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High-Throughput Microplate Enzymatic Assays for Fast Sugar and Acid Quantification in Apple and Tomato

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In this article, we report on the use of miniaturized and automated enzymatic assays as an alternative technology for fast sugar and acid quantification in apples and tomatoes. Enzymatic assays for D-glucose, D-fructose, sucrose, D-sorbitol/xylitol, L-malic acid, citric acid, succinic acid, and L-glutamic acid were miniaturized from the standard 3 mL assays in cuvettes into assays of 200 μL or lower in 96 or 384 well microplates. The miniaturization and the automation were achieved with a four channel automatic liquid handling system in order to reduce the dispensing errors and to obtain an increased sample throughput. Performance factors (limit of detection, linearity of calibration curve, and repeatability) of the assays with standard solutions were proven to be satisfactory. The automated and miniaturized assays were validated with high-pressure liquid chromatography (HPLC) analyses for the quantification of sugars and acids in tomato and apple extracts. The high correlation between the two techniques for the different components indicates that the high-throughput microplate enzymatic assays can serve as a fast, reliable, and inexpensive alternative for HPLC as the standard analysis technique in the taste characterization of fruit and vegetables. In addition to the analysis of extracts, the high-throughput microplate enzymatic assays were used for the direct analysis of centrifuged and filtered tomato juice with an additional advantage that the sample preparation time and analysis costs are reduced significantly.

KEYWORDS: Taste; high-throughput analysis; enzymatic assays; microplate; apple; tomato

INTRODUCTION

The quality of horticultural products is defined by organoleptic parameters such as flavor, texture, and appearance, which all have an influence on the preference of consumers (*1*−*3*). Flavor is characterized by the interaction of volatile components, which define the aroma (*4*−*7*), and nonvolatile components, which define the taste. Because the dry matter of fruit and vegetables is composed mainly of sugars and acids, those analytes are considered as the most important taste-active components (*8*, *9*). Hence, cheap, fast, and robust standard analysis techniques to determine the sugar and acid contents in fruit and vegetables are of great importance toward taste or chemical profiling of new cultivars (*10*), the determination of picking dates (*11*, *12*), or the monitoring of the postharvest storage behavior (*13*).

Trained sensory panels are commonly used for taste analysis but have as a disadvantage that panelists have to be screened and trained in advance, must operate in standardized conditions, and have a taste potential that is limited in time (*14*). The determinations of titratable acidity (TA) and the soluble solid content (°Brix) determination only give indicative values of total acid and total sugar contents, respectively. Till now, the most frequently used analytical techniques for sugar and acid determination in fruit and vegetables are based on chromatography such as gas chromatography (*14*) and high-pressure liquid chromatography (HPLC) (*9*, *15*). Those methods, however, suffer from the drawback that they are too time-consuming and too work laborious to be implemented in experiments in a high-throughput context. The use of biomimetic sensors, such as the electronic tongue (E-tongue), has been investigated as an alternative standard technique in taste analysis (*16*, *17*). A major disadvantage, however, of the E-tongue is the occurrence of drift, which complicates a quantitative analysis of horticultural products.

Attenuated total reflectance infrared spectroscopy is another promising technique for fast taste characterization of food (*18*). It has been applied to the classification of food samples according to corresponding sugar and acid profiles (*19*, *20*). Also, quantification of individual sugar and acid components is possible up to detection limits in the millimolar range.

As an alternative for the methods mentioned before, biosensors and bioanalytical systems are generally considered as an emerging technology in food analysis because of their specificity, simplicity, and short analysis times (*21*). For a general overview of different biosensor systems in food analysis, the reader is referred to refs *22* and *23*. Although most biosensor

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and bioanalytical systems are designed for the quantification of one specific component, a few multianalyte sensors measuring both sugars and acids are reported in the literature (*24*−*27*).

Although enzymatic assays are commonly used as a reference technique (*9*, *28*) in food analysis, their potential in a high-throughput context has never been assessed. High-throughput analysis can be defined as the implementation of assays in the wells of microplates in combination with liquid handling robotics (*29*). Such a multianalyte bioanalytical system may result in a considerable theoretical cost reduction up to 98% when the traditional analysis in 3 mL can be carried out in a volume of 50 μL.

The objective of this paper is to develop a multianalyte bioanalytical system for fast taste profiling in food samples based on accurate quantification of sugars and acids. Hereto, the combined effect of miniaturization and automation of enzymatic assays for D-glucose, D-fructose, sucrose, D-sorbitol, citric acid, L-malic acid, and succinic acid will be studied on the assay performance factors in 96 well (200, 100, and 80 μL) and in 384 well (80 and 55 μL) microplates. Also, L-glutamic acid is incorporated as a major component representing the umami taste in tomatoes (*9*). Subsequently, the bioanalytical platform will be validated on tomato and apple extracts, as well as on tomato juices, using HPLC and the standard addition method as validation techniques.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical reagent grade. D-Glucose and D-sorbitol were purchased from Merck (Leuven, Belgium), D-fructose and sucrose were obtained from AppliChem (Darmstadt, Germany), and L-malic acid and succinic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Citric acid and L-glutamic acid were obtained from Acros (New Jersey, United States).

Apparatus. An automated liquid handling system (Multiprobe II Plus, Perkin-Elmer, Boston, MA) with four channels was programmed (Winprep Software, Perkin-Elmer) to dispense all of the different reagents in the wells of the microplates. The Multiprobe had an open configuration composed of 16 different stations to accommodate the different reagent troughs, microplate holders, and racks for the eppendorf samples. The four channel pipet arm, housing fixed tips, moved in three different directions, and the four channels worked independently of each other. The automatic liquid handling system can dispense volumes from 0.5 up to 500 μL with an accuracy of <1% coefficient of variation (CV). The absorbance at the specified wavelengths was read with a Multiskan Spectrum (Thermo Electron Corp., Waltham, United States) controlled with Thermo Labsystems software Multiskan Spectrum vs.1. Two different types of flat-bottomed non-treated polystyrene microplates were used in this study: 96 well microplates (NUNC, Roskilde, Denmark) and 384 well microplates (Corning, New York).

Sample Preparation. Two types of sample preparation procedures were included in this research: the preparation of extracts (apple and tomato samples) and the preparation of juices (tomato samples).

Apple Extracts. Apples (*Malus sylvestris*) of different cultivars were obtained from a local grocery. The apple samples were cut in small slices and immediately cooled with liquid nitrogen. The frozen tissue was ground to a powder using a MM200 Mixer Mill (Retsch GmbH, Haan, Germany). The pulverized samples were stored at −80 °C until analysis. For the extraction, 0.1 (±0.01) g of the powder was dissolved in 500 μL of 80% ethanol (w/w) and incubated for 10 min at 78 °C and 850 rpm in a Thermomixer Comfort (Eppendorf, Hamburg). The samples were then centrifuged for 5 min at 25000*g* at 4 °C (MSE HAWK 15/05R centrifuge, Sanyo, Bensenville, United States). This extraction step was repeated three times, and (at the end) about 1500 μL of supernatant was collected. For the analysis of acids and sugars, respectively, 800 and 600 μL of supernatant were transferred to 1 mL eppendorfs and dry centrifuged at 30 °C (Eppendorf Concentrator 5301, Eppendorf) overnight. The residues were stored at −20 °C until further analysis was executed.

Tomato Extracts. Tomatoes (*Lycopersicon esculentum* Mill.) of different cultivars were obtained from a local grocery. One tomato per sample was mixed in a blender (PowerBlend, MX250, Braun GmbH, Kronberg, Germany), and the collected juice was cooled with liquid nitrogen. Subsequently, the frozen tissue was ground to a powder. The pulverized samples were stored at −80 °C until extraction. The extraction procedure for tomato samples was identical to that for apple samples except that extraction with 80% ethanol was repeated only once. Amounts of 300 and 100 μL of the obtained extract were, respectively, used for the analysis of acids and sugars.

Tomato Juices. When tomato juice was analyzed directly, tomatoes were mixed with the blender and stored at −80 °C. On the day of analysis, the samples were thawed in a warm (35 °C) water bath. Thereafter, 2 mL of juice was centrifuged at 25000*g* for 10 min at 4 °C. The supernatants were guided through a 0.45 μm filter (Millipore, Billerica, United States) immediately before analysis.

HPLC Analysis. Acid and sugar analyses were carried out on two separate HPLC devices (Agilent HP 1100, Agilent Technologies, Palo Alto, CA) following different protocols. The residues from the extraction step were dissolved in 400 μL of HPLC-grade water (Fisher Scientific, Leicestershire, United Kingdom) and lead over a filter with a 0.45 μm pore size (Millipore). The pH of the sugar samples was adjusted with KOH (0.25 M) to obtain a final pH between 5 and 9. No pH corrections were carried out on the acid samples. Finally, the samples were transferred into vials, which were placed in the temperature-controlled (4 °C) autosampler of the HPLC. Only one repetition per sample was executed due to the long runtime of the different protocols.

Sugar Analysis. The different sugars were separated on an Aminex HPX-87 C Carbohydrate column 300 mm × 7.8 mm (Bio-Rad Laboratories Inc., Hercules, CA) and detected with a refractive index detector (RID). Operating conditions were as follows: HPLC water as the mobile phase at 0.600 mL/min; detector temperature of 35 °C; column temperature of 80 °C; and injected volume, 20 μL. The total analysis time for the sugars was 26 min. The retention times for sucrose, glucose, fructose, and sorbitol were 8, 10, 13, and 23 min, respectively.

Acid Analysis. Organic acids were separated on a Prevail Organic Acid column, 5 μm, 250 mm × 4.6 mm (Alltech Associates Inc., Deerfield, United States) and detected with a diode array detector (DAD) on a wavelength of 200 nm with a reference wavelength of 360 nm. HPLC water, adjusted to pH 2.5 with formic acid, was used as the mobile phase, with a flow rate of 0.800 mL/min during 4 min followed by 1.2 mL/min during 8 min. The detector and column were kept at room temperature, and 20 μL of sample was injected. The total analysis time for the acids was 12 min. The retention times for malic acid, citric acid, and succinic acid were 5, 8, and 9 min, respectively.

Enzymatic Assays. Enzymatic assays for the quantification of D-glucose, D-fructose, sucrose, D-sorbitol/xylitol, L-malic acid, citric acid, succinic acid, and L-glutamic acid were purchased from R-Biopharm (Darmstadt, Germany). The assays were based on an increase/decrease in absorbance, at a specific wavelength, caused by a change in NAD(P)H (340 nm) or formazan concentration (492 nm). The change was stoichiometrically related to the concentration of the component of interest through a cascade of several enzymatic reactions. The absorbance at the specific wavelength of the chromogenic molecules was measured before (A_1) and after (A_2) the addition of the substrate specific enzyme and corrected for the δ absorbance of blank values according to the next equation:

$$\Delta A = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{blank}} \quad (1)$$

The absorbance values were obtained immediately after addition of the enzyme and then repeated every 5 min until the final absorbance values were observed. The calculated ΔA values were used for further data analysis. NAD(P)H-based enzymatic assays included the assays for D-glucose, D-fructose, sucrose, L-malic acid, citric acid, and succinic acid. The assays for D-sorbitol and L-glutamic acid were also based on a change in NADH concentration, but the formed NADH was removed in a subsequent reaction, catalyzed by diaphorase (DIA) with iodonitrotetrazolium chloride (INT), which resulted in formazan production. An overview of the different applied enzymatic reactions is given in

Table 1. Overview of the Total Concentrations of Enzyme and NAD(P)H, Used in the Different Enzymatic Assays in a Volume of 200 μL[a]

	amount of enzyme (mU/μL)							
assay	enzyme 1		enzyme 2		enzyme 3		detection molecule (μg/μL)	
D-glucose	HK	1.95	G6P-DH	0.95			NADPH	0.81
D-fructose	HK	1.90	G6P-DH	0.90	PGI	4.65	NADPH	0.79
sucrose	HK	1.95	G6P-DH	0.95	β-FRU	10.80	NADPH	0.81
D-sorbitol	SDH	2.75	DIA	0.11			NADH	0.74
L-malic acid	L-MDH	27.00	GOT	1.80			NADH	3.16
citric acid	CL	0.05	L-MDH	3.75	L-LDH	7.75	NADH	0.14
succinic acid	SCS	0.06	PK	8.15	L-LDH	7.50	NADH	0.15
L-glutamic acid	GIDH	9.00	DIA	0.11			NADH	0.74

[a] Amounts were reduced proportionally for the lower volumes.

the Appendix. For the analysis of sucrose and succinic acid, the microplates were incubated at a temperature of 37 °C in the spectrophotometer. The different reagents of the assays were used in the same proportion as described in the manuals of the different enzymatic assays. The only exception was the sucrose assay, for which the first reaction step, the decomposition of sucrose into glucose and fructose, took place in an additional 384 well microplate in a total volume of 90 μL. Miniaturization of the assays in volumes of 200 and 80 μL resulted in a theoretical cost reduction of, respectively, 93 and 97%. **Table 1** gives an overview of the different concentrations of enzymes and chromogenic molecules in 200 μL assays. When analyses were executed in lower volumes, the concentrations decreased proportionally.

Analysis with the Enzymatic Assays. *Standard Solutions.* In the experiments with standard solutions, four repetitions per concentration were measured. Calibration curves for the different analytes were obtained for a minimum of eight different concentrations in volumes of 200 (96 well microplate), 100 (96 well microplate), 80 (96 well microplate), 80 (384 well microplate), and 55 μL (384 well microplate).

Extracts. Extracts were analyzed in triplicate. For each component, a calibration curve based on four different concentrations with three repetitions per concentration, and three blank samples, was always included on the microplate. Sample analysis was performed directly on the dissolved apple or tomato extracts or on the centrifuged and filtered tomato juice without pH correction or other special pretreatment procedures. Depending on the specific investigated component, 5 μL up to a maximum of 30 μL of extract was needed to perform the analysis. Sample dilution with distilled water was necessary to obtain concentrations that were within the linear range of the calibration curve. This was achieved automatically with the liquid handling system. The high-throughput microplate enzymatic assays were used, together with the HPLC reference protocols, for the quantification of the main sugars in apples (D-glucose, D-fructose, sucrose, and D-sorbitol) and tomatoes (D-glucose and D-fructose) and for the most abundant acids in apples (L-malic acid) and tomatoes (citric acid and L-malic acid). The validation analysis was executed on 24 apple samples of three cultivars (Jonagold, Jonagored, and Pink Lady; eight apples per cultivar) and 30 tomato samples of three cultivars (Tricia, Clotilde, and Bonaparte; 10 tomatoes per cultivar). The concentration of L-glutamic acid was not quantified in extracts since no reference HPLC protocol was available. The enzymatic assays were executed first in a volume of 200 μL in a 96 well microplate. The enzymatic analysis for the sugars in apple extracts was repeated in a volume of 80 μL in a 384 well microplate.

Juices. To compare the two sample preparation techniques, 30 tomatoes of three cultivars (Tricia, Clotilde, and Bonaparte; 10 tomatoes per cultivar) were analyzed. The tomatoes were cut in half; one-half was used for the preparation of a juice, and the other half was used for the preparation of an extract. The juices were diluted automatically by the liquid handling system. To obtain sample concentrations in the linear range of the enzymatic assays, the samples were diluted 550× for the L-glutamic acid quantification, 400× for the D-glucose and D-fructose quantifications, 320× for the citric acid quantification, and 20× for the L-malic acid quantification. Protocols for the automated liquid handling system were optimized to ensure the exact dilution for the different components. The optimized protocols were checked by the analysis of a model tomato solution, composed of D-glucose, D-fructose, citric acid, L-malic acid, and L-glutamic acid. Subsequently, the direct analysis on tomato juice was validated by conducting a standard addition experiment. Three samples, originating from three cultivars, were analyzed on three different manners: a normal analysis, a dilution analysis (50% sample−50% distilled water), and an addition analysis whereby a second model tomato solution (50%), composed of D-glucose, D-fructose, citric acid, L-malic acid, and L-glutamic acid, was added to the tomato juice (50%). Normal, dilution, and addition analysis took place on the same microplate.

Sensor Performance Characteristics. The sensitivity of the bioanalytical system for a specific component was defined as the slope of the linear part of the calibration curve. The limit of detection (LOD) was set at three times the standard deviation (SD) of the blank values (S_b) divided by the slope of the calibration curve, whereas the limit of quantification (LOQ) was set to 10 S_b divided by the slope of the calibration curve (*30*). Repeatability was defined by the CV, which is calculated as the SD of the absorbance values divided by the average of the absorbance values. The average CV value (CV_a) of a calibration curve is defined as the average CV value of the points of the linear part of the calibration curve.

Statistical Analysis. Calibration curves were built by linear regression analysis using the SAS statistical software package (version 8.02, SAS Institute Inc., Cary, NC). General recommendations for method comparison studies, given by refs *31* and *32*), were included in the statistical analysis. The correlation analysis was carried out in SAS to compare the results from the enzymatic assays with the corresponding HPLC reference measurements.

Partial least squares−discriminant analysis (PLS-DA) was used to discriminate between the taste profiles of the different cultivars. These multivariate statistical analyses were executed in The Unscrambler (version 9.6, CAMO technologies Inc., Woodbridge, United States).

RESULTS AND DISCUSSION

Standard Solutions. *Assay Optimization.* Experiments were conducted to optimize the automation of the different enzymatic assays with regard to the reaction kinetics and fluid transfer and mixing. The miniaturization implies low enzyme volumes (mostly ≤2 μL) to be transferred from the reagent recipients to the wells of the microplate. Care must be taken to ensure the transfer of the correct amount of enzyme into the microplate and proper mixing of the enzyme−sample solution. When low volumes are dispensed with the liquid handling system, huge dead volumes are standard programmed in the transfer protocols. The "dead volume" is the volume that is aspirated from the pure enzyme solution but that is not dispensed to the microplate and is expressed as the percent of the volume that has to be transferred. Minimization of this volume results in less expensive analyses. Therefore, different percentages of dead volumes were examined in combination with and without a postdispense mixing step for the D-glucose assays with standard solutions. In this postdispense mixing step, the liquid handling system is programmed to aspirate and redispense at high speed a volume from the wells of the microplate to enhance mixing. Kinetic experiments indicate that, without postdispense mixing, the

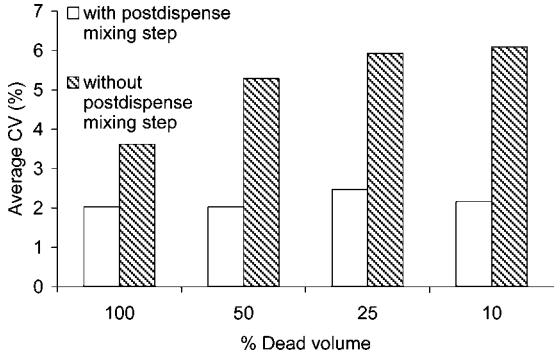

Figure 1. Influence of percentage dead volume on the CV_a with and without a postdispense mixing step for the glucose assay. The CV_a value was based on six different concentrations, which were analyzed in fourfold.

Table 2. LOD, Linear Range, R^2 Values, and CV_a Values for the Calibration Curves of the Microplate Assays for D-Glucose, D-Fructose, Sucrose, D-Sorbitol, L-Malic Acid, Citric Acid, Succinic Acid, and L-Glutamic Acid for Assays, Executed in a Volume of 200 μL[a]

assay	LOD (mg/L)	linear range (mg/L)	R^2 value	CV_a value (%)
D-glucose	1.4	4.5–150	1.00	1.1
D-fructose	1.1	3.6–120	1.00	2.6
sucrose	55	184–2500	1.00	2.7
D-sorbitol	0.09	0.3–14	1.00	1.3
L-malic acid	1.5	4.9–160	1.00	1.3
citric acid	1.3	4.4–34	1.00	2.6
succinic acid	0.7	2.2–30	1.00	3.1
L-glutamic acid	0.03	0.09–6	1.00	1.5

[a] The CV_a value is based on a minimum of eight different concentrations, which were analyzed in four-fold.

analysis time increases substantially since the reaction between substrate and enzyme is then diffusion limited. Postdispense mixing also has a beneficial influence on the repeatability of the assays, which is observed in a decrease of the CV_a (**Figure 1**). This figure also indicates that lowering the % dead volume to 10% has almost no influence on the repeatability of the glucose assays. Therefore, further experiments were carried out always with the most optimal working condition including the postdispense mixing step and a dead volume of 10%. In addition to postdispense mixing, the microplates were shaken in the microplate reader just before the reading, since kinetic experiments have indicated that regularly shaking the microplate is necessary to obtain fast final absorbance values.

Assay Performance Characteristics. Calibration curves for the assays for D-glucose, D-fructose, sucrose, D-sorbitol, L-malic acid, citric acid, succinic acid, and L-glutamic acid were established in volumes of 200, 100, and 80 μL in 96 well microplates and in volumes of 80 and 55 μL in standard 384 well microplates. The calibration curves, based on a minimum of eight different concentrations, were examined on linearity, LOD, sensitivity, and repeatability. In **Table 2**, the LOD, the linear range, the R^2 values, and the CV_a values are presented for the different assays executed in a total volume of 200 μL. The correlation coefficients near 1 and the CV_a values $\leq$ 3% indicate that excellent calibration curves are produced with the microplate enzymatic assays. The linear ranges for the different components are in agreement with the values given by the manuals of the enzymatic kits. However, lower LODs were found with the proposed method as compared to the values reported for 3 mL assays. Similar results were obtained for the assays in volumes of 100 and 80 μL (96 well microplates) and

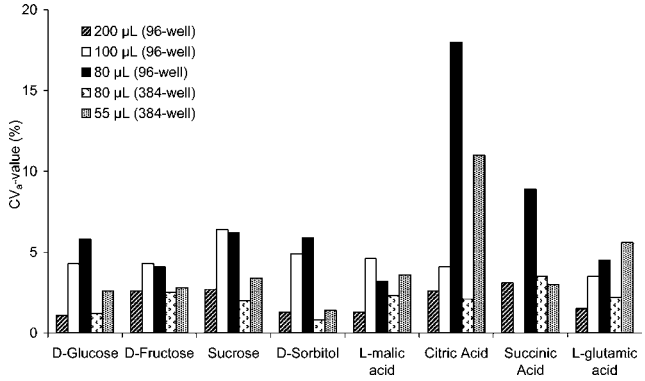

Figure 2. Overview of the CV_a values for the different investigated volumes for the different enzymatic assays. The CV_a value is the average of the CV values of the points included in the calibration curve. CV_a values lower than 5% are accepted as reasonable.

80 and 55 μL (384 well microplates) (data not shown). Only the LOD (and the LOQ) increased slightly when analyses were executed in microplates with lower volumes, but this is due to the decrease in path length.

The sensitivity of the assays was determined from the slopes of the calibration curves. The sensitivity of the high-throughput microplate enzymatic assays follows the law of Lambert−Beer. The highest theoretical path length and, as a consequence, the highest sensitivity are obtained when assays are executed in volumes of 80 μL in a 384 well microplate. The path length and, as a consequence, the sensitivity for the different setups are ranked from large to small: 80 (384 well) > 200 (96 well) > 55 (384 well) > 100 (96 well) > 80 μL (96 well).

Repeatability is defined by the CV_a value of the calibration curve, and a limit of 5% is considered as acceptable. **Figure 2** represents the CV_a values of the calibration curves of the microplate enzymatic assays for the different volumes. It is observed that a decrease in volume from 200 to 100 and 80 μL in a 96 well microplate results in an increase in the CV_a value. A decrease in path length causes an increase in CV_a value. The same phenomenon is observed for the CV_a values of the assays executed in a volume of 80 μL in 384 well microplates, which are a lot smaller than those of the assays with the same volume in 96 well microplates. Despite the difference in path length, still many assays have CV_a values below 5%, indicating the high accuracy and repeatability of the high-throughput microplate enzymatic assays.

The interday repeatability of the high-throughput microplate enzymatic assays was examined by comparing the slopes of the calibration curves of the different enzymatic assays on four different days. The CV values of the different slopes were 2.53% for D-glucose and D-fructose, 4.44% for sucrose, 4.22% for D-sorbitol, 1.24% for L-malic acid, 3.69% for citric acid, 3.34% for succinic acid, and 1.88% for L-glutamic acid. From the experiments with the standard solutions, we can conclude that the best results are obtained with a setup in which the analysis is executed in a volume of 80 μL in a 384 well microplate or in a volume of 200 μL in a 96 well microplate.

Apple and Tomato Samples. *HPLC Analysis.* The results of the HPLC measurements for the different apple and tomato cultivars are shown in **Table 3**. Tukey tests were executed to find significant ($\alpha = 0.05$) differences in the concentrations of the main sugars and acids for the different investigated apple and tomato cultivars. Significant differences in glucose, sucrose, and sorbitol concentrations were found for the investigated apple cultivars. In the tomato samples, higher glucose and fructose concentrations are found in samples, originating from the

Table 3. Results of the HPLC Analysis for the Different Apple and Tomato Cultivars[a]

		glucose	fructose	sucrose	sorbitol	malic acid	citric acid
apple	Jonagold	2.70 ± 0.67 a	6.78 ± 1.89 a	0.88 ± 0.37 a	0.27 ± 0.08 a	0.88 ± 0.28 a	<LOD
	Jonagored	1.86 ± 0.60 b	5.85 ± 2.10 a	1.13 ± 0.40 a	0.31 ± 0.11 ab	1.01 ± 0.38 a	<LOD
	Pink Lady	1.00 ± 0.36 c	4.89 ± 2.08 a	4.87 ± 2.17 b	0.47 ± 0.20 b	1.01 ± 0.34 a	<LOD
tomato	Tricia	1.81 ± 0.11 a	2.00 ± 0.08 a	<LOD	<LOD	0.09 ± 0.01 a	0.48 ± 0.06 a
	Bonaparte	2.46 ± 0.21 b	2.32 ± 0.21 b	<LOD	<LOD	0.19 ± 0.04 b	0.36 ± 0.04 b
	Clotilde	2.28 ± 0.43 b	2.43 ± 0.37 b	<LOD	<LOD	0.09 ± 0.01 a	0.55 ± 0.06 a

[a] The amounts are expressed as g/L. Significant differences between the cultivars for each individual component are indicated by different letters.

Table 4. Validation Analysis for High-Throughput Enzymatic Assays, Executed in a 96 Well Microplate in a Volume of 200 μL for D-Glucose, D-Fructose, Sucrose, D-Sorbitol, L-Malic Acid, and Citric Acid in Tomato and Apple Extracts[a]

assay		intercept (±95% CI)	slope (±95% CI)	n	R^2 value	CV_a value (%)
D-glucose	apple	–0.01 ± 0.11	0.97 ± 0.05	24	0.99	1.0
	tomato	–0.03 ± 0.01	1.01 ± 0.03	30	0.99	1.4
D-fructose	apple	–0.07 ± 0.24	0.99 ± 0.04	24	0.99	0.9
	tomato	0.01 ± 0.02	0.97 ± 0.03	30	0.99	2.3
sucrose	apple	–0.14 ± 0.13	1.01 ± 0.04	24	0.99	1.3
D-sorbitol	apple	–0.03 ± 0.05	1.17 ± 0.15	24	0.93	1.2
L-malic acid	apple	0.01 ± 0.02	1.00 ± 0.04	24	0.99	1.8
	tomato	0.03 ± 0.03	0.95 ± 0.05	30	0.99	0.7
citric acid	tomato	0.02 ± 0.02	0.96 ± 0.03	30	0.99	1.6

[a] The different samples were analyzed in triplicate (CI, confidence intervals; n = sample size).

cultivars Bonaparte and Clotilde. In addition, these cultivars are characterized by a significant difference in malic acid and citric acid concentration.

The citric acid and succinic acid concentrations in apples and the sorbitol, sucrose, and succinic acid concentrations in the tomato samples were below the LOD of the HPLC reference method. Hence, these analytes were not measured with the high-throughput microplate enzymatic assays either. Because these analytes are identified as minor components (*35*, *36*) in the different species, they will not severely contribute to the taste profile of the investigated species.

Analysis in 96 Well Microplates. Next, the D-glucose, D-fructose, sucrose, D-sorbitol, L-malic, and citric acid concentrations were measured in apple and tomato extracts with the HPLC reference method and with the high-throughput enzymatic assays in a 96 well microplate with a total volume of 200 μL. The results of the validation analysis are presented in **Table 4**. The correlation coefficients between the HPLC and the enzymatic assays are close to one for all sugars and acids tested. Moreover, the intercept and slope parameters resulting from the regression of the HPLC data on the assay data are close to 0 and 1, respectively. However, the validation analysis for sorbitol reveals that the analysis with the enzymatic assays results in higher sorbitol concentrations than with the HPLC analysis. This is due to the lack of specificity of the sorbitol dehydrogenase (SDH) enzyme (*33*), which oxidizes also xylitol, another polyalcohol present in minor amounts in apples (*34*). Besides the good correlation of the validated enzymatic assays, the CV_a values indicate that the repeatability of the high-throughput microplate enzymatic assays on samples is satisfactory with values <2%.

Because no standard HPLC procedure was available for the validation of the L-glutamic assay, this assay was validated by means of the standard addition method. Four different L-glutamic acid concentrations were added to the tomato samples, originating from four different tomato cultivars. With recovery coefficients between 97.1 and 103.8%, the proposed method can be judged as validated.

Analysis in 384 Well Microplates. D-Glucose, D-fructose, sucrose, and D-sorbitol/xylitol concentrations were measured in the same 24 apple extracts as in the previous experiment, but the analysis was now executed in a total volume of 80 μL in a 384 well microplate. **Figure 3** depicts the scatter plots for the four different components, and **Table 5** represents the results from the correlation analysis between the two different investigated volumes. From these plots and the statistical analysis, it follows that there is almost no difference between the two different volumes for the assays of D-glucose, D-fructose, sucrose, and D-sorbitol/xylitol. Only for the sucrose assay, there was some (not systematic) scattering observed in the low (<1.5 g/L) sucrose range. The repeatability of the assays, executed in a volume of 80 μL, was assessed as very good with CV_a values of 1.5% for D-glucose, 1.7% for D-fructose, 4.4% for sucrose, and 1.1% for D-sorbitol.

Analyses on Tomato Juices. Because HPLC analysis creates the necessity to have an extensive sampling preparation, we examined the possibility to analyze directly the centrifuged and the filtered tomato juice by means of the high-throughput microplate enzymatic assays. First, dilution protocols to ensure the exact dilution of the samples by the liquid handling system were optimized and tested on a model tomato solution. With the optimized protocols, recoveries of 98% for D-glucose, 94% for D-fructose, 97% for citric acid, 99% for L-malic acid, and 94% for L-glutamic acid were obtained.

Subsequently, these protocols were used for the validation experiment on tomato juices. The recovery coefficients for the different components for the dilution experiment and the standard addition experiment are shown in **Table 6**. This table indicates that the analysis on tomato juices with the microplate enzymatic assays becomes possible and, as a consequence, eliminates the time-consuming sampling preparation, which results in additional cost savings and an increased throughput.

Analysis of Tomato Juices vs Tomato Extracts. Because a successful validation was achieved for the tomato extracts as well as for the tomato juices, it now becomes possible to study the effect of the difference in sample preparation. The results originating from the experiments with the extracts were expressed as mg/g fresh weight (FW), whereas the results from the analyses on juices were expressed as g/L. Therefore, the different concentration values were standardized by dividing these values by the average value of a specific analysis. The validation results on the normalized standard concentrations between the two different methods are shown in **Table 7**.

This table shows that the correlation between the different sample preparation methods is rather low for D-glucose, D-fructose, and citric acid with R^2 values of, respectively, 0.70, 0.51, and 0.62. The R^2 values for the other investigated components, L-malic acid and L-glutamic acid, are higher with values of, respectively, 0.93 and 0.86, but this difference is due

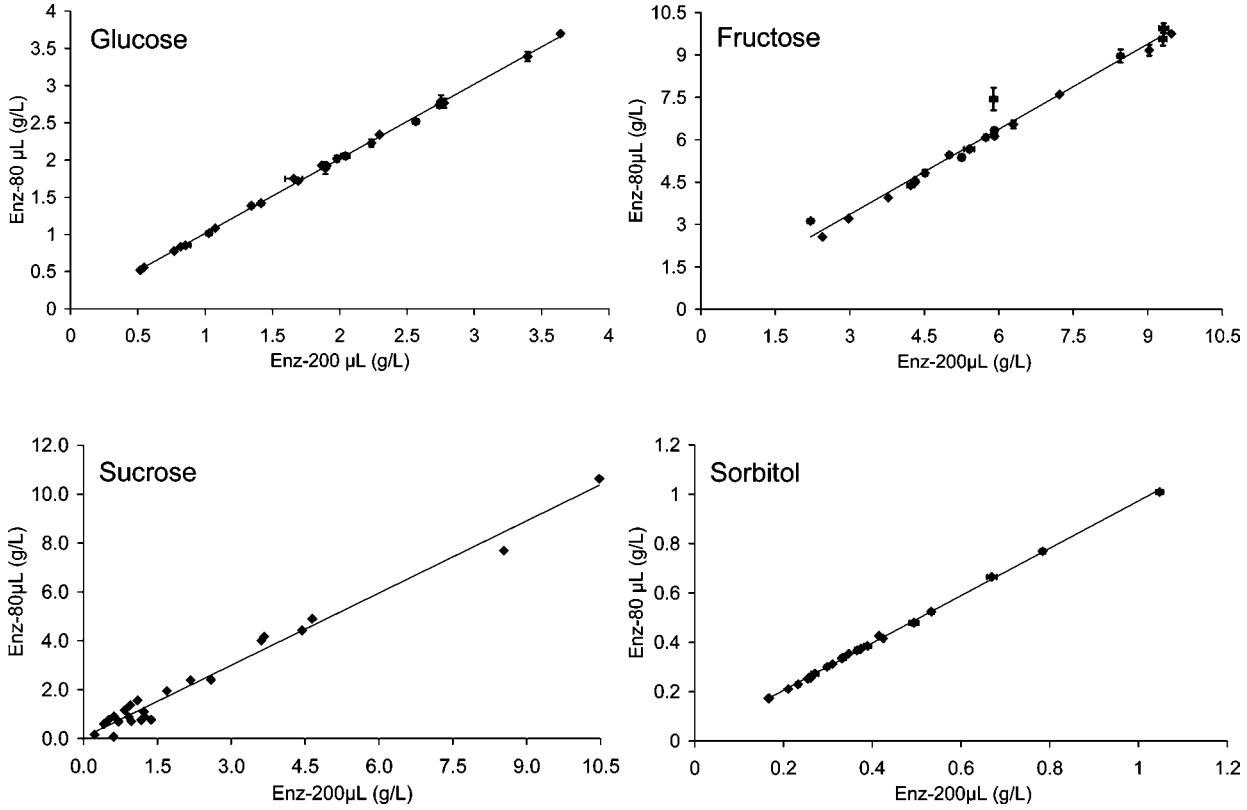

Figure 3. Validation analysis between enzymatic assays for D-glucose, D-fructose, sucrose, and D-sorbitol, executed in 96 well microplates (200 μL) and 384 well microplates (80 μL). Error bars represent the SD on the *Y*-axis as well as on the *X*-axis.

Table 5. Correlation Analysis between the Enzymatic Assays on Apple Extracts for D-Glucose, D-Fructose, Sucrose, and D-Sorbitol/Xylitol, Executed in Volumes of 200 (96 Well Microplate) and 80 μL (384 Well Microplate) (CI, Confidence Intervals)[a]

assay	intercept (±95% CI)	slope (±95% CI)	R^2 value
D-glucose	0.01 ± 0.03	1.00 ± 0.01	0.99
D-fructose	0.29 ± 0.25	1.00 ± 0.04	0.99
sucrose	0.09 ± 0.20	0.98 ± 0.06	0.98
D-sorbitol/xylitol	0.01 ± 0.01	0.97 ± 0.02	1.00

[a] Twenty-four different samples per component were analyzed.

Table 6. Range of the Recovery Coefficients for the Validation Experiments, Performed on Tomato Juices[a]

	dilution (%)	standard addition (%)
D-glucose	93–103	88–102
D-fructose	93–107	90–97
citric acid	90–104	89–102
L-malic acid	93–104	88–102
L-glutamic acid	90–106	91–109

[a] Nine samples were analyzed per component whereby on one microplate the normal sample, the diluted sample, and the sample with the standard addition method were analyzed.

to the more extended concentration range of these components. However, no significant difference in sample preparation method was observed for D-glucose, D-fructose, citric acid, L-glutamic acid, and L-malic acid. The low correlation between the two different methods is probably caused by the variability that comes into play when extensive sample preparation methods are used instead of simple preparation methods.

Therefore, the preparation of a juice is preferred above the preparation of an extract as a sample preparation method because it is much easier to execute and the time between preparation and analysis is reduced significantly, which allows the more accurate quantification of the different components in the samples.

Table 7. Correlation Analysis for the Standardized Results of the Analysis on Tomato Juices and Tomato Extracts with the High-Throughput Microplate Enzymatic Assays[a]

	intercept (±95% CI)	slope (±95% CI)	R^2 value
D-glucose	0.18 ± 0.20	0.82 ± 0.20	0.70
D-fructose	0.38 ± 0.24	0.62 ± 0.23	0.51
citric acid	0.42 ± 0.19	0.58 ± 0.18	0.62
L-malic acid	–0.03 ± 0.11	1.09 ± 0.12	0.93
L-glutamic acid	–0.07 ± 0.18	1.07 ± 0.17	0.86

[a] Thirty different samples per component were analyzed.

Fast Taste Profiling of Tomato Cultivars. To evaluate the potential of the high-throughput microplate enzymatic assays in fast taste profiling, the results from the experiments with the tomato samples were analyzed with PLS-DA. The dependent variables for the tomato samples were D-glucose, D-fructose, citric acid, L-malid acid, and L-glutamic acid. The results of the PLS-DA on the obtained concentrations from the two experiments are presented in **Figures 4** and **5**. **Figure 4** shows clearly the advantage of using juices instead of extracts since a more clear discrimination between the cultivars Tricia and Clotilde was obtained for juices. The correlation loadings for the PLS-DA on juices in **Figure 5** show that samples from the cultivar Tricia are characterized by low concentrations of D-glucose, D-fructose, and L-glutamic acid and as such is separated from the other two cultivars. These components determine the principal component (PC1) along the *X*-axis. Discrimination between cultivars Bonaparte and Clotilde is due to the difference in organic acid composition (PC2). Samples

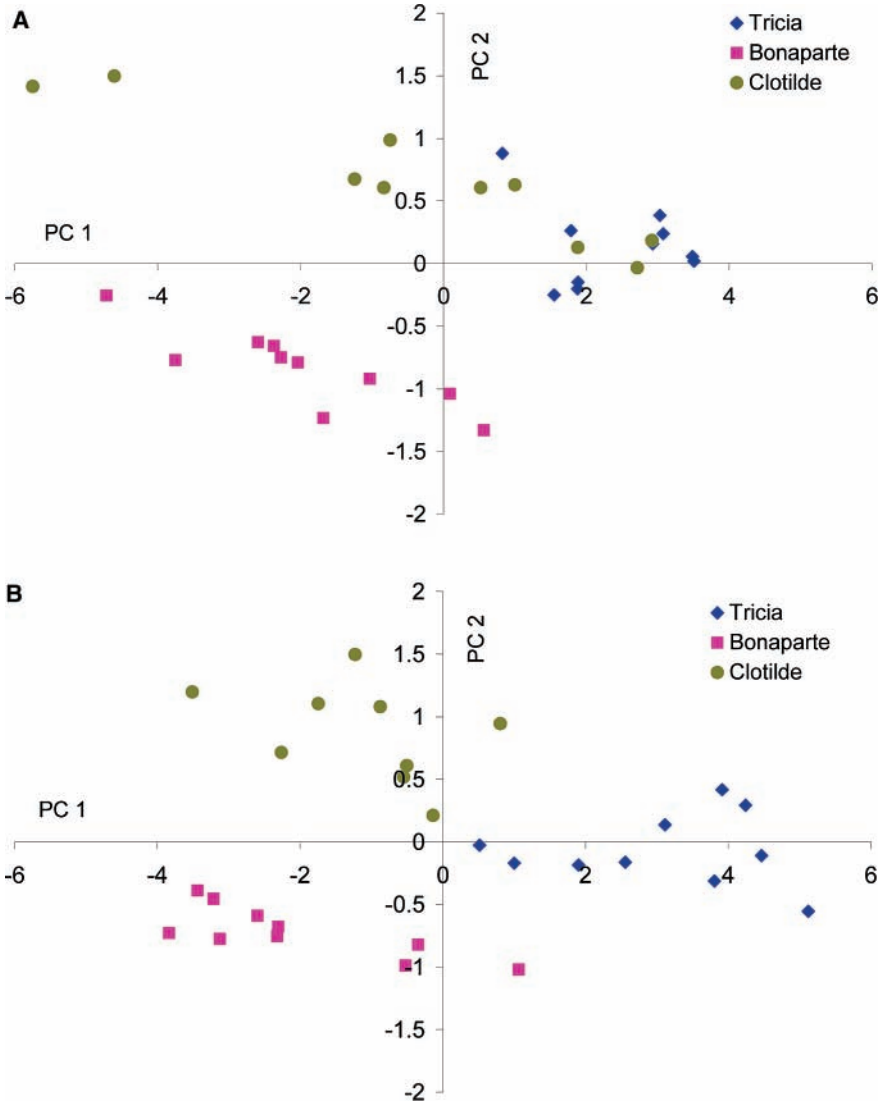

Figure 4. Score plots of the PLS-DA for the extracts (**A**) and juices (**B**), executed with the high-throughput microplate enzymatic assays.

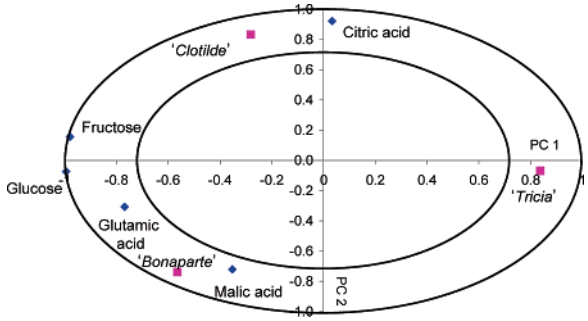

Figure 5. Correlation loadings for the PLS-DA for the juices.

from the cultivar Bonaparte are characterized by lower concentrations of citric acid and higher concentrations of L-malic acid as compared to the samples originating from the cultivar Clotilde.

Time Issues of the High-Throughput Microplate Enzymatic Assays. Depending on the quantified component, it takes 1−2 h to complete a full analysis cycle, including the filling procedure of the microplate and the reading of the plate. In a 96 well microplate, 84 samples (one repetition per sample) can be analyzed simultaneously, which results in total analysis times of 45−85 s per sample per component. As compared to HPLC analysis, data analysis occurs also much faster with the enzymatic assays since templates can be designed easily in spreadsheet software where the results can be pasted. Hereby, the results are obtained in a few minutes time, whereas HPLC analysis includes the time laborious peak integration, which can be done only by trained personnel.

As a conclusion, we can distinguish several advantages of the high-throughput microplate enzymatic assays above common standard analysis techniques such as HPLC: (i) the overall time of analysis, including sample preparation and data analysis, is much shorter; (ii) more accurate information can be obtained when juices are analyzed directly instead of extracts; and (iii) cost of analysis is reduced due to the increased sample throughput and the decreased labor costs. The total equipment cost for the microplate enzymatic assays (liquid handling system and microplate reader) is in the same order of magnitude as one HPLC device. Therefore, the high-throughput microplate enzymatic assays become a cost-effective competitor to replace HPLC as a reference technique for the quantification of the individual sugars and acids in food products. The developed bioanalytical platform can be used in a broad range of applications going from fast taste profiling of new fruit and vegetable cultivars to quality control of fruit juices or other beverages.

ABBREVIATIONS USED

CL, citrate lyase; CV, coefficient of variation; DIA, diaphorase; DAD, diode array detector; FW, fresh weight; β-FRU, β-fructosidase; G6P-DH, glucose-6-phosphate dehydrogenase; GIDH, glutamate dehydrogenase; GOT, glutamate-oxaloacetate transaminase; HK, hexokinase; L-LDH, L-lactate dehydrogenase; LOD, limit of detection; LOQ, limit of quantification; L-MDH, L-malate dehydrogenase; NAD, nicotinamide-adenine dinucleotide; NADP, nicotinamide-adenine dinucleotide phosphate; PC, principal component; PLS-DA, partial least squares−discriminant analysis; PGI, phosphoglucoisomerase; PK, pyruvate kinase; RID, refractive index detection; SD, standard deviation; SDH, sorbitol dehydrogenase; SCS, succinyl-CoA synthetase.

APPENDIX

Enzymatic reactions for the different miniaturized and automated assays are as follows.

D-Glucose:

$$\text{(1) D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{D-glucose-6-phosphate} + \text{ADP}$$

$$\text{(2) D-glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

D-Fructose:

$$\text{(1) D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{D-glucose-6-phosphate} + \text{ADP}$$

$$\text{(2) D-fructose} + \text{ATP} \xrightarrow{\text{HK}} \text{D-fructose-6-phosphate} + \text{ADP}$$

$$\text{(3) D-glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

$$\text{(4) D-fructose-6-phosphate} \xrightarrow{\text{PGI}} \text{D-glucose-6-phosphate}$$

Sucrose:

$$\text{(1) D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{D-glucose-6-phosphate} + \text{ADP}$$

$$\text{(2) D-glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

$$\text{(3) sucrose} + \text{H}_2\text{O} \xrightarrow{\beta\text{-FRU}} \text{D-glucose} + \text{D-fructose}$$

D-Sorbitol:

$$\text{(1) D-sorbitol} + \text{NAD}^+ \xrightarrow{\text{SDH}} \text{D-fructose} + \text{NADH} + \text{H}^+$$

$$\text{(2) xylitol} + \text{NAD}^+ \xrightarrow{\text{SDH}} \text{D-xylulose} + \text{NADH} + \text{H}^+$$

$$\text{(3) NADH} + \text{INT} + \text{H}^+ \xrightarrow{\text{DIA}} \text{NAD}^+ + \text{formazan}$$

Citric acid:

$$\text{(1) citrate} \xrightarrow{\text{CL}} \text{oxaloacetate} + \text{acetate}$$

$$\text{(2) oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-MDH}} \text{L-malate} + \text{NAD}^+$$

$$\text{(3) pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + \text{NAD}^+$$

L-Malic acid:

$$\text{(1) L-malate} + \text{NAD}^+ \xrightarrow{\text{L-MDH}} \text{oxaloacetate} + \text{NADH} + \text{H}^+$$

$$\text{(2) oxaloacetate} + \text{L-glutamate} \xrightarrow{\text{GOT}} \text{L-aspartate} + \text{2-oxoglutarate}$$

Succinic acid:

$$\text{(1) succinate} + \text{ITP} + \text{CoA} \xrightarrow{\text{SCS}} \text{IDP} + \text{succinyl-CoA} + \text{P}_\text{i}$$

$$\text{(2) IDP} + \text{PEP} \xrightarrow{\text{PK}} \text{ITP} + \text{pyruvate}$$

$$\text{(3) pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + \text{NAD}^+$$

L-Glutamic acid:

$$\text{(1) L-glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{GIDH}} \text{2-oxoglutarate} + \text{NADH} + \text{NH}_4^+$$

$$\text{(2) NADH} + \text{INT} + \text{H}^+ \xrightarrow{\text{DIA}} \text{NAD}^+ + \text{formazan}$$

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