete deactivation would require a more sensitive approach, such as the semi-quantitative pasteurization test of IFU method no. 46 (IFU, 1972, 2005). The A_{PE} records obtained for the four

Tab. 4: Pectin methylesterase activities (A_{PE}) of commercial *Citrus* juices distributed in cold chain.

Product code	JA	JB	JC	JD
Package size	1 L	1 L	1 L	1 L
Citrus species processed	*C. sinensis*	*C. sinensis*	*C. sinensis*	*C. reticulata*
Source: retail markets in	France	France	Germany	France
Preservation as labeled on the package	Pressurized	Pasteurized	Gently pasteurized	Pasteurized
A_{PE} [units g^{-1} of juice]	0.030 ± 0.016	nd	tr	0.014 ± 0.014

nd, not detected; tr, traces.

commercial products of **Tab. 4** were similar to those reported by HIRSCH al. (2008, 2011) for juices after continuous thermal processing of FSCJ at 72-92 °C (partly also at 62 °C) with dwell times of 12 s in a tube-type Actijoule® heating system, which was integrated in a tubular heat exchanger (**Fig. 2**). If soft treatments like the non-thermal and thermal ones indicated by **Tab. 4** for the commercial juices are applied to NFC products, incomplete deactivation of thermostable PE fractions can be assumed (NIENHABER and SHELLHAMMER, 2001; SENTANDREU et al., 2005; CARBONELL et al., 2006), but the residual total activities can though be expected to be low enough under chilled conditions to extend the shelf life relative to FSCJ due to significant retardation of juice clarification until consumption (HIRSCH et al., 2008). The A_{PE} differences between FSCJ and minimally processed juices were discussed in detail by HIRSCH et al. (2011) for different thermal and non-thermal mild preservation techniques. The abrupt selectivity loss below the LOI (**Fig. 1**) enabled clear and reliable distinction of FSOJ from chilled juices like those of **Tab. 4** (**Fig. 2**).

4. Conclusions

Freshness evaluation requires reliable appraisal of the PE activity for unknown *Citrus* juice samples. A_{PE} quantitation via the tested method was considered reproducible and accurate within the linear range, but complete recovery obviously required the presence of at least ~0.009 units in the titration jar. Although analysis was mostly very repeatable, extreme results that were sporadically observed overall limited the intra-assay precision to ~13 %. This suboptimal repeatability, which resulted from unidentified factors constricting robustness, constituted the greatest weakness of the method. Hence, strict consideration of standardized operation conditions is necessary.

However, the validated method overall proved suitable for verification of *Citrus* juice freshness via the activity of PE in the product because of two reasons: (1) The wide cultivar-independent A_{PE} range of FSCJ (HIRSCH et al., 2011) conformed to the linear range of the method, which was limited by the LOI (0.42 units g^{-1} of juice). (2) The fact that the standardized method involving an assay with an enzyme extract volume of 1 mL per 60 mL abruptly became insensitive below the LOI ensured reliable distinction of FSCJ from other chilled juices having comparative advantages due to enhanced convenience. Thermal or non-thermal treatments of the latter group had inactivated at least the thermo-labile PE fractions almost completely and thus extended the shelf life significantly. Hence, limited sensitivity of the analytical method, which prevented applications as to specification of pasteurization results, conversely enabled its use as regards authentication of freshness for *Citrus* juices. Samples that could not be distinguished from FSCJ solely by A_{PE} comprised juices with residual activities > LOI, which may be caused by minor partial reduction of initial A_{PE} via preservation techniques or by blending of pasteurized juice with FSCJ. But for such products, the risk of clarification (HIRSCH et al., 2008) and limitation of shelf life would be the same as for FSCJ (HIRSCH et al., 2011). If a highly active FSCJ like the one of this study from 'Navelina' orange fruit was used for blending with a juice reconstituted from concentrate, addition of ~22 % of FSOJ would cause a residual activity equal to LOI.

In conclusion, 3 complete runs of the standard procedure according to 2.4 were recommended for authentication of juice freshness. When the accurate A_{PE} values are needed for samples with activities near LOI for unambiguity or to evaluate the risk of clarification more precisely, 3 further runs with extract volumes ensuring ≥ 0.009 units in the titration jar additionally become necessary.

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Abbreviations used

a, ordinate intercept of the regression line (cf. 2.5.4, Fig. 1); A_{PE}, pectin methylesterase activity; *b*, slope of the regression line (cf. 2.5.4, Fig. 1); CI, half two-sided confidence interval; DE, degree of esterification; FSCJ, freshly squeezed *Citrus* juice; FSMOJ, freshly squeezed model orange juice (derived from FSOJ by dilution); FSOJ, freshly squeezed orange juice; FC, product from concentrate; LOD, limit of detection (cf. 2.5.4); LOI, limit of identification (cf. 2.5.4); LOQ, limit of quantification (cf. 2.5.4); NFC, product not from concentrate; PE, pectin methylesterase; PVPP, polyvinylpolypyrrolidone; RSD, relative standard deviation; RSD_{day}, time-dependent intermediate precision based on analyses under day-to-day conditions (cf. 2.5.1); RSD_R, reproducibility as the analyst-dependent intermediate precision (cf. 2.5.2); $RSD_{r,i}$, intra-assay precision based on the analyses under repeatability conditions on day *i* (cf. 2.5.1); $RSD_{r,p}$, intra-assay precision based on the analyses of a given analyst under repeatability conditions (cf. 2.5.2); $RSD_{R,run}$, total precision as deduced from all individual runs performed by all analysts (cf. 2.5.2); RSD_{run}, total precision as deduced from all individual runs performed by the same analyst irrespective of the time of analysis (cf. 2.5.1); SD, standard deviation; $SD_{y,x}$, residual standard deviation of the measured values used for regression (cf. 2.5.4); SD_{xo} (= $SD_{y,x}/b$), standard deviation of the procedure (cf. 2.5.4); SE, standard error; *x*, expected values (predicted PE activities) of the regression line (cf. 2.5.4, Fig. 1); x_{LOD}, expected PE activity of a sample ranging at the limit of detection; *y*, responses (measured PE activities) of the regression line (cf. 2.5.4, Fig. 1); y_c (= $a + bx_{LOD}$), critical minimum PE activity that is unequivocally measurable for the sample ranging at the limit of detection (cf. 2.5.4).

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