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Comparison of derivatization and chromatographic methods for GC–MS analysis of amino acid enantiomers in physiological samples

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ABSTRACT

GC–MS analysis of fluorinated and non-fluorinated chloroformate and anhydride derivatives of amino acid (AA) enantiomers on two different chiral columns was compared for the direct quantification of free L- and D-AAs in human serum and urine in a single analytical run. Best sensitivity was achieved with pentafluoropropionic anhydride/heptafluorobutanol derivatives separated on a Chirasil-L-Val column. However, the occurrence of racemization during derivatization precluded accurate quantification of AA enantiomers. Derivatization with methyl chloroformate/methanol and separation on an Rt-γDEXsa column did not exhibit racemization and yielded ten baseline separated racemates of proteinogenic AAs with resolution values greater than 2.4. However, protein and peptide hydrolysis occurred in serum and urine during the highly exothermal derivatization reaction under alkaline conditions. Removing serum proteins by precipitation before derivatization and performing the reaction at neutral pH enabled the determination of accurate free AA enantiomer concentrations. Accuracy of quantification was validated by an established nonchiral GC–MS method for AA analysis. Reliable quantification was achieved using stable-isotope labeled L-AAs as internal standards. Limits of detection (LOD) and lower limits of quantification (LLOQ) for the D-AAs were in the range of 3.2–446 nM and 0.031–1.95 μM, respectively. Relative standard deviations ($N = 6$) for the measurement of AAs in urine and serum ranged from 0.49–11.10% to 0.70–3.87%, respectively. The method was applied to the analysis of urine from 19 patients with renal insufficiency. In comparison to healthy probands, *D*-ratios of Ala, Val, Pro, Thr, Asp, and Asn were significantly increased.

1. Introduction

D-Amino acids (AAs) are excluded from the ribosomal biosynthesis of proteins and, for some time, were not thought to have any biological functions. However, they are found in all species ranging from bacteria to humans and plants [1–6]. This sparked a continued interest in the development of chromatographic and electrophoretic methods for enantioselective AA analysis [7].

The presence of D-configured AAs in prokaryotic and eukaryotic organisms is well established [8–10]. Especially bacteria have a distinct D-AA metabolism [1]. Consequently, foods treated with bacteria in fermentation and maturation processes, such as cheese, yogurt, and wine, contain high levels of D-AAs and play a central role in D-AA ingestion [4–6,11]. Additionally, bacteria in the human oral and gastrointestinal flora are a rich source of free D-AAs [2]. There is no intestinal barrier for the absorption of D-AAs and about 80–90% of the ingested D-enantiomers are absorbed by sodium-dependent transport systems and metabolized by D-amino acid oxidase (DAO) and D-aspartate oxidase in the liver and kidney [12]. With the growing knowledge of human D-AA metabolism interest arose in diagnostic applications, including their measurement in plasma and urine of patients suffering from renal insufficiency and short bowel syndrome, respectively [13,14]. Such measurements require quantification methods that offer not only appropriate chiral resolution, but also high reliability and low LOQs. Gas chromatography coupled to mass spectrometry (GC–MS) excels in robustness and selectivity, whereas HPLC methods are at the forefront of enantioselective resolution and capture the highest number of analytes.

Here, we compared systemically methods for the generation of fluorinated and non-fluorinated AA derivatives using combinations of various alcohols with anhydrides or alkyl chloroformates and their subsequent separation by GC on two different enantioselective stationary phases. Of the two most promising methods in terms of sensitivity and number of AA racemates resolved, derivatization with heptafluorobutanol and pentafluoropropionic

Abbreviations: AA, amino acids; CD, cyclodextrin; DAO, D-amino acid oxidase; GC–MS, gas chromatography–mass spectrometry; HFB, heptafluorobutanol; IPA, isopropanol; LLOQ, lower limits of quantification; LOD, limits of detection; MCF, methyl chloroformate; MeOH, methanol; PCF, propyl chloroformate; PFPA, pentafluoropropionic anhydride; PrOH, propanol; TFAA, trifluoroacetic anhydride.

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Table 1
GC methods used.

Method	Temperature program	Injection parameters	He flow (mL/min)	Derivatives analyzed and column used
A	70 °C (1 min)–10 °C/min–190 °C (30 min)	1 μL, splitless (0.5 min)	1	Phenomenex AA mix on Chirasil-L-Val after propanol/PCF and MeOH/MCF derivatization
B	70 °C (5 min)–8 °C/min–190 °C (20 min)	1 μL, splitless (0.5 min)	1	Fluka standard on Chirasil-L-Val after derivatization with HFB/MCF and all anhydride protocols
C	70 °C (5 min)–4 °C/min–150 °C (15 min)	1 μL, split (1:10)	2	Mastermix I on Chirasil-L-Val column using all derivatization protocols
D	70 °C (1 min)–4 °C/min–150 °C (5 min)–3 °C/min–190 °C (30 min)	1 μL, split (1:10)	2	Mastermix I on RT-γDEXsa using all derivatization protocols
E	70 °C (1 min)–4 °C/min–150 °C (5 min)–3 °C/min–190 °C (5 min)	1 μL, splitless (1 min)	2	MeOH/MCF derivatives on RT-γDEXsa

anhydride proved ultimately unsuited for the quantification of D-AAs because of uncontrollable racemization of L-AAs during derivatization. On the other hand, derivatization with methanol (MeOH)/MCF resulted in overquantification of AAs in physiological fluids due to the hydrolysis of proteins and peptides, which required prior protein precipitation, respectively derivatization at neutral pH before reproducible and accurate quantification of AA enantiomers could be achieved in human serum and urine specimens by means of GC–MS employing a γ-cyclodextrin (CD) based stationary phase.

2. Experimental

2.1. Chemicals

Isooctane, pyridine, heptafluorobutanol (HFB), methyl chloroformate (MCF), 1 M HCl, 1 M NaOH, D-norvaline, all solids of L and D-configured AAs, racemates of proteinogenic AAs, and an L-AA standard solution (Fluka) containing 17 proteinogenic AAs at 1 mM each in 0.1 M HCl were purchased from Sigma–Aldrich (Taufkirchen, Germany). Stock solutions in water with concentrations between 11 and 60 mM were prepared from the L- and D-AA solids. Racemic stock solutions contained enantiomers in the range of 7.0–46.5 mM. From these solutions two master mixtures were prepared. Master mix I contained all enantiomers of proteinogenic AAs in concentrations of 0.5 or 1 mM. Master mix II was prepared for the MeOH/MCF method, consisting of enantiomers detectable by the method at 1 mM each. The Phenomenex EZ:faast GC kit (Phenomenex Inc., Torrence, CA, USA) was used for the derivatization of AAs with propyl chloroformate (PCF) and propanol (PrOH). The kit included 3 L-AA standard solutions that in sum contained all proteinogenic AAs at 66.67 μM each when equal volumes of the solutions were combined. The pentafluoropropionic anhydride (PFPA)/isopropanol (IPA) and the trifluoroacetic anhydride (TFAA)/IPA kit were from Alltech (Rottenburg-Hailfingen, Germany). The [U–^{13}C, U–^{15}N] cell free amino acid mix was obtained from Euriso-top (Saint-Aubin Cedex, France). Methanol (MeOH, LC–MS grade) and chloroform (HPLC grade) were purchased from Fisher Scientific GmbH (Ulm, Germany). The Department of Organic Chemistry, University of Regensburg, provided solutions of 4 M HCl in dioxane and ether, respectively.

2.2. Biological samples

Urine specimens from 19 patients suffering from renal failure and 23 healthy donors, as well as serum from patients with minor liver damage and control serum were provided by collaborators from the University Hospital of Regensburg with the approval of the institutional review board.

2.3. GC–MS analysis

An Agilent (Waldbronn, Germany) model 6890 gas chromatograph equipped with a split/splitless injector, and a quadrupole mass selective detector model 5973 A with an EI source was used. Two different columns were tested: a Chirasil-L-Val (L-Val-tert-butylamide modified polydimethylsiloxane) column (25 m × 0.25 mm ID, 0.16 μm film thickness) from Alltech, and a RT-γDEXsa (2,3-di-acetoxy-6-O-tert-butyl-dimethylsilyl gamma CD doped into 14% cyanopropylphenyl/86% dimethyl polysiloxane) column (30 m × 0.25 mm ID, 0.25 μm film thickness) from Restek GmbH (Bad Homburg, Germany), respectively. Each column was coupled to a fused silica deactivated pre-column (10 m × 0.25 mm ID) from Agilent. The GC methods tested for the different derivatives are summarized in Table 1. The temperature of the injector and the transfer line to the mass spectrometer was kept at 250 °C except for method E, where the transfer line was operated at 230 °C. One μL of each sample was injected into a Phenomenex AGO 4680 liner. The MS detector was used either in scan mode (50–500 *m*/*z*) for preliminary experiments or in SIM mode for racemization investigations and quantification. For the SIM mode, two characteristic mass fragments were chosen for each AA detected.

2.4. Derivatization with PrOH/PCF

Derivatization with PrOH/PCF was performed as described by Kaspar et al. [15]. For initial experiments on the Chirasil-L-Val column, 200 μL of the Phenomenex AA mix were derivatized. Enantioselectivity of both chiral columns was tested with the Master mix I that contained glycine and the 19 racemic proteinogenic AAs at concentrations of 1 or 0.5 mM. For derivatization, 50 μL of Master mix I were used.

2.5. Derivatization with MeOH/MCF

Derivatization with MeOH/MCF was performed following essentially the protocol described by Villas-Bôas et al. [16]. Briefly, 100 μL of a 1 M NaOH solution were added to varying volumes (see below) of an aqueous AA solution, followed by 167 μL of MeOH and 34 μL of pyridine. Finally, MCF was added in two portions of 20 μL each. After every addition the solution was vortexed for 10 s. Villas-Bôas described completion of the reaction within 30 s after

addition of MCF [16]. The derivatives were extracted by the addition of 300 μL of chloroform. After vortexing for 15 s, the organic layer was transferred into a GC vial with a 100-μL micro-insert (VWR, Cat. No. 548-0006). For initial experiments on the Chirasil-L-Val column, 200 μL of the Phenomenex AA mix were derivatized. Fifty μL of Master mix I were prepared to investigate enantiomer separation on both chiral columns. To check for racemization, aqueous stock solutions (10 μL) of L-Ala (14.2 mM), D-Leu (34.0 mM), L-Asn (16.4 mM), and L-Met (31.0 mM) were derivatized and analyzed separately. One hundred and fifty μL of each Master mix I serial dilution were employed for calibration and 110–150 μL for the analysis of urine. The occurrence of peptide and protein hydrolysis in biological samples during derivatization required modification of the above protocol as described below.

2.6. Derivatization with HFB/MCF

Following the protocol by Zampolli et al. [17], 75 μL of HFB were added to 50 μL of an aqueous 1 mM AA standard solution, followed by 30 μL each of pyridine and MCF. The solution was vortexed for 10 s. The derivatives were extracted by the addition of 400 μL of chloroform and 40 μL of a saturated NaCl solution and vortexing for 10 s [17]. The organic phase was transferred into a GC vial with insert. Two hundred μL of Fluka AA standard solution were derivatized to investigate the total number of detectable AAs. For checking enantioselectivity of the two columns, 50 μL of Master mix I were used for derivatization.

2.7. Derivatization with IPA/PFPA or IPA/TFAA

The derivatization protocol of the PFPA–IPA AA derivatization kit from Alltech was optimized in preliminary experiments to obtain a higher and less variable derivative yield. Briefly, 30 μL of a 0.1 M HCl solution was added to an aqueous 1 mM AA standard solution, before the analytes were dried under nitrogen. Twenty μL of a mixture of IPA and acetyl chloride (5:1.25, v/v) were added to the dry residue. The vial was capped and incubated at 70 °C for 50 min. After the vial had been cooled, the excess reagent was removed at room temperature under a stream of nitrogen. In a second step, 20 μL each of isooctane and anhydride (PFPA or TFAA) were added. The vial was capped and heated at 60 °C for 20 min. After the cooled solution had been dried, the products were redissolved in 100 μL of isooctane and transferred into a vial with insert. For preliminary experiments, 100 μL of the Fluka AA standard were derivatized and the enantioselectivity of the chiral columns was tested by analyzing 50 μL of Master mix I. This protocol was implemented for both IPA/PFPA and IPA/TFAA derivatives.

2.8. Derivatization with MeOH/PFPA and MeOH/TFAA

The derivatization was performed according to the procedure described above. For the first reaction step, 20 μL of MeOH–acetyl chloride (4:1, v/v), which had been prepared at 0 °C under continuous stirring by the dropwise addition of 1.25 mL of acetyl chloride to 5 mL of MeOH, were added to the dried AA residue. Hundred μL of the Fluka standard mixture were derivatized with both reagent combinations as well as 50 μL of the AA Master mix I.

2.9. Derivatization with HFB and PFPA or TFAA

These two types of derivatives were generated in a one-step procedure. While the reagent combination of HFB and TFAA had been described originally by Zampolli et al. [18], the one-step procedure with HFB/PFPA used here has not been described previously to the best of our knowledge. The protocol includes the acidification of the aqueous AA solution with 30 μL of 0.1 M HCl before the solution was dried with nitrogen. Then, 50 μL of HFB and 100 μL of PFPA or TFAA were added in a single step to the sample. The vial was capped and heated at 95 °C for 70 min. Afterwards the solution was dried at room temperature. The products were dissolved in 100 μL of isooctane and transferred into a GC vial with insert. Hundred μL Fluka standard and 125 μL of the AA Master mix I were derivatized with both reagent combinations. The derivatization was also tested in a two-step protocol as described above.

2.10. Racemization during HFB/PFPA derivatization

Racemization during the HFB/PFPA one-step derivatization was initially investigated using enantiomer standard mixtures for Phe and Ala stereoisomers with D- to L-AA ratios of 0.3, 0.1, 0.05, 0.03, and 0.01, respectively. The final AA concentration of the solutions was 1 and 0.5 mM, respectively, for Ala and Phe. Hundred μL of each enantiomer mixture and 50 μL of a stable-isotope labeled AA internal standard mixture were derivatized and analyzed using the one-step procedure. Following these preliminary experiments, pH, temperature and anhydride concentration were varied to further investigate the cause of racemization.

One hundred μL each of five Fluka AA standard solutions ranging in concentration from 6 to 263 μM were mixed with 50 μL of stable-isotope labeled AA standards and derivatized using either 30 μL of 0.1 M HCl as catalyst or no HCl.

Twenty μL of a L-Leu stock solution (15.0 mM) and 20 μL of an L-Ala solution (56.8 mM) were derivatized six times using reaction temperatures of 65, 80 and 95 °C, respectively. At each temperature two experiments were performed using either 100 μL PFPA and 50 μL HFB or 20 μL PFPA and 20 μL HFB. To investigate the influence of the amount of anhydride employed, 20 μL of an L-Ala solution (14.2 mM) were derivatized at 95 °C with 100, 50, and 20 μL of anhydride, respectively. The alcohol volume was kept constant (50 μL) and each experiment was performed in triplicate. In addition, three aliquots were derivatized with 20 μL of PFPA kept strictly under nitrogen.

Two-step HFB/PFPA derivatization resulted in very low reaction yields probably due to the low miscibility of the aqueous HCl solution with HFB. Since HCl is required as a catalyst, organic hydrochloric acid solutions were prepared by passing gaseous HCl into dioxane and ether, respectively. Hundred μL of undiluted Fluka standard and 50 μL of internal standard were dried under nitrogen, before 20 μL each of HFB and either organic HCl solutions were added. The vial was capped and heated at 70 °C for 50 min. Further derivatization steps were performed as above.

2.11. Peptide and protein hydrolysis during MeOH/MCF derivatization

Nineteen serum samples collected from patients with minor liver damage were analyzed by both the MeOH/MCF method on a RT-γDEXsa column and the automated propanol/PCF derivatization method on a nonchiral ZB-AAA column from Phenomenex, as published recently [15].

As a result of this method comparison, the MeOH/MCF derivatization procedure was modified to avoid protein and peptide hydrolysis during the highly exothermal reaction. The addition of 100 μL NaOH (1 M) was replaced with the addition of 100 μL of water. A similar method comparison was performed for 10 urine samples using the modified MeOH/MCF derivatization protocol. Five of the urine samples were additionally prepared with the common protocol and all results were compared to those of the nonchiral automated AA method as described above.

For the derivatization of serum AA enantiomers, four different preparation strategies were performed in triplicate including the original derivatization protocol, the omission of NaOH, and with

Table 2
Number and identity of chloroformate and anhydride AA derivatives detected on a Chirasil-L-Val column using GC method A for the Phenomenex AA mix and GC method B for the Fluka standard solution, respectively.

AA	PrOH/PCF[a]	MeOH/MCF[a]	HFB/MCF	IPA/PFPA	IPA/TFAA	HFB/PFPA	HFB/TFAA	MeOH/PFPA/	MeOH/TFAA
Ala	X	X	X	X	X	X	X	X	X
Gly	X	X	X	X	X	X	X	X	X
Val	X	X	X	X	X	X	X	X	X
Leu	X	X	X	X	X	X	X	X	X
Ile	X	X	X	X	X	X	X	X	X
Pro	X	X	X	X	X	X	X	X	X
Met	X	X	X	X	X	X	–	X	–
Phe	X	X	X	X	X	X	X	X	X
Ser	–	–	–	X	X	–	–	X	X
Thr	–	–	–	X	X	–	–	X	–
Asp	X	X	–	X	X	X	X	X	X
Asn	X	–	–	–	–	–	–	–	–
Glu	–	X	–	X	X	X	X	X	X
Gln	–	–	–	–	–	–	–	–	–
Tyr	–	–	–	X	X	X	X	X	X
Trp	–	–	–	–	–	–	–	–	–
Lys	–	–	–	X	X	X	X	X	X
Arg	–	–	–	–	–	–	–	–	–
His	–	–	–	–	–	–	–	–	–
C–C	–	–	–	X	X	–	–	–	–
Total	10	10	8	15	15	12	11	14	12

[a] Derivatives only tested with the Phenomenex AA standard solution.

or without removal of proteins prior to derivatization. To precipitate proteins, 600 μL of cold methanol (−20 °C) were added to 150 μL of serum, 20 μL of internal stable-isotope labeled standard mix and 5 μL of D-norvaline standard (5.7 mM). The sample was vortexed and centrifuged at 1431 × *g* and 5 °C for 4 min. The supernatant was removed and the precipitated proteins were washed twice with 250 μL of a cold (0° C) methanol–water mixture (4:1). The supernatants were combined and evaporated to complete dryness (CombiDancer, Hettich AG, Bäch, Switzerland). Afterwards the analytes were dissolved in 175 μL of water and the derivatization was performed as described above.

The 12 aliquots were measured with method E on the Rt-γDEX column. As described below, removal of protein and derivatization without NaOH showed the best performance and was eventually used for the analysis of serum.

2.12. Calibration for the HFB/PFPA method

Calibration was performed with Master mix I that contained 19 racemic proteinogenic AAs and Gly. The stock solution contained 1 mM for the enantiomers of Ala, Ser, Thr, Gln, His, and non-racemic Gly, respectively, and 0.5 mM of all remaining enantiomers. A serial 15-point dilution of Master mix I was performed in the range of 1 mM to 0.98 μM for the high abundant and 500–0.49 μM for the low-abundant analytes, respectively. Hundred μL of standard solution were analyzed together with 50 μL of the uniformly ^{13}C, ^{15}N-labeled AA mix. The aqueous internal standard mix contained the labeled AAs in a range of 0.35–2.83 mM. The AAs were normalized by the area of the labeled AAs for the generation of calibration curves. Analysis was performed on the Chirasil-L-Val column using GC method C. A standard with a concentration of 31.25 μM for the lower and 62.5 μM for higher concentrated enantiomers was derivatized and analyzed five times to evaluate method reproducibility.

2.13. Validation of the MeOH/MCF method

Calibration was performed using a 16-point serial dilution of Master mix II prepared for the MeOH/MCF method on the γ-CD based column. It contained only AAs that could be detected by that method in a concentration range of 31 nM to 1 mM each. One hundred and fifty μL of the standard dilution and 20 μL of the internal standard mix were used. Analysis was performed on the RT-γDEXsa column using method E. Additionally, 7 method blank samples containing only internal standard solution were analyzed, because small amounts of unlabeled L- and D-AAs were found in the method blank. The internal standard mix originated from algae, which might explain the blank levels. The LODs were calculated with the following equation:

$$c(\mathrm{LOD}) = \mathrm{mean}_{\mathrm{blank}} + 3\mathrm{SD}_{\mathrm{blank}}$$

A human serum and a human urine sample were analyzed six times to test method reproducibility.

2.14. Determination of creatinine levels in urine by NMR

Urinary creatinine was measured by 2D ^{1}H–^{13}C HSQC spectra generated on a Bruker Avance III 600 MHz NMR spectrometer [19].

3. Results and discussion

3.1. Preliminary experiments

Preliminary experiments were performed using enantiopure L-AA standards from Phenomenex and Fluka, respectively, to evaluate the number of the different AA derivatives amenable to detection on the Chirasil-L-Val column, which has been commonly recommended for the separation of chiral AA derivatives [8–10,14,17,20]. Three chloroformate and 6 anhydride derivatization reactions were tested. The results are summarized in Table 2. Four amino acids (Gln, Trp, Arg, and His) were not detected on the Chirasil-L-Val column with any of the derivatization methods tested. These higher molecular weight AAs might be retained indefinitely on the column due to the fairly low maximum oven temperature of 190 °C employed to avoid degradation of the stationary phase. Alternatively, the significant column bleeding that had occurred nevertheless might have masked late eluting analytes. Among the chloroformate derivatives, Thr, Ser, Lys, Tyr, and C–C were also not detected with any derivatization reaction tested. Thr and Ser are aliphatic amino acids with a hydroxyl group that is not derivatized by the chloroformate reaction, possibly causing strong retention of these polar analytes. In contrast, anhydride derivatiza-

Table 3
Resolution of chloroformate and anhydride derivatized AA racemates on a Chirasil-L-Val column using GC method C. Master mix I was employed for derivatization and the injection volume was 1 μL. Enantiomers were considered baseline resolved with $R_s \geq 1.5$.

AA	PrOH/PCF	MeOH/MCF	HFB/MCF	IPA/PFPA	IPA/TFAA	HFB/PFPA	HFB/TFAA
Ala	1.41	1.81	2.28	3.86	6.84	4.04	5.22
Val	0.86	1.57	1.98	5.44	3.88	2.47	3.26
Leu	0	1.97	2.87	6.16	9.66	6.25	7.11
Ile	0.70	1.37	1.60	3.66	4.84	2.45	3.40
Pro	0	0	0	0	0	0	0
Met	–	0.86	1.59	3.12	–	3.17	–
Phe	–	0	0.64	2.16	2.38	2.08	2.34
Ser	–	–	–	2.28	4.32	–	–
Thr	–	–	–	1.79	0	–	–
Asp	0	0	–	0.88	1.34	0	0
Asn	0	–	–	–	–	–	–
Glu	–	0	–	2.42	3.20	2.78	2.90
Gln	–	–	–	–	–	–	–
Tyr	–	–	–	1.62	1.16	1.49	1.87
Trp	–	–	–	–	–	–	–
Lys	–	–	–	–	–	1.22	–
Arg	–	–	–	–	–	–	–
His	–	–	–	–	–	–	–
Cys	–	–	–	–	–	–	–
Total with $R_s \geq 1.5$	0	3	5	10	7	8	7

tion also derivatizes alcohols, which might explain the successful analysis of these amino acids on the Chirasil-L-Val column after IPA/PFPA, IPA/TFAA and MeOH/PFPA derivatization. Overall, of the alcohol/alkyl chloroformate combinations tested, the propanol/PCF and the methanol/MCF reaction offered the highest yield of analytes with 10 detected AAs each.

Among the anhydrides, IPA/PFPA and IPA/TFAA derivatization yielded the best results with 15 detected analytes each. Asn and Gln were not detected with either anhydride reaction, because they are hydrolyzed to form Asp and Glu during the reaction. Methanol/PFPA and methanol/TFAA derivatization allowed the detection of 14 and 12 AAs, respectively, but the peak abundances were low in comparison to the other methods. These two reactions were therefore not investigated further.

All other derivative types were investigated with regard to chiral resolution of 19 pairs of AA enantiomers. In addition to the Chirasil-L-Val column, a RT-γDEXsa column was tested. The latter could be run at a higher maximum temperature while displaying less column bleeding. The temperature program was changed for the analysis on both columns to lower heating rates and lower final temperatures. This resulted in improved enantiomer separation (data not shown) in accordance with previous literature reports [17]. The number of baseline separated AA enantiomer pairs and values of chromatographic resolution are listed in Tables 3 and 4 for all types of derivatives and both columns. Neither column showed superior performance. The advantages of Chirasil-L-Val were short run times and better selectivity for different AA types, but it offers low enantioselectivity for chloroformate derivatives. Derivatization with IPA/PFPA and separation on the Chirasil-L-Val yielded the highest number of baseline separated enantiomers (10) on this column. However, peak intensities were in general low for Ala, Val, Leu, Ile, and Met; they did not exceed a S/N ratio of 5. In contrast, derivatization with HFB/PFPA resulted in sufficient peak intensities and 8 resolved enantiomer pairs. The RT-γDEXsa column enabled reliable analysis with high peak intensities due to the absence of column bleeding. It offered the highest number of baseline separated enantiomer pairs (10) for the MeOH/MCF derivatives. However, this method was not at first investigated further because of the coelution of L-Thr/L-Asp and L-Leu/D-Ile, respectively. For the latter two analytes, separate quantification is not possible, because they are isobaric and show an identical fragmentation pattern.

Table 4
Resolution of chloroformate and anhydride derivatized AA racemates on an RT-γDEXsa column using GC method D. Master mix I was employed for derivatization and the injection volume was 1 μL. Enantiomers were considered baseline resolved with $R_s \geq 1.5$.

AA	PrOH/PCF	MeOH/MCF	HFB/MCF	IPA/PFPA	IPA/TFAA	HFB/PFPA	HFB/TFAA
Ala	1.34	4.79	0	6.68	10.27	2.08	0
Val	1.70	9.48	0	0	0	2.38	2.33
Leu	0	2.45	0	2.44	1.99	0	0
Ile	0.86	5.90	0	–	–	1.05	0.41
Pro	0	14.06	0.98	1.57	3.69	1.58	0
Met	0	3.81	0.74	0	–	2.00	–
Phe	0	0	–	1.46	1.98	2.63	5.48
Ser	–	11.33	–	6.85		8.34	–
Thr	2.13	10.64	–	0.66	5.60	2.42	–
Asp	0	7.89	–	0	0	–	–
Asn	8.90	5.99	–	–	–	–	–
Glu	0	–	–	0	0	10.16	–
Gln	–	–	–	–	–	–	–
Tyr	–	–	–	0.98	–	1.39	–
Trp	–	–	–	–	–	–	–
Lys	–	–	–	1.44	–	3.59	–
Arg	–	–	–	–	–	–	–
His	–	–	–	–	–	–	–
Cys	–	–	–	–	–	–	–
Total with $R_s \geq 1.5$	3	10	0	5	5	9	2

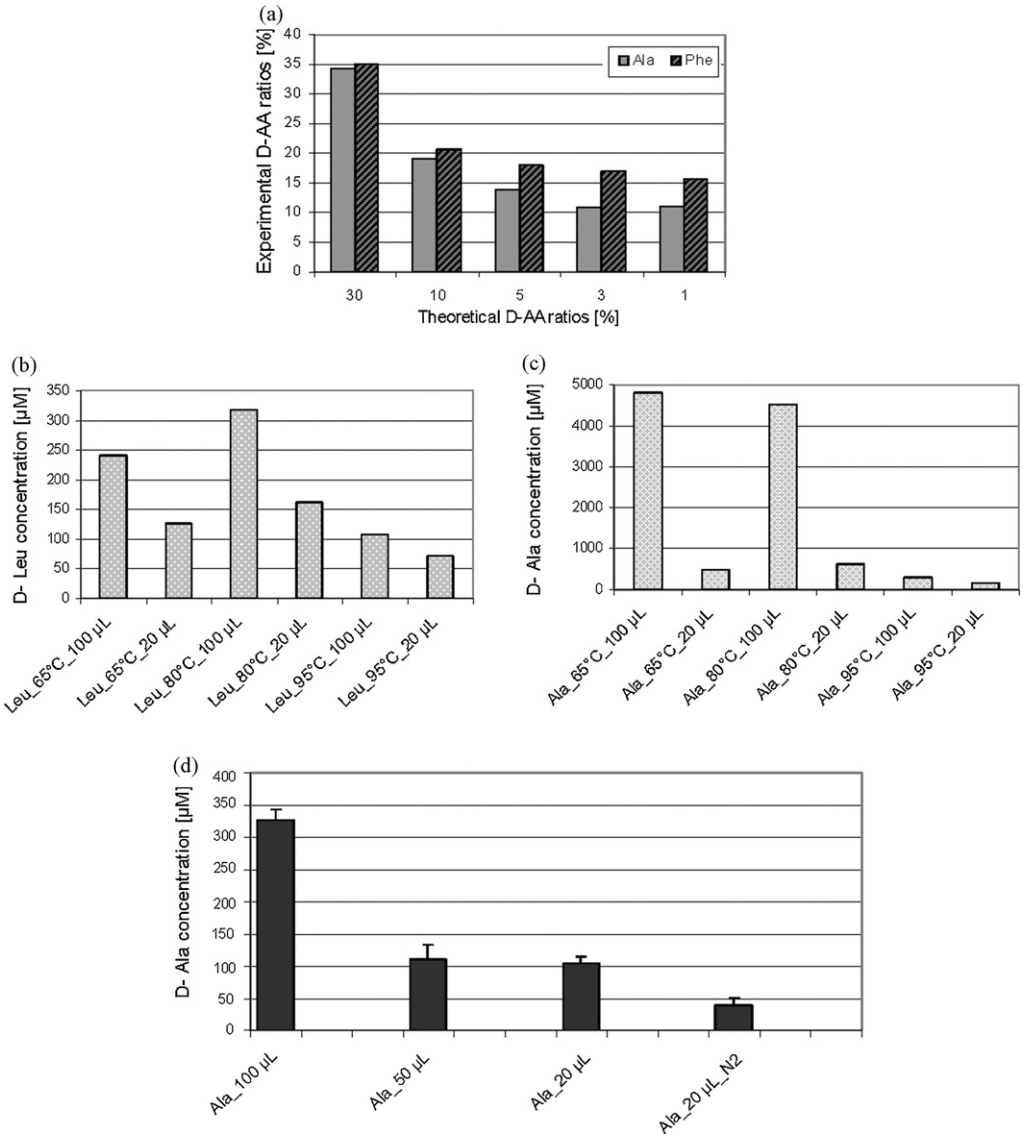

Fig. 1. Racemization during one-step derivatization with HFB/PFPA: experimentally determined D-AA ratios of Ala and Phe (a) are plotted against the theoretical D-AA ratios. The influence of temperature and anhydride volume on racemization rate was tested by derivatizing L-Leu (b) and L-Ala (c) under various conditions. (d) Dependency of racemization on PFPA volume and moisture.

3.2. HFB/PFPA method on Chirasil-L-Val

HFB/PFPA derivatization followed by separation on Chirasil-L-Val was chosen as a suitable method for quantification, because no overlapping peaks occurred and the highly volatile fluorinated derivatives exhibited best sensitivity. Eight types of AAs (Ala, Val, Leu, Ile, Met, Phe, Glu and Tyr) were baseline separated into their enantiomers within 27 min applying GC method C. Calibration in the range of 0.49 (0.98) µM to 0.5 (1) mM yielded good linearity ($0.9940 \leq R^2 \leq 0.9992$). Reproducibility was tested by derivatizing and analyzing a standard five times; relative standard deviations (RSDs) were below 5%. Next the method's applicability to correctly determine low amounts of D-AAs in the presence of excess L-AAs was investigated. Standard solutions containing defined D- to L-AA ratios of Ala and Phe were analyzed and a remarkable isomerization rate upon derivatization was observed (Fig. 1a). The lower the original D-AA ratio the larger was the bias of the determined ratios, because at low D-AA fractions the isomerization reaction proceeded faster. In general, the deviation observed for D-Phe was higher than that for D-Ala, which demonstrated the influence of the AA moiety on racemization rate. This preference of AA type for racemization was previously described in the literature [21]. Due to the method's promising linearity and good reproducibility further efforts were made to identify the cause of racemization. First, the influence of the catalyst HCl on isomerization was examined, because high racemization rates of free AAs during acetic hydrolysis of peptides had been reported [21]. Omission of the addition of HCl neither reduced formation of D-isomers nor overall derivative yields. This might be explained by the low amount of HCl (30 µL 0.1 M) used for the HFB/PFPA derivatization in contrast to the concentration (1 mL 6N) used for peptide hydrolysis [21]. More surprising was the missing catalytic effect of HCl on esterification, because no obvious decrease in D + L derivative amounts was detected in the absence of HCl.

This led to the assumption that PFPA hydrolyzes to pentafluoropropionic acid, which acidifies the solution and, thereby, catalyzes the esterification. Indeed the decomposition of fluorinated anhydrides had been already mentioned in the literature in the context of the one-step derivatization strategy [18]. To investigate the hypothesis that moisture from the air hydrolyzes PFPA to pentafluoropropionic acid that catalyzes not only esterification but also analyte isomerization, the derivatization was carried out with PFPA

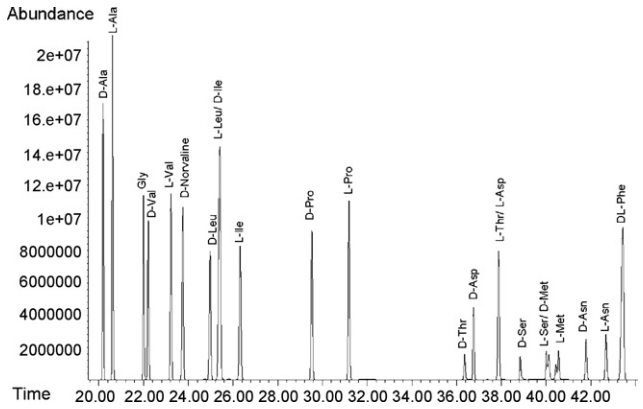

Fig. 2. Characteristic GC–MS chromatogram of AA racemates derivatized with MeOH/MCF on a Rt-γDEXsa column, applying method E.

volumes of 100 and 20 μL. In addition, the influence of temperature on isomerization was examined. The experiments were performed with L-Ala and L-Leu and the determined concentrations of the respective D-AAs are shown in Fig. 1b and c. Overall, the amount of D-AAs decreased with lower PFPA volume in the reaction mixture. The highest levels of D-enantiomer were formed at 80 °C for Leu and at 65 °C for Ala both using 100 μL PFPA. In both cases the usual reaction temperature of 95 °C yielded the lowest D-enantiomer amounts. To verify the derivatization reagent PFPA as the cause of racemization, further derivatization of L-Ala was performed in triplicate using 100, 50, and 20 μL of PFPA as well as 20 μL of PFPA that was kept under nitrogen to prove the influence of moisture. The relative amounts of D-Ala decreased with decreasing anhydride volume used for derivatization from 11.5% to 3.7%. Keeping PFPA and the reaction strictly under nitrogen resulted in a further reduction to 1.3%. This corroborated the hypothesis, that racemization is caused by pentafluoropropionic acid formed by PFPA hydrolysis in the presence of moisture. Since an excess of PFPA is required to guarantee high reaction yield, all attempts to avoid racemization by reducing anhydride volume will end in an impasse. The effort to perform the reaction under strict air exclusion was considered too complex and time-consuming so that the one-step derivatization was abandoned.

Since no racemization occurred during the two-step derivatization with anhydrides and non-fluorinated alcohols (data not shown) this approach was tested for the reagent combination of HFB and PFPA. This proved to be challenging, because the catalyst HCl was not soluble in HFB. Using HCl dissolved in dioxane or ether allowed to introduce the acid into the HFB phase. However, distinct lower peak abundances were obtained for the L-AA derivatives compared to the one-step derivatization of the Master mix I. Besides, Asp and Glu were not converted into their HFB/PFPA derivatives neither with the help of dioxane dissolved HCl nor by using acidic ether solution. These AAs are probably not soluble in HFB/dioxane and HFB/ether because of their high polarity. As the two-step approach lacked detection sensitivity, the HFB/PFPA method was finally abandoned and the focus switched to the methanol/MCF method.

3.3. MeOH/MCF derivatives on Rt-γDEXsa

Fig. 2 shows a representative chromatogram of MCF derivatized AA racemates on a RT-γDEXsa column. No D-AAs were detected upon derivatization of L-AAs with MeOH/MCF. Calibration yielded good linearities with regression square values between 0.9912 and 0.9999. Sensitivity was satisfactory with LLOQs in the nanomolar range for all D-AAs except for D-Ser (1.95 μM). Calibration of L-Ser was impeded by the overlapping peak of D-Met. No characteristic mass fragment was found for L-Ser that did not also occur in the spectrum of D-Met. Thus L-Ser occurred as a double peak, which made reliable integration difficult, as reflected by the poor linearity and a remarkably high LLOQ (31.25 μM). All determined LLOQs, LODs, regression square values and ion traces used for SIM acquisition are listed in Table 5. D-Ile and L-Leu were both separated perfectly from their mirrored molecules but could not be quantified, because they coeluted and had identical fragmentation patterns. Consequently, the method allowed the determination of 9 D-AA (Ala, Val, Leu, Pro, Thr, Asp, Ser, Met and Asn). From generated data D-AA ratios of eight different AA types could be calculated by dividing the D-AA amount by the complete amount of the AA type (D + L).

Table 5
Regression square values, LODs, LLOQs, as well as quantifier and qualifier ion traces, respectively, for the MeOH/MCF method on an Rt-γDEXsa column.

AA	R^2	LOD [μM]	LLOQ [μM]	Ion traces quantifier + qualifier
D-Ala	0.99957	0.0229	0.031	102 + 161
L-Ala	0.99916	0.0966	0.122	102 + 161
Gly	0.99924	0.0237	0.977	88 + 147
D-Val	0.99977	0.0452	0.244	130 + 98
L-Val	0.99979	0.0178	0.244	130 + 98
D-Leu	0.99993	0.0899	0.244	144 + 88
L-Ile	0.99962	0.0029	0.061	144 + 88
D-Pro	0.99967	0.0032	0.061	128 + 187
L-Pro	0.99987	0.0468	0.061	128 + 187
D-Thr	0.99930	0.0791	0.977	147 + 132
L-Thr	0.99751	0.7380	1.953	147 + 132
D-Asp	0.99959	0.0098	0.122	160 + 219
L-Asp	0.99872	0.2764	0.977	160 + 219
D-Ser	0.99972	0.4459	1.953	118 + 86
L-Ser	0.99118	16.4172	31.250	118 + 86
D-Met	0.99979	0.0402	0.488	162 + 221
L-Met	0.99911	0.0854	0.244	162 + 221
D-Asn	0.99978	0.0199	0.244	127 + 95
L-Asn	0.99990	0.0190	0.244	127 + 95
D + L-Phe	0.99891	0.0415	0.061	162 + 178

To check method accuracy, 19 serum samples were analyzed both by the enantioselective MeOH/MCF method and an established GC–MS method for achiral AA analysis [15]. Comparison of results revealed that the enantioselective MeOH/MCF method overquantified the sum of free AAs (D + L). Averaged recoveries ranged between 116.6% for Asn and 176.1% for Phe using the achiral AA method as a reference. Consequently, we hypothesized that proteins and peptides were partly hydrolyzed during the highly exothermal MCF derivatization reaction performed under alkaline conditions. To test this hypothesis, derivatization was performed at alkaline or neutral pH with or without removal of proteins before derivatization. As shown in Fig. 3a, the removal of proteins led to a distinct decrease in detected L-AA amounts, whereas replacing the base by water in the reaction mixture in the presence of proteins showed a slight increase in concentrations. Nevertheless, skipping the addition of NaOH after removal of proteins caused a further decrease in the L-AA concentrations determined. This supported the hypothesis, that protein and peptide hydrolysis had occurred during the derivatization reaction.

Prior to the second addition of MCF, the solution was slightly basic in the presence of NaOH (pH 8); afterwards, it was slightly acidic (pH 5–6). Replacement of the base with water resulted in a neutral pH-value (6–7) after the first addition of MCF and acidic conditions after the second addition (pH 1). The relatively low pH-values can be explained by the reaction of MCF with water yielding HCl. Protein hydrolysis seemed to proceed faster at acidic pH, whereas the decomposition of peptides seemed to be faster at higher pH-values >5. If the underlying mechanism were an acetylation of the N-terminus followed by an alcoholysis, both

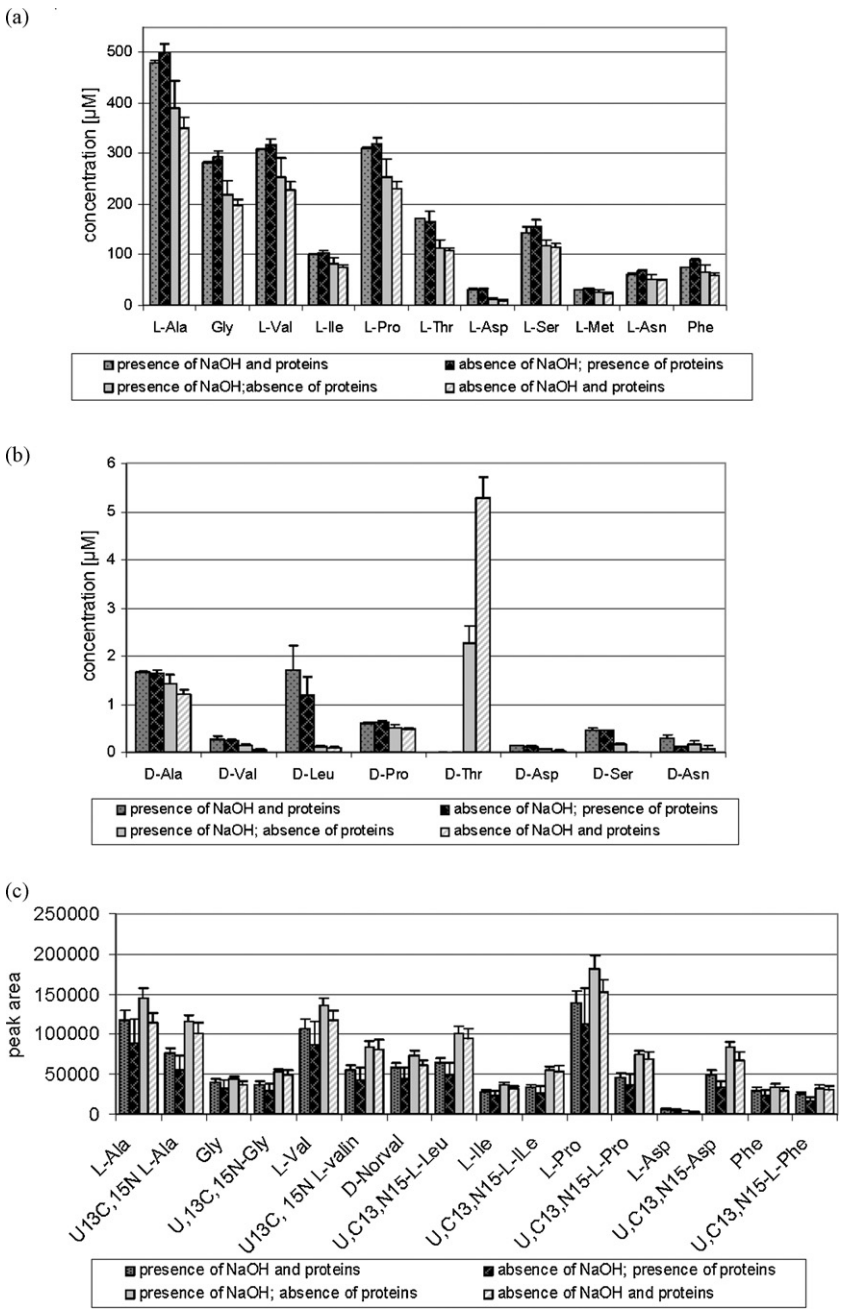

Fig. 3. Influence of NaOH and proteins on quantification of free (a) L-AA and (b) D-AA in serum by the MeOH/MCF method. (c) Reaction yields of the four different preparation strategies are shown by absolute peak areas.

derivatization reagents (MCF and methanol) would be protagonists of protein and peptide degradation. Albeit related reactions had been reported, no further efforts were made to ascertain an underlying mechanism [22].

D- and L-AAs showed similar trends for the four different preparation strategies (Fig. 3b). L-AAs contained in proteins are stronger affected by racemization than free AAs [23]. Furthermore, Frank at al. had observed a higher rate of racemization during the initial hydrolysis than several hours later for AAs such as Asp and Val [21]. They had also described an elevated isomerization rate of protein-bound Leu that was explained by a thiazoline intermediate. In contrast, the racemization rate for Thr was very low [21]. This is consistent with our results. As can be seen in Fig. 3b, the highest bias was observed for Leu, while D-Thr was the only analyte that did not decrease when proteins were removed. Consequently, derivatization had to be performed after protein precipitation and without NaOH. Analyzing the 19 serum samples again with the modified protocol, yielded recoveries between 87.2% and 119.3% using the achiral AA method as a reference.

Contrary to serum, which contains high amounts of proteins, urine of healthy volunteers contains no proteins but peptides. Here, the protein precipitation can be skipped, but base hydrolysis of the peptides can still occur. The method comparison in 10 urine samples confirmed that accurate concentrations could be obtained by the enantioselective method if the addition of base was skipped. Averaged recoveries are listed for 6 analytes in Table 6. When NaOH was used for derivatization, AA amounts were overquantified. However, the chiral method yielded accurate AA concentrations upon performance of the MeOH/MCF derivatization in the absence of proteins and NaOH.

Table 6
Method comparison between the MeOH/MCF method and an achiral GC–MS method for AA analysis in urine and serum using two different MCF derivatization procedures. Relative recoveries were calculated by dividing the combined concentrations of D- and L-amino acids obtained by the chiral method by the concentration value of the achiral method and multiplication by 100.

Averaged recovery [%]	Ala	Gly	Val	Pro	Asn	Phe
Serum (N = 19) derivatization in presence of proteins and NaOH	144.9	152.5	145.5	138.1	116.6	176.1
Serum (N = 19) derivatization in absence of proteins and NaOH	98.3	104.7	87.2	97.8	94.9	119.3
Urine (N = 5) derivatization with NaOH	105.9	125.8	117.4	116.4	120.6	110.7
Urine (N = 10) derivatization w/o NaOH	90.4	105.2	92.3	77.2	95.8	92.7

Finally, the influence of the omission of NaOH on reaction yield and, consequently, sensitivity was evaluated. Peak areas obtained with the four different preparation strategies were compared (Fig. 3c). The lower sensitivity in the analysis of urine is caused by replacing NaOH by water. Contrary to expectations the sensitivity for serum analytes stayed the same or was even improved by the removal of proteins. This might be caused by reduced analyte inclusion into precipitated proteins. In the original procedure proteins precipitated during the first addition of MCF because of the very high reaction temperature. In the optimized procedure proteins are precipitated before derivatization and the protein pellet is washed two times with a methanol/water mixture to release included analytes, which might explain the slightly increased sensitivity that also compensates the effect of omitting NaOH.

The optimized sample preparation showed excellent method reproducibility with RSDs between 0.49–11.1% and 0.70–3.87% for the replicate analyses (N = 6) of a urine and a serum sample, respectively. The RSD of D-Pro (11.1%) in urine was much higher compared to the other analytes (0.49–4.25%), most likely due to its low level in the analyzed sample, which was close to the LLOQ. In general, for the low-abundant D-AAs higher deviations were observed than for the L-AAs. D-Val, D-Leu, D-Met, D-Ser and L-Ser were not quantified above LLOQ in the serum sample. In the urine sample, only D-Met could not be quantified above its LLOQ.

3.4. Analysis of urine samples from patients with renal failure

As a diagnostic application, D-AA levels in urine specimens from patients suffering from renal failure were compared with those of healthy controls. To that end, 19 patient and 23 control samples were analyzed. Six of the eight amenable D-AA ratios were significantly increased (two-tailed t-test, $P \leq 0.05$) in urine specimens of patients with renal failure in comparison to the respective ratios determined for the healthy volunteers (Fig. 4). The P-values for Ala, Val, Pro, and Asp ranged from 0.0027 to 0.041, while those for D-Thr and D-Asn were even ≤0.001. The D-ratio of Met also appeared significantly increased, but with only three samples from the patient group exhibiting D-Met levels above LLOQ the significance of a t-test is diminished. The differences in D-AA ratios were mainly the result of significant decreases in L-AA concentrations (data not shown) normalized by creatinine levels. Relative L-AA amounts of Thr, Asn, and Gly were significantly decreased (P = 0.0035–0.032) in the patient group, while for Ala, Ile and Met the relative L-enantiomer levels were even highly significantly (P = 0.00026–0.00066) decreased. As reflected by the low urinary creatinine levels in the patient group as a consequence of reduced glomerular filtration rates, the filtration and excretion of AAs is reduced in patients with renal failure [24]. However, excretion of D-AA was not decreased. Relative D-Asp amounts seemed to be even increased, most likely due to reduced DAO and D-aspartate oxidase activities.

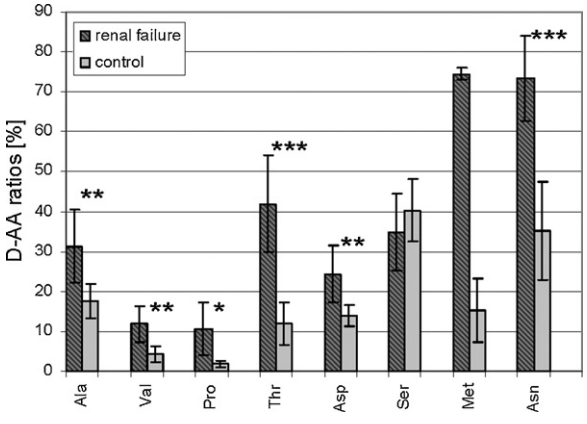

Fig. 4. Averaged D-AA ratios in urine of 19 patients suffering from renal failure compared to respective values of 23 healthy probands (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

3.5. Comparison with published methods

Comparing the chromatographic performance of 7 derivative types on two different chiral columns revealed that previously reported GC methods for the separation of anhydride derivatives on a Chirasil-L-Val column had actually not been well suited for D-AA analysis in urine and serum [9,10,14]. With 9 [9,14] or 10 [10] baseline resolved racemates of proteinogenic AAs, these methods presented comparable enantioselectivity to the HFB/PFPA method; however, resolution values of the MeOH/MCF method were superior (>2.4). Applying TFAA or PFPA for acetylation, and MeOH, propanol or IPA for esterification impeded quantification of several AAs (Asn, Asp, Gln, Glu, Ser, Thr and Tyr) [9,10,14]. Asn degraded to form Asp and, analogously, Gln degraded to form Glu. IPA/TFAA derivatives of AA carrying hydroxy groups lacked stability and thus hampered quantification [14]. With the MeOH/MCF method quantification of D-Asn, D-Asp, D-Ser, and D-Thr posed no problem. Another drawback of common methods is a rising baseline caused by strong bleeding of the Chirasil-L-Val column at elevated temperatures. Consequently, we used 150 °C instead of the commonly used 190 °C as the maximum temperature for Chirasil-L-Val, which yielded a low and constant baseline. The one-step HFB/PFPA method showed superior reproducibility and sensitivity. Nevertheless, due to racemization during derivatization, the method was not suited for D-AA quantification. Albeit the preparation of serum including removal of proteins was complex, the MeOH/MCF method exceeded in efficiency compared to formerly reported GC quantification approaches. Prior to chiral analysis of anhydride derivatives on Chirasil-L-Val, conventional achiral analysis applying ion exchange chromatography were performed to either determine D + L AA quantities [9,14] or to isolate AAs from the biological matrix [10]. Apart from Pätzold et al. [10], who used an internal standard for quantification, D-AA quantities were calculated from D + L amounts using D-ratios estimated from peak areas of the enantiomers [9,14]. Peak areas are an unreliable measure in case of the Chirasil-L-Val column, because the rising baseline masks later eluting L-AAs more than the corresponding D-AAs. Thus, accuracy of this approach is likely to be inferior to the MeOH/MCF method. Moreover, the latter method is the only technique available to date that has been shown to allow in a single analytical run the direct quantification of D-AAs in both human serum and

urine. Among the LC methods applied to physiological fluids, the one by Nagata et al. showed the best performance [13]. With 12 D-AAs amenable to quantification, applying Marfey's reagent and a conventional RP-column, the method yielded superior enantioselectivity. Nevertheless, detection limits (4–10 μM) were much higher than those for the MeOH/MCF method (3.2–446 nM). Therefore, only D-Ala, D-Ser and D-Pro were detected in plasma [13], whereas the MeOH/MCF method enabled accurate quantification of 5 D-AAs (Ala, Pro, Thr, Asn and Asp) in serum. Applying two-dimensional thin-layer chromatography for the pre-separation of AAs from the biological matrix rendered the method of Nagata et al. more elaborate, but is the pre-separation was required due to the low selectivity of fluorescence detection [13]. Further, several two-dimensional HPLC approaches using chiral columns and fluorescence detection were also not as efficient as the MeOH/MCF method, because they did not allow to separate more than one racemate per run because of disturbing matrix compounds [25–27]. Only the multi-loop two-dimensional HPLC system of Hamase et al. enabled resolution of 4 racemates of branched AAs in one run [28]. LLOQs of two-dimensional LC methods were similar to LLOQs of the MeOH/MCF method [25–28], but the GC–MS method excelled in selectivity, robustness and efficiency, because no matrix compounds appeared in the chromatogram due to mass spectrometric detection in the SIM mode. However, there were three pairs of overlapping peaks, which disturbed quantification of L-Ser, L-Leu and D-Ile. Performing the method on GCxGC-TOF-MS might resolve these overlapping peaks [29]. Overall, chiral GC columns do not allow the profiling of all D-enantiomers of proteinogenic AAs in physiological fluids, as they do not elute every AA type. Therefore, LC–MS methods should be considered for enlarging the analyte spectrum.

Acknowledgements

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