Fast mass spectrometry-based enantiomeric excess determination of proteinogenic amino acids

Heidi Fleischer · Kerstin Thurow

Abstract A rapid determination of the enantiomeric excess of proteinogenic amino acids is of great importance in various fields of chemical and biologic research and industries. Owing to their different biologic effects, enantiomers are interesting research subjects in drug development for the design of new and more efficient pharmaceuticals. Usually, the enantiomeric composition of amino acids is determined by conventional analytical methods such as liquid or gas chromatography or capillary electrophoresis. These analytical techniques do not fulfill the requirements of high-throughput screening due to their relative long analysis times. The method presented allows a fast analysis of chiral amino acids without previous time consuming chromatographic separation. The analytical measurements base on parallel kinetic resolution with pseudoenantiomeric mass tagged auxiliaries and were carried out by mass spectrometry with electrospray ionization. All 19 chiral proteinogenic amino acids were tested and Pro, Ser, Trp, His, and Glu were selected as model substrates for verification measurements. The enantiomeric excesses of amino acids with non-polar and aliphatic side chains as well as Trp and Phe (aromatic side chains) were determined with maximum deviations of the expected value less than or equal to 10ee%. Ser, Cys, His, Glu, and Asp were determined with deviations lower or equal to 14ee% and the enantiomeric excess of Tyr were calculated with 17ee% deviation. The total screening process is fully automated from the sample pretreatment to the data processing. The method presented enables fast measurement times about 1.38 min per sample and is applicable in the scope of high-throughput screenings.

Keywords Amino acids · Enantiomeric excess · Laboratory automation · Parallel kinetic resolution · Mass spectrometry

Introduction

Amino acids are ubiquitous in biologic systems since they are the building blocks of proteins and consequently the building blocks of the life. Of the 20 genetically encoded α-amino acids, 19 are chiral compounds. Enantiomers may differ in their biologic effects (Smith 2009). The investigation of the influence of amino acid enantiomers to plants (Robinson 1976; Forsum et al. 2008), animals (Sasamura et al. 1998; Esteban et al. 2010), and humans (Tsai et al. 1998; Bhushan and Kumar 2010) is very important in different scientific fields, especially in drug development (Stinson 2001; Rouhi 2002a). In nature, proteins are mainly built from amino acid L-enantiomers. However, D-amino acids and their derivatives can also be found in living organisms. Some functions of them are well researched, but there still remain a lot of interesting scientific issues. D-amino acids are distributed in food such as dairy products (e.g., fresh and fermented milk), sourdough (resulting from fermentation using lactic acid bacteria or yeast), fruits and vegetables, and in a wide variety of processed foods (Friedman 1999). In the field of neuroscience, Hudson et al. offer a promising approach for the treatment of insomnia (Hudson et al. 2005). This small selection of examples represents the wide spectrum of applications and shows the

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requirement of fast analysis methods for enantiomeric excess determination, especially in the scope of high-throughput screenings.

Commonly, no universal single sensors are available for the analysis of chiral compounds. Suitable single sensors for enantiomeric excess determination have only been developed for special purposes. Pernot et al. presented a yeast D-amino acid oxidase microbiosensor for the in vitro and in vivo determination of D-serin in rat brain (2008). Usually, for the determination of chiral substances, the concept of pre-, intra-, and post-sensoric selectivity of material measurement engineering is applicable (Thurow 1999). Pre-sensoric selectivity includes the first separation of the substantial measuring object or a single compound from complex matrix in front of the sensor. This can be performed by mechanical, thermal, electrophoretic, chemical, or chromatographic techniques. The intra-sensoric selectivity is achieved by sensors with suitable selectivity concerning the substances to be analyzed. Therefore chemical, physical or biologic single sensors or complex sensor systems such as mass spectrometry can be applied. In the last step, the post-sensoric selectivity, the interpretation and evaluation of the measured data are performed. These include qualitative and quantitative analysis as well as the visualization of the results.

In general, structural information of amino acids was mostly obtained by classical analytical measurement techniques in the pre-sensoric step such as HPLC, GC, or CE. Brückner et al. describe in their studies liquid chromatographic methods (HPLC) for analyzing food (1992, 1995), biologic samples (1994), and samples with reference to medicine (1995). The total analysis is consisted of the time for a linear gradient of 75 min and following equilibration time of 10 min. A further LC–MS method for analyzing proteinogenic amino acids is described by Gördes et al. (2011). A GC–MS-based method for the detection of D-amino acids has been described by Pätzold and Brückner (2005) which requires a time frame about 45 min. Waldhier et al. describe different methods for determining amino acid enantiomers by CE, HPLC ,and GC (2009). Analysis times in the range of 15–30 min are reported (Waldhier et al. 2009). Kirschner et al. document various techniques for the analysis of D-amino acids in biologic samples (2009). An example for rapid D-Ser detection reported in this paper is the technique used in enzyme-linked assays adapted in vivo or in vitro, which enables short response times about only a view seconds. The authors describe further analytical methods by GC/MS with analysis times about 11 min, HPLC/fluorescence with total times in the range of 29 min up to 75 min, HPLC/MS/MS with injection cycle times from 3.5 min up to 50 min, and CE-based techniques which enable total times in a range of view seconds up to 42 min (Kirschner and Green 2009).

Some of the analytical techniques mentioned above are characterized by relative long analysis times, high costs of chiral columns if used by direct separation, and a high usage of solvents and/or chiral additives.

In contrast, mass spectrometry enables short analysis times and a high degree of automation. Various methods for chiral determination of amino acids have been reported such as ion–molecule reactions (Grigorean et al. 2000) and the kinetic method by collision-induced decay of diastereomeric complexes (Tao et al. 2000; Zhang et al. 2001; Schug and Lindner 2005). The parallel kinetic resolution is a further method for enantiomeric analysis (Horeau 1977; Vedejs and Chen 1997; Guo et al. 1999). A screening procedure of entirely automated sample preparation and determination of enantiomeric excess by parallel kinetic resolution and MS with electrospray ionization (ESI–MS) has been recently developed (Thurow and Gördes 2006). An additional software module for subsequent fast data evaluation was implemented and various substrates such as carboxylic acids, alcohols, and amino alcohols as well as further auxiliaries were investigated (Fleischer et al. 2009). The technique was also applied in the enantiomeric excess determination of the amino acid proline (Fleischer and Thurow 2011).

Amino acids and parallel kinetic resolution

The principle of parallel kinetic resolution is based on different reaction kinetics between chiral substances (Guo et al. 1999; Vedejs and Chen 1997). In a derivatization procedure, the chiral analytes are derivatized with suitable pseudoenantiomeric mass-tagged auxiliaries which differ in their chiral configuration and in their molecule mass. If the analytes have one chirality center in the molecule, four reaction products are formed with two m/z-values detected by mass spectrometry. As a result of different reaction velocities, the ratio of these characteristic m/z-values is related to the enantiomeric ratio of the chiral substrates and is used for the enantiomeric excess calculation.

Bhushan and Bückner described a classic HPLC method for the determination of amino acid enantiomers using Marfey's reagent for the formation of diastereomers and subsequent separation on an achiral stationary phase (2004). Based on this method, two variants of Marfey's reagent N_α-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA) and N_α-(5-fluoro-2,4-dinitrophenyl)-D-leucinamide (D-FDLA) were selected in this study as adequate auxiliaries for parallel kinetic resolution. These substances are characterized by a mass difference of $m/z = 14$ and opposed chiral configuration. Figure 1 exemplarily shows the reaction equation for the derivatization of Val. The
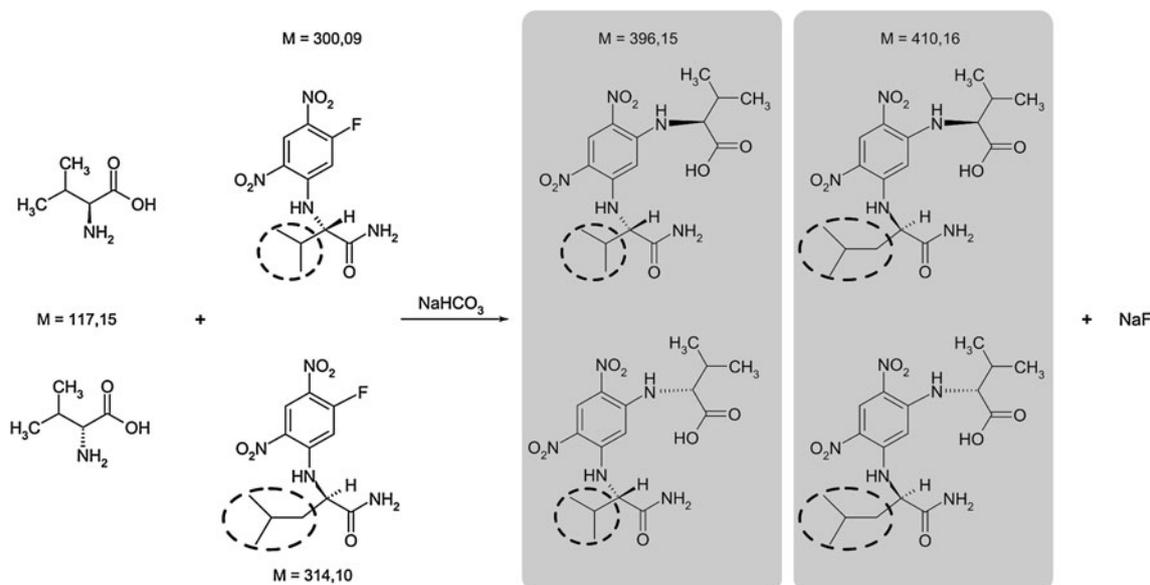
Fig. 1 Structural equation for derivatization of D- and L-Val using two variants of Marfey's reagent

mass differences of the auxiliaries and the corresponding reaction products are highlighted.

The derivatization leads to characteristic *m/z*-values of the resulting diastereomers for each amino acid. Table 1 summarizes these characteristic masses of the derivatives of 19 proteinogenic amino acids. Thereby, the m/z-values of the [M-H]⁻-ions are listed, which can be observed by ESI–MS. The ratio of the intensities (peak areas) of these masses listed in Table 1 corresponds with the chiral composition of the substrates.

With regard to the intended application as a high-throughput screening procedure, a compromise is to be found between the exactness of the results and the total analysis time. For fast screening procedures, measuring inaccuracies are allowed up to 10 % which enables a good classification of the samples in categories such as poor, middle, or very well (Rouhi 2002b). In this study, the purpose was to develop a high-throughput analytical technique for fast enantiomeric excess determining of amino acids by one or an easily adaptable method with minimal material effort and a maximum of automation. The method presented is by means of parallel kinetic resolution and ESI–MS. Based on the works of Guo, Vedejs, and Thurow et al., the technique of parallel kinetic resolution was extended to the determination of the amino acids (Guo et al. 1999; Vedejs and Chen 1997; Thurow and Gördes 2006; Fleischer et al. 2009; Fleischer and Thurow 2011). The present paper demonstrates the extension to the whole set of proteinogenic amino acids. Our aim was the development of a universal rapid screening method for enantiomeric excess determination of chiral compounds.

Experimental

The analysis of amino acids by the fast method of parallel kinetic resolution was performed with chemicals and reagents listed in Sect. "Chemicals and reagents," using sample preparation methods described in Sect. "Sample preparation" and with the equipment specified in Sect. "Instrumentation and analysis." The determination of enantiomeric excess additionally was performed by classic techniques such as HPLC–MS, HPLC–DAD, as well as GC–FID. These experiments enable an evaluation as well as the determining of advantages and limitations of the method presented. The method parameters are described in the sections below.

Chemicals and reagents

The following amino acids used as chiral substrates were purchased from Sigma Aldrich (Steinheim, Germany): D-Ala (99 + %), L-Ala (99 %), D-Ser (98 %), L-Ser (>99 %), D-Pro (>99 %), L-Pro (99 + %), D-Val (98 + %), L-Val (99 %), D-Thr (98 %), L-Thr (98 %), D-Cys (≥99.0 %), L-Cys (≥99.5 %), D-Ile (98 %), L-Ile (99 %), D-Leu (99 %), L-Leu (99 %), D-Asn (99 %), L-Asn (99 %), D-Asp (99 %), L-Asp (98 %), D-Gln (≥98.5 %), L-Gln (≥99.5 %), D-Lys (≥96.0 %), L-Lys (≥98 %), D-Glu (99 + %), L-Glu (99 %), D-Met (99 + %), L-Met (>99,0 %), D-His (≥99.0 %), L-His (≥99.5 %), D-Phe (99 + %), L-Phe (99 %), D-Arg (≥99 %), L-Arg (≥99.5 %), D-Tyr (99 %), L-Tyr (99 + %), D-Trp (99 + %), and L-Trp (99 %). The non-chiral amino acid

Table 1 Characteristic m/z-values of the [M-H]⁻ ions of the amino acid derivative pairs

Amino acid	Detected [M-H]⁻ ions (m/z)	
	m/z-value 1	m/z-value 2
Gly (non-chiral)	354.10	368.13
Ala	368.12	382.13
Ser	384.11	398.13
Pro	394.13	408.15
Val	396.15	410.16
Thr	398.13	412.14
Cys	400.09	414.10
Ile	410.16	424.18
Leu	410.16	424.18
Asn	411.12	425.14
Asp	412.11	426.12
Gln	425.14	439.16
Lys	425.18	439.19
Glu	426.12	440.14
Met	428.12	442.13
His	434.14	448.15
Phe	444.15	458.16
Arg	453.18	467.20
Tyr	460.14	474.16
Trp	483.16	497.17

glycine (>99 %), acetone (99 + %), and formic acid (~98 %) are also from this supplier. N_α-(2,4-dinitro-5-fluorophenyl)-D-valinamide (D-FDVA) (≥98 %) and N_α-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA) (≥98 %) were purchased from Fluka (Buchs, Switzerland) as well as N_α-(5-fluoro-2,4-dinitrophenyl)-D-leucinamide (D-FDLA) (98 %) and N_α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA) (98 %) from ABCR (Karlsruhe, Germany). Methanol (HPLC Gradient Grade), hydrochloric acid (37 %), and isopropanol (99,9 %) were purchased from Roth (Karlsruhe, Germany), sodium bicarbonate (≥99.5 %) from AppliChem (Darmstadt, Germany) as well as trifluoroacetic acid anhydride (TFAA (>99 %) from Fluka (Buchs, Switzerland).

Sample preparation

For each amino acid, enantiomer stock solutions of 10 mL were prepared in a concentration of 50 mmol/L. Therefore, the crystalline amino acids were solved in 1 N hydrochloric acid, neutralized with 1 N sodium bicarbonate and filled up to 10 mL with ultrapure water. D- and L-Tyr are slightly soluble, wherefore these amino acids were solved with an excess of hydrochloric acid. If sodium bicarbonate is added to L-Trp, a fine precipitate is formed. To avoid this, L-Trp was also prepared with an excess of hydrochloric acid. A volume of 2 mL of these solutions was

filled in small vials and stored in the refrigerator at 4 °C (39.2 °F) for the following experiments. The remaining solutions were stored at −18 °C (−0.4 °F). Owing to the low stability of Cys, the stock and standard solutions for this compound should be prepared shortly before the measurements.

Parallel kinetic resolution and ESI–MS

Before the derivatization, the amino acid stock solutions were diluted (1:50, v/v) to a concentration of 1 mmol/L with ultra pure water. Only the stock solution of Ser was prepared in a concentration of 10 mmol/L. The auxiliary solution contained L-FDVA and D-FDLA dissolved in acetone each in a concentration of 2.5 mmol/L. For the calibration, the 1 mmol/L amino acid solutions (chiral substrates) were used to prepare five mixtures with defined enantiomeric excesses of −100, −50, 0, 50, and 100ee%. The following definition of enantiomeric excess (Faber 1997) was used, whereby ee% means the enantiomeric excess in percent, A the volume (mL) of the L-enantiomer of an amino acid, and B the volume of the D-enantiomer. In the case of enantiopure L-enantiomer, the resulting enantiomeric excess is 100ee%. For the racemate the enantiomeric excess is 0ee% and for the enantiopure D-enantiomer −100ee%

$$ee\% = \frac{(A - B)}{(A + B)} \cdot 100.$$

The derivatization was performed in 1 mL GC-vials with screw caps (Agilent, Waldbronn, Germany) as well as in 96-well master blocks with a well volume of 500 μL (Greiner-BioOne, Essen, Germany) with sealing mats. For the derivatization, 50 μL of the chiral substrates, 100 μL of the auxiliary solution, and 20 μL of sodium bicarbonate (1 M) were added. The solutions were mixed in a thermo shaker (Thermomixer comfort, Eppendorf, Hamburg, Germany) at 750 rpm for 1 h at 20 °C. Subsequently, 10 μL hydrochloric acid (2 M) was added and the samples were mixed for further 5 min. Finally, 320 μL of methanol was added and the samples were shortly mixed for a few seconds. If GC-Vials were used, the solutions were filled in 500-μL micro vials. This is the optimized method which has been used in the verification experiments. The measurements for optimizing are described in Sect. "Pre sensoric selectivity."

HPLC–MS

This technique is based on an indirect analysis on an achiral stationary phase with a precolumn derivatization of the samples forming diastereomers. Before the derivatization, the amino acid stock solutions were diluted (1:100,

v/v) to a concentration of 500 μmol/L with HCl (0.075 M). The auxiliary solution contained 1 % of L-FDVA in acetone. The derivatization was performed in 1 mL GC-vials with screw caps (Agilent, Waldbronn, Germany). 50 μL of the chiral substrates, 100 μL of the auxiliary solution, and 20 μL of sodium bicarbonate (1 M) were added. The samples were derivatized in an incubator for 2 h at 37 °C. Subsequently, 10 μL hydrochloric acid (2 M) and 320 μL of methanol were added. Pro was analyzed with this technique.

HPLC–DAD

This method is a direct analysis on a chiral stationary phase without sample pretreatment. The crystalline amino acids were solved in perchloric acid (pH 1.5) in a concentration of 1 mmol/L. After a dilution in a ratio of 1:10 (v/v) with perchloric acid the samples can be analyzed. By this technique, Trp and His were analyzed.

GC–FID

The principle of this technique is the direct analysis on a chiral stationary phase and a previous achiral derivatization of the samples. Therefore, an alkylating reagent is required, which is generated from 250 μL acetyl chloride slowly added to ice cooled isopropanol. A volume of 200 μL of the amino acid stock solutions with a concentration of 50 mmol/L was completely vaporized using compressed air at a temperature at 40 °C (104 °F). After taking up the residue in 500 μL alkylating reagent, the closed GC crimp vials are heated for one hour at 95 °C (203 °F) on a heating plate. The samples were cooled to room temperature and the solvent completely vaporized. For the following acylation, 200 μL methylene chloride and 100 μL trifluoroacetic anhydride were added to the residue. After this, the samples were heated for 1 h at 65 °C (149 °F) on a heating plate. After cooling, the solvent was vaporized using a compressed air stream. Finally, the residue was taken with 100 mL methylene chloride and filled in a micro-vial (vol. 200 μL) for subsequent GC analysis. Ser and Glu were analyzed by this technique.

Instrumentation and analysis parameter

Parallel kinetic resolution and ESI–MS

The mass spectrometric analyses were carried out on an Agilent LC–MS system with the following units: G1379B vacuum degasser, G1312B binary pump, G1367C high-performance automated liquid sampler, and G1969A time-of-flight mass spectrometer (TOF–MS) with an electrospray ion source (ESI). Five microliters of the sample solution were injected with a prior needle wash. Methanol with 0.1 % formic acid and water with 0.1 % formic acid in a ratio of 90:10 (v/v) has been used as mobile phase for the sample injection and had a flow of 0.35 mL/min. The stop time was set of 0.5 min. The auto sampler was operated in the high-throughput mode starting overlapping injections after 0.3 min. The TOF–MS was operated in negative ion mode with the following parameters: nitrogen as nebulizer and drying gas, nebulizer pressure of 35 psig, drying gas flow of 10 L/min, drying gas temperature of 300 °C, capillary voltage of 4000 V, fragmentor voltage of 215 V, skimmer voltage of 60 V, and the octopol voltage was set at 250 V. The data acquisition, the extraction of the peak areas of the required masses and the integration of these peak areas were performed using "Mass Hunter Data Acquisition" as well as "Mass Hunter Qualification" (Agilent Technologies, Waldbronn, Germany). The data evaluation, in detail the calculation of the calibration, the enantiomeric excess determination, and the visualization of the results, was realized with the developed software module "Chiral MS" implemented for these special tasks using Microsoft Excel VBA.

HPLC–MS

The HPLC–MS analyses were carried out on the same Agilent LC–MS system mentioned above additionally equipped with a G1316B column compartment. Five microliters of the sample solution were injected with a prior needle wash. Water with 0.1 % formic Acid and acetonitrile was used as mobile phase in a ratio of 90:10 (v/v) for 6 min, of 20:80 (v/v) for 1 min, and of 90:10 (v/v) for the remaining run time. The flow rate was set of 1 mL/min and the stop time of 10 min. The auto sampler was operated in a simple injection mode with disabled overlapped injection. The separation was performed using a Zorbax SB-C18 Rapid Resolution column with 4.6 × 50 mm and 1.8 μm (Agilent Technologies, Waldbronn, Germany) at a column temperature of 45 °C left and right. The TOF–MS was operated in the positive ion mode with the following parameters: nitrogen as nebulizer and drying gas, nebulizer pressure of 50 psig, drying gas flow of 10 L/min, drying gas temperature of 350 °C, capillary voltage of 4000 V, fragmentor voltage of 250 V, skimmer voltage of 60 V, and octopol voltage was set at 250 V. The data acquisition, the extraction of the peak areas of the required masses, and the integration of these peak areas were performed using "Mass Hunter" modules as mentioned above. The enantiomeric excess was manually calculated using the values of the peak areas of the enantiomers.

HPLC–DAD

The HPLC–DAD analysis was carried out on an Agilent LC system with the following parts: G1322A vacuum degasser, G1311A binary pump, G1313A high-performance automated liquid sampler, G1316A column compartment, and G1315A diode array detector (DAD). The separation was performed with a Daicel Crownpak CR(+) column with $0{,}4 \times 15$ cm (Daicel, Illkirch Cedex, France). The signal of 200 nm with a bandwidth of 8 nm was stored; the reference signal was at 360 nm with a bandwidth of 100 nm. For analyzing Trp, the mobile phase is composed of perchloric acid (pH 1.5) and perchloric acid (pH 1.5) with 15 % methanol in a ratio of 34:66 (v/v) and had a flow rate of 1.2 mL/min. The temperature on both sides of the column was 25 °C (77 °F) and the stop time was 25 min. The injection volume was 10 μL. For analyzing His, the mobile phase was of perchloric acid (pH 1.5) and had a flow rate of 0.4 mL/min. The temperature on both sides of the column was 10 °C (50 °F) and the stop time was 10 min. The injection volume was 2.5 μL. The data acquisition and data evaluation were performed using the Agilent LC/MSD Chemstation. The enantiomeric excess was manually calculated using the peak areas.

GC-FID

The GC-FID analyses were carried out on an Agilent GC-System with a 6890 N (G1540 N) device and a prep and load system PAL (CTC Analytics AG, Zwingen, Switzerland). The GC oven used the following temperature program: 5 min from 80 °C (176 °F) to 128 °C (262.4 °F), 4 min at 128 °C (262.4 °F), 4 min from 128 °C (262.4 °F) to 160 °C (320 °F), 5 min from 160 °C (320 °F) to 190 °C (374 °F), and 17 min at 190 °C (374 °F). The total run time was 48.60 min. The samples were injected using the split mode at 220 °C (428 °F), 0.422 bar, a split ratio of 15.1:1 as well as a split flow of 15.1 mL/min and a total flow of 23.8 mL/min of hydrogen. The column was a L-Chirasil-Val from Macherey and Nagel (Düren, Germany) with a nominal length of 25 m and a nominal diameter of 250 μm. The initial flow was 1.0 mL/min, the nominal initial pressure 0.422 bar, the average velocity 33 cm/s, and the outlet pressure was ambient. The detector operated at a temperature of 250 °C (482 °F), a hydrogen flow of 45 mL/min, an air flow of 450 mL/min and a constant makeup gas (nitrogen) flow of 45 μL/min.

Results and discussion

The proteinogenic amino acids were classified according to their side chains in five groups. One amino acid was selected as reference substance for each group and for optimizing and verification measurements. Pro is the reference substance for amino acids with non-polar and aliphatic charged side chains, Ser for the group with polar and uncharged side chains, Trp for aromatic amino acids as well as His and Glu for amino acids with positive and negative charged side chains. A different set of reaction parameters was determined to optimize the derivatization reaction (see the following section). The method was verified in various test measurements with the five reference amino acids mentioned above (see Sect. "Intra sensoric selectivity"). Figure 2 shows the mass spectra of the both Val derivatives for various enantiomeric excesses and visualizes the change of the mass intensities depending on the enantiomeric excess of the substrate. Hence, the ratio is used for establishing a calibration curve and for subsequent enantiomeric excess calculation for samples with an unknown chiral composition.

In Fig. 3, a calibration curve of the derivatization of valine in five enantiomeric excesses is shown. The enantiomeric excess determination of D- and L-Pro has been published earlier (Fleischer and Thurow 2011). Finally, the method (especially the analysis time) was compared with the parameters of common HPLC and GC methods (see Sect. "Post-sensoric selectivity").

Pre-sensoric selectivity

Selectivity in analytical measurements can be reached by the principle of the measuring system/sensor itself (intra sensoric) or by suitable sample preparation methods before the measurement (pre sensoric). For the determination of the proteinogenic amino acids, a derivatization of the chiral substrates with mass-tagged pseudoenantiomeric auxiliaries has been used to reach a sufficient selectivity. The progress of a chemical reaction can be influenced by the derivatization temperature, the reaction time, and the time between the finish of the sample pretreatment and the subsequent mass spectrometric analysis. Pro was used as model substrate for investigation in these reaction parameters. For each test, 15 standard samples were prepared with five enantiomeric excesses (-100, -50, 0, 50, and 100ee%). The resulting calibration curves and their parameters were used for comparing the experiments. Reaction times of 0.5, 1, 2, 3, 6, 18, 24, 48, 72, and 96 h were tested. The resulting calibration curves showed no significant differences. To insure that one method can be applied to all 19 proteinogenic amino acids, the reaction time was set to 1 h for further experiments. Furthermore, reaction temperatures of 10 °C (50 °F), 20 °C (68 °F), 30 °C (86 °F), 37 °C (98.6 °F), 40 °C (104 °F), and 50 °C (122 °F) were tested. As a result, nearly identical calibration curves were obtained. Consequently, a reaction

Fig. 2 For different ee% values of the substrate Val (100, 0, −100ee%), changing mass intensities of the two derivatives with characteristic m/z-values can be observed in the mass spectra
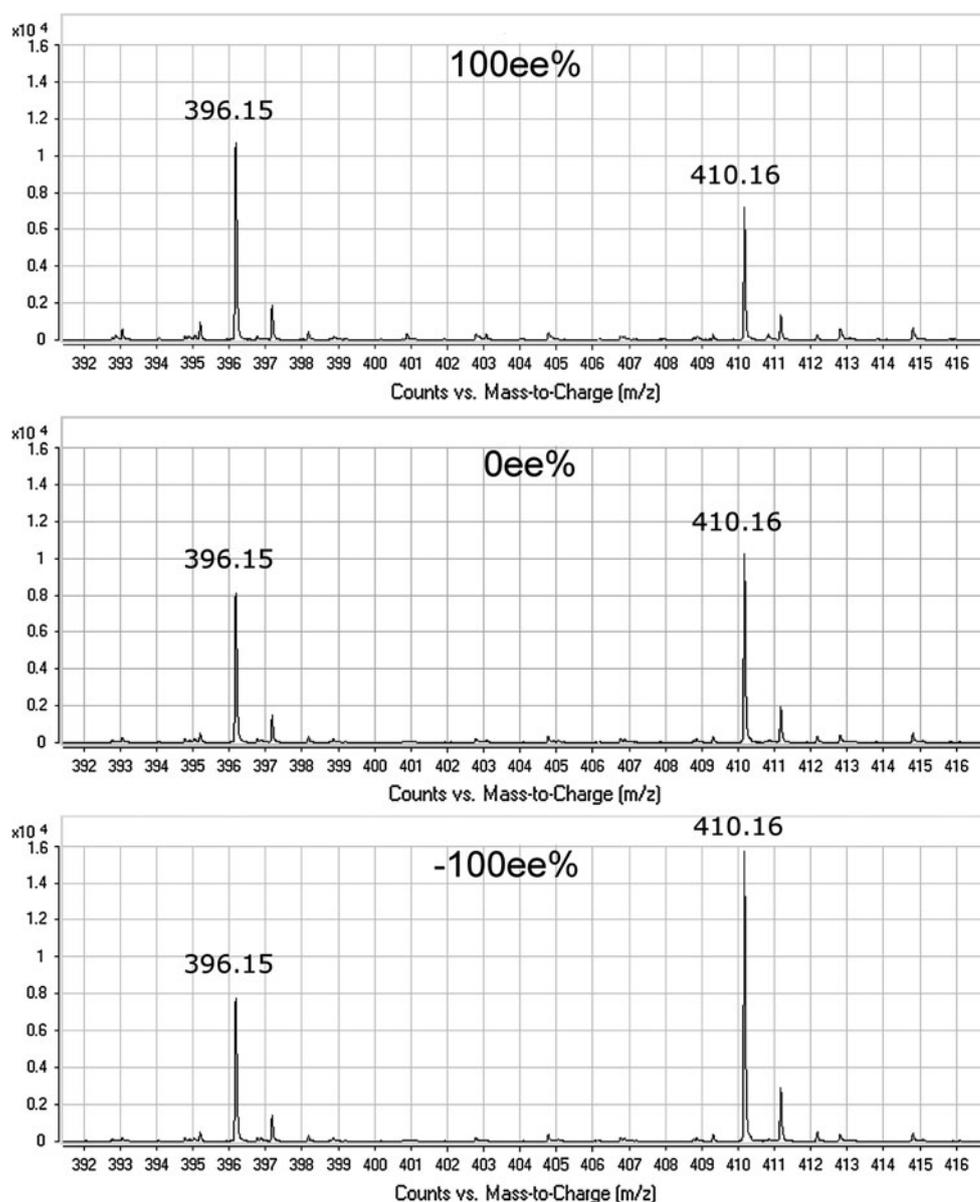
temperature of 20 °C (68 °F) was used in further experiments since it is somewhere nearing the room temperature and requires a minimal energy effort for tempering the samples. The third parameter which was investigated for the sample pretreatment is the time between the end of the sample preparation and the measurement. Therefore, the 15 Pro calibration samples were measured immediately after the sample preparation, 30 min later, after 1, 2, 3, and 4 h. The results show nearly identical calibration curves. However, it is usually recommended to measure the samples directly after the sample pretreatment without time delay. The results of these experiments show the stability of the reaction products under various conditions. Furthermore, it can be concluded that the reaction time, the reaction temperature, as well as the time between sample

preparation and measurement have no significant influence to the ratio of the diastereomeric reaction products. However, all verification measurements were performed using the parameters listed in Sect. "Sample preparation."

Intra-sensoric selectivity

In the case of the method presented, intra-sensoric selectivity was achieved by mass spectrometric analysis of the amino acid derivatives. To validate the method, various experiments were performed using the model substrates of each amino acid class. For all test series, a set of 15 calibration solutions with five enantiomeric excesses (data points) of the amino acid substrate in the range from −100, −50, 0, 50, and 100ee% and three replicate samples were
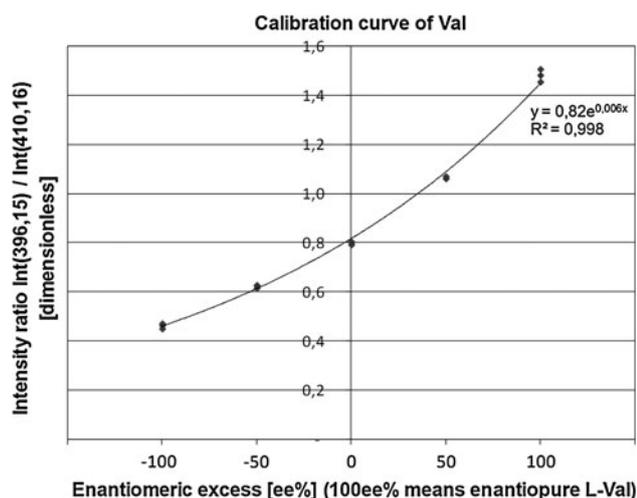
Calibration curve of Val

Fig. 3 Typical calibration curve of Val with five data points and three replicate measurements for each value

prepared. For creating the calibration curve, the intensity ratios from the characteristic masses (*m/z*-values) of the derivatives were calculated for each standard solution. The average of the intensity ratio of the three replicate samples delivers one data point in the calibration curve. Data evaluation was done according to Horwitz (1982). He defined the between-laboratory and the within-laboratory coefficient of variation (CV) based on the inter-laboratory tests and acceptable limit values. In our case, the within-laboratory CV for the calculated intensity ratio for the both derivatives of Pro did not exceed 5.2 %, of Ser max 5.3 %, of Trp max 4.8 %, of His max 5.0 %, and of Glu not exceeding 5.0 %.

Measurements for the determination of precision

The repeatability testing (intraday precision) was performed with five data points and 25 replicate samples for each data point. The measurement values of these five model substrates tested fulfill the criterion of Horwitz. The non-chiral amino acid Gly was also tested with 25 replicate samples. The results showed a relative standard deviation (RSD) of the intensity ratio of the both measured mass intensities of 3.9 %. The within-laboratory reproducibility (inter day precision) was tested with five data points and three replicate samples for each data point. At five consecutive days, one sample set was prepared and measured. As a result, every day, one calibration curve was created and finally compared with the curves of all 5 days. The calibration curves of all model substrates tested showed a good correlation concerning their points of intersection with the ordinate, their slope, and their coefficients of determination. The criterion from Horwitz was fulfilled in the measurements of Pro. The measurements of Ser and

Trp show CV values only slightly over the limit (see Table 2). The method stability was evaluated using one sample set with five data points and three replicate samples. This sample set was divided in five parts. One part was immediately measured and the remaining parts were frozen at -18 °C (-0.4 °F). At the following days, one sample part was defrosted at room temperature and measured. The results show a good correlation of the calibration curves for Pro, Ser, Trp, His, as well as Glu. The measurement precision was performed using five calibration solutions with one sample for each enantiomeric excess of the amino acid should be tested. Every sample was measured ten times. Table 2 shows the measured CV values for the five model substrates in the experiments mentioned above. The recovery rate concerning the ee% value was determined with 20–25 samples of each amino acid and enantiomeric excess values of 100, 50, 0, -50, and -100ee% on 96-well microtiter plates. The recovery rate is calculated by $RR = \overline{x}/x_R * 100$ % (with RR: recovery rate, $\overline{x}$ measured average, x_R right value/expected value). For 100, 50, -50, and -100ee% the recovery rate can be calculated, but not for the racemate with 0ee% (zero division). The recovery rates for Pro, Trp, Glu, His, and Ser were in the range between interval 79–123 % (average 99–100 %). González et al. describe the analyte recovery ranges in % depending on the concentration of the analyte (1999).

Selectivity and sensitivity

The selectivity of the auxiliaries was tested using four combinations of them. In the standard method, an equimolecular mixture of L-FDVA and D-FDLA is used. In this selectivity test, an additional set of calibration samples with five enantiomeric excess values and three replicate samples was prepared with the switched auxiliaries (D-FDVA and L-FDLA). Two calibration sets were prepared with auxiliary solutions in the same chiral configuration. For Pro and Ser, a mirror-reversed shape of the calibration curves at a usage of auxiliary solutions containing (L-FDVA and D-FDLA as well as D-FDVA and L-FDLA) was observed. A usage of auxiliaries with the same chiral configuration (L-FDVA and L-FDLA as well as D-FDVA and D-FDLA) leads to horizontal calibration curves. Identical reaction velocities between the chiral substrates and the auxiliaries with the same configuration are the reason for the lacking selectivity. In addition, the achiral amino acid Gly was tested with the four auxiliary combinations. The results show nearly the same intensity ratios for each auxiliary combination because glycine is non-chiral and the reaction kinetics between the substrate and the auxiliaries is identical. The sensitivity of the method is limited by the deviation from the expected value

Table 2 Values of the coefficient of variation (CV) for the intensity ratios for five model substrates in repeatability testing, within-laboratory reproducibility, method stability, and measurement precision

Coefficient of variation (%)	Repeatability testing		Within-laboratory reproducibility		Method stability		Measurement precision	
	min	max	min	max	min	max	min	max
Ser	2.1	4.1	0.2	5.5	0.6	5.4	1.2	3.0
Pro	1.9	2.9	0.1	3.3	0.1	2.9	0.8	1.3
Glu	3.4	5.5	2.8	24.9	3.5	22.8	1.8	5.6
His	1.4	2.4	0.4	7.1	0.8	8.2	1.1	2.9
Trp	1.5	2.6	0.2	4.2	0.1	4.2	0.8	3.0

and the standard deviations (see Sect. "Post-sensoric selectivity"). The exactness of the ee% value is without decimal places. Furthermore, the sensitivity is limited by the concentration of the chiral substrates (Pro 0.05 mmol/L-Ser 1 mmol/L-Trp 0.1 mmol/L-His 0.3 mmol/L-Glu 0.6 mmol/L) (see Sect. "Limitation of MS method"). If the chiral substrates are in lower concentrations, no proper determination of the enantiomeric excess is possible.

Application to further proteinogenic amino acids

The verification measurements mentioned above are performed with one model substrate for each amino acid class. Furthermore, the repeatability testing with 25 replicate samples was performed with each of the 19 proteinogenic amino acids. Table 3 shows the minimum and maximum determined intensity ratios, calibration equations, and coefficients of determination (R^2) for all amino acids tested. Usually, concentration-based calibrations show a linear fit. In the case of parallel kinetic resolution, no simple standard solutions are used for calibration. The calibration solutions were generated in a chemical reaction of the chiral substrates with pseudoenantiomeric mass tagged auxiliaries. The reaction kinetics between chiral substrates and auxiliaries has an important influence to the resulting reaction products, which is depending on the chemical structure of the substrates. The highest coefficients of determination were observed for amino acids with non-polar and aliphatic side chains (Ala, Pro, Val, Leu, Ile, and Met). The coefficients of variation were for Ala in the interval of 2.3–3.7 %, for Val 1.4–5.1 %, for Leu 1.7–2.9 %, for Ile 2.1–6.5 %, and for Met 1.8–3.4 %. In the group of amino acids with polar uncharged side chains, slightly lower R^2 values were determined. For Gln, no calibration could be created ($R^2 = 0.380$). The CV values were for Thr between 5.0 and 8.6 %, for Cys 4.0–6.8 %, for Asn 6.4–9.4 %, and for Gln 3.8–8.2 %. The calibrations of amino acids with aromatic side chains were created with good results. The CV values were for Phe between 1.8 and 3.1 % and for Tyr between 2.4 and 4.1 %. The calibrations of amino acids with positive and negative charged side chains delivered calibrations with R^2 values in the range of 0.955–0.990. The CV values were between 4.0 and 5.8 % for Lys and 7.5–11.2 % for Arg. The CV values were for Gln 3.4–5.5 %, and for Asp 5.3–10.6 %.

Post-sensoric selectivity

Additional selectivity can be reached in analytical measurement by a suitable data analysis and interpretation after the measurement (post-sensoric selectivity). This includes the creation of a calibration curve, the enantiomeric excess calculation for samples with an unknown chiral composition, and the data visualization. For each amino acid, standard solutions with five enantiomeric excesses and three replicate samples were prepared. The absolute standard deviation (SD) related to the average of the three replicate measurements as well as the deviation of the average from the expected value (DEV) were the main criteria for evaluation the mass spectrometric method. Both values have ee% as unit. The classic determination of the recovery rate cannot be used here since the main measuring principle is not the determination of concentrations. The method is calculating the ratios of both enantiomers instead of the exact concentrations. The standard solutions were prepared with a defined enantiomeric excess of 100, 50, 0, −50, and −100ee% with the unit ee%. The enantiomeric excess defines the x-values in calibration and the expected value. The classic calculation of the recovery rate leads in case of the enantiomeric value of the race mate (0ee%) to a division through zero, which is not defined. For this reason, the maximum DEV is given. This enables to report the recovery rate for an enantiomeric excess of 0ee%. The results are given for measurements with three replicates with the DEV in Table 4. The enantiomeric excess of amino acids with non-polar and aliphatic side chains was determined with maximum deviations of the expected value less than or equal to 10ee%. In the group of amino acids with aromatic side chains, the determination of the enantiomeric excess of Trp and Phe delivered DEV values lower and equal 10ee%. Figure 4 shows the relation between the expected value of

Table 3 Minimum and maximum determined intensity ratios, calibration equations, and coefficients of determination for all amino acids in repeatability testing with 25 replicate samples and five data points

Amino acid	Intensity ratio		Calibration equation	Coefficient of determination (R^2)
	min	max		
Gly (non-chiral)	0.72		No calibration	–
Ala	0.61	0.90	$y = 0.74e^{0.002x}$	0.997
Ser	0.58	0.86	$y = -0.001x + 0.71$	0.997
Pro	0.40	1.04	$y = 0.64e^{0.005x}$	0.999
Val	0.46	1.47	$y = 0.82e^{0.006x}$	0.998
Thr	0.85	0.91	$y = 0.88e^{-0.001x}$	0.907
Cys	0.31	1.44	$y = 0.61e^{-0.008x}$	0.978
Ile	0.38	1.29	$y = 0.72e^{0.006x}$	0.999
Leu	0.40	1.29	$y = 0.70e^{0.006x}$	0.999
Asn	0.27	0.33	$y = 0.30e^{-0.001x}$	0.999
Asp	0.50	0.77	$y = 0.65e^{-0.002x}$	0.957
Gln	1.13	1.27	$y = 1.12e^{-0.001x}$	0.380
Lys	0.86	0.98	$y = 0.92e^{0.001x}$	0.981
Glu	0.63	1.52	$y = 0.94e^{-0.004x}$	0.988
Met	0.49	1.19	$y = 0.74e^{0.004x}$	0.988
His	0.73	1.02	$y = 0.84e^{0.002x}$	0.955
Phe	0.43	1.72	$y = 0.82e^{0.007x}$	0.993
Arg	0.52	0.73	$y = 0.61e^{-0.002x}$	0.990
Tyr	0.74	1.28	$y = 0.94e^{0.003x}$	0.984
Trp	0.72	1.33	$y = 0.95e^{0.003x}$	0.982

Table 4 Values of absolute standard deviation (SD) and maximum deviation from the expected value (DEV) of selected amino acids according to the characteristics of their side chains

Amino acid	Absolute standard deviation (SD) (ee%)		Maximum deviation from expected value (DEV) (ee%)
	min	max	
Amino acids with non-polar, aliphatic side chains			
Pro	0.22	2.60	2
Leu	1.80	7.99	1
Ile	1.70	13.67	6
Val	1.16	4.79	6
Ala	11.96	23.28	7
Met	2.39	7.80	10
Amino acids with polar, uncharged side chains			
Ser	8.96	40.61	14
Cys	3.51	9.99	14
Amino acids with aromatic side chains			
Trp	0.56	14.48	3
Phe	0.90	6.50	10
Tyr	4.65	22.03	17
Amino acids with positive charged side chains			
His	6.38	22.09	11
Amino acids with negative charged side chains			
Glu	27.80	43.27	12
Asp	4.53	82.19	12

enantiomeric excess as well as the measured and calculated enantiomeric excess for the model substrates Pro and Trp.

Amino acids with polar and non-charged side chains were determined with somewhat higher SD values resulting from lower mass intensities of the derivatives of these compounds. The calibration curves of Thr, Asn, and Gln showed relatively low sensitivities (low slope of the calibration curve) and the calibration of Thr and Gln showed
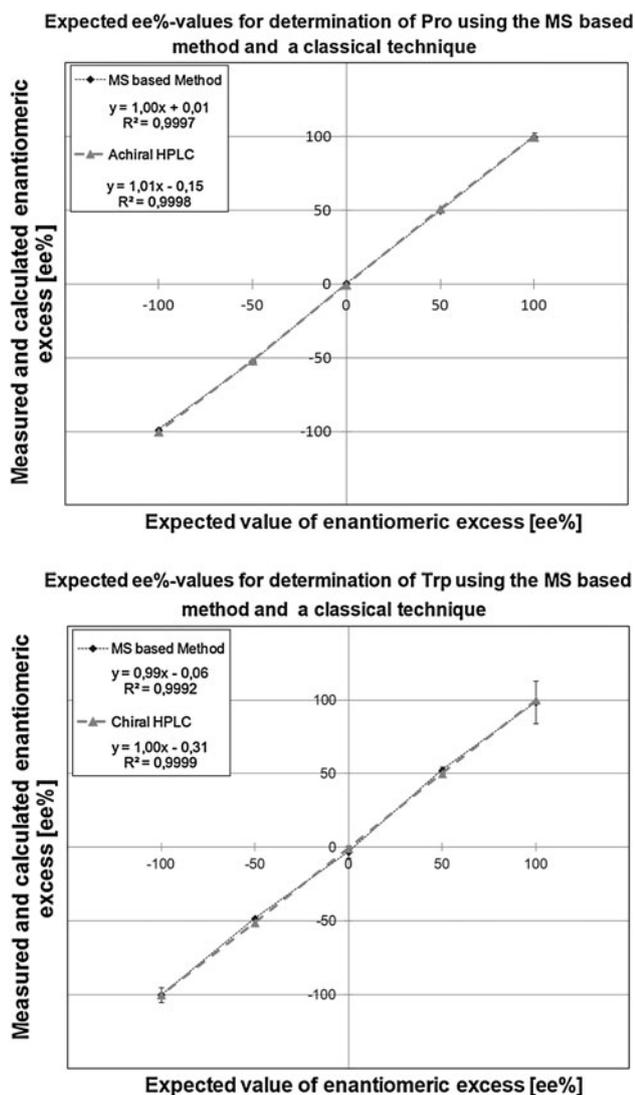
Expected ee%-values for determination of Pro using the MS based method and a classical technique

MS based Method
y = 1,00x + 0,01
R² = 0,9997

Achiral HPLC
y = 1,01x - 0,15
R² = 0,9998

Expected ee%-values for determination of Trp using the MS based method and a classical technique

MS based Method
y = 0,99x - 0,06
R² = 0,9992

Chiral HPLC
y = 1,00x - 0,31
R² = 0,9999

Fig. 4 Relation between the expected value of enantiomeric excess as well as the measured and calculated enantiomeric excess for the model substrates Pro and Trp

low coefficients of determination. This is the reason for relative high deviations in enantiomeric excess calculation for these three amino acids by the mass spectrometric

method. The results of the enantiomeric excess determination of amino acids with positive charged side chains showed acceptable results for His. The determination of Lys and Arg results in higher deviations, thus a reliable determination of the chiral composition of these amino acids requires the usage of classical analytical methods. In the group of amino acids with negative charged side chains, the enantiomeric excess determination delivered acceptable results with a DEV about 12ee% for Glu and Asp, but the SD values were in a relatively high range. Table 4 gives an overview about these values for selected amino acids.

Comparison with classical analytical methods

For comparison of the mass spectrometry-based method with classical analytical techniques, each model substrate of the five amino acid classes was measured by a suitable HPLC or GC method. Therefore, a set of amino acid standard solutions with five enantiomeric excesses and three replicate samples was prepared. The determination of the enantiomeric excess of Pro was performed by achiral HPLC–MS with a previous derivatization using Marfey's reagent (Bhushan and Brückner 2004). The enantiomeric excess of proline was calculated with a maximum SD of 1.82ee% and the maximum DEV was 2ee% (Fleischer and Thurow 2011). The total method run time with 10 min (retention times: 4.3 min for L-Pro and 4.6 min for D-Pro) per sample is more than five times higher than the run time using the chiral mass spectrometry, which requires only about 1.38 min. Table 5 gives an overview about the measurement times and total analysis times by using 96-well microtiter plates for the applied methods.

The enantiomeric excess of Ser was determined by a well-known GC-FID method with a previous achiral derivatization of the samples. The substrates were alkylated and subsequently analyzed using a chiral GC column. The observed maximum SD values were not exceeding 1.84 % and the DEV not exceeding 6ee%. The total run time for one sample was about 48.60 min, which is more than 35 times in comparison with the MS method. Trp of the group

Table 5 Comparison of the time required for the mass spectrometric-based method and various classical analytical techniques

Measurement technique	Amino Acid	Derivatization (h)	Measurement time per sample (min)	Total time per 96-well MTP (h)	Time saving (%)
Chiral MS	19 AA	1.00	1.38	3.21	
Achiral HPLC–MS	Pro	2.00	10.00	18.00	82.18
Chiral GC-FID	Ser	3.50	48.60	81.26	96.05
	Glu	3.50	48.60	81.26	96.05
Chiral HPLC–DAD	Trp	–	25.00	40.00	91.98
	His	–	10.00	16.00	79.95

Table 6 Results of enantiomeric excess determination by the MS-based method and by a classical technique for the five model substrates measured with three replicates

Substrate	MS based method		Classical technique	
	Maximum deviation from expected value (DEV)(ee%)	Maximum standard deviation (SD) (ee%)	Maximum deviation from expected value (DEV) (ee%)	Maximum standard deviation (SD) (ee%)
Pro	2	2.6	2	1.80
Ser	14	40.6	6	1.84
Trp	3	14.5	2	<0.1
His	11	17.7	2	<0.1
Glu	12	43.3	8	2.86

of amino acids with aromatic side chains was directly analyzed by HPLC–DAD using a chiral column. The observed SD values were not exceeding 0.11ee% and the DEV not exceeding 2ee%. The total analysis time was about 25 min. This is more than 18 times compared with the MS method. The analysis of His (positive charged side chain) was performed using the same technique as for Trp. The results at a total runtime of 10 min showed maximum SD values of 0.41ee% and a DEV not exceeding 2ee%. In comparison with the MS-based method, HPLC is required more than five times. The enantiomeric excess determination of Glu was performed with the same technique used in Ser analysis with the same parameters. The results delivered maximum SD values of 2.86ee% and a DEV not exceeding 8ee%. Table 6 summarizes the results of enantiomeric excess determination by the MS-based method and by a classical technique.

Limitations of the MS method

The method presented is limited in the analysis of amino acids which have the same molecular mass such as Leu and Ile. Furthermore, the limit of analytical determination was calculated using ten blank measurements and for Pro experimentally revised using various concentrations, whereby the limit was found at 0.05 mmol/L (Fleischer and Thurow 2011). The analysis of Ser delivered evaluable measurement values for concentrations higher than 1 mmol/L. For Trp, a limit of 0.1 mmol/L was calculated, for His a limit of 0.3 mmol/L, and for Glu a limit of 0.6 mmol/L. Another limitation of the method presented is found in the analysis of amino acids, which deliver identical m/z-values of the derivatives. For example, the heavier derivative of Ser and the lighter one of Thr have the same mass, also the heavier derivative of Val and the lighter ones of Ile and Leu. These amino acids cannot be analyzed using the auxiliary pair mentioned above. To address this challenge, other variants of the Marfey's reagent with another suitable mass difference can be used.

Concluding remarks

The method presented enables a rapid enantiomeric excess determination of amino acids without a previous time consuming chromatographic separation. The analytical principle is based on parallel kinetic resolution. Therefore, the amino acids were derivatized with pseudoenantiomeric mass-tagged auxiliaries to form diastereomeric reaction products, which can be measured by ESI–MS. The ratio of the mass intensities of the derivatives is used for enantiomeric excess calculation. The comparison with common HPLC and GC methods was done in the scope of analysis time. The screening procedure presented in this paper enables very short analysis times and this can be used for the screening of large amounts of samples. Samples with a positive screening result (e.g., high ee) can be analyzed in a second step using common techniques for exactly determining of the enantiomeric excess which require longer analysis times. Common methods show a higher precision than the screening method presented. The measured deviations are typical for screening procedures as described by Rouhi (2002b). The method presented enables time reduction between 79.95 % up to 96.05 % using of 96-well microtiter plates. In addition to the method development, a high-throughput screening-suitable data processing network was constructed, which allows post-run data manipulation, subsequent data evaluation, and visualization by means of commercial software and additionally implemented software with a maximum of automation. This also includes a fully automated sample preparation using liquid handlers. The method presented allows efficient high-throughput screening processes.

Conflict of interest The authors declare that they have no conflict of interest.

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