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A New *N*-Acyl Derivative of (*S*)-Cysteine for Quantitative Determination of Enantiomers of Amino Compounds by HPLC with a Precolumn Modification with *o*-Phthalaldehyde

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Abstract—*N*-Phenylacetyl-(*R*)-phenylglycyl-(*S*)-cysteine (NPPC) was used for the determination of enantiomers of primary amines by rpHPLC with a precolumn modification with *o*-phthalaldehyde. NPPC was compared with the classic SH reagent *N*-acetyl-(*S*)-cysteine (NAC) in the analysis of stereomers of nonfunctionalized amines and amino alcohols. After the NAC-modification, the resulting diastereomeric isoindoles were difficult to separate by HPLC, and satisfactory resolution was achieved only for some aliphatic amino alcohols. The use of NPPC improved the chromatographic analysis of stereomeric amino alcohols and, in addition, allowed the enantiomeric analysis of the nonfunctionalized amines. Similarity between the side radicals of the amino component and the thiol reagent favored the diastereomer separation. This method was used for determination of the absolute concentration of individual enantiomers of amines in the course of stereoselective enzymatic reactions. The optically active NPPC was prepared with a high yield by a chemoenzymatic synthesis based on a regioselective acylation of the (*S*)-cysteine amino group in aqueous medium by the action of penicillin acylase.

Key words: *amines, chemoselective acylation, chiral analysis, penicillin acylase from Alcaligenes faecalis, thiol reagent*

INTRODUCTION

The reversed phase HPLC with a precolumn modification of amino groups by a reagent composed of *o*-phthalaldehyde and optically active thiol-containing compound is one of the well-known methods of indirect determination of enantiomers of amino compounds.[2] This approach is based on a preliminary modification of the primary amino groups by the chiral reagent and subsequent separation of the resulting diastereomeric indoles on an achiral stationary phase. This method is used for enantiomeric analysis of a wide number of amino compounds, including α-amino acids [1, 2], their esters and amides [3–5], amino alcohols [5–7], arylalkylamines [8], α-alkyl-α-amino acids [3–5, 9], and nucleoamino acids [10]. For the first time, the possibility of stereomer separation was demonstrated when NAC was used as an SH-reagent [1, 2]. Further, other chiral compounds were also applied for the formation of diastereomeric adducts with OPA: various *N*-acyl derivatives of cysteine [2, 6, 11–14], thiosugars [7, 15], and 3-mercapto-2-methylpropionic acid [5]. A comparative analysis demonstrated that the structure of mercapto compound used for the modification of primary amino groups mainly determines both the diastereoselectivity of separation [2, 5–7, 16] and the stability of resulting isoindoles [5, 17]. Interpretation of the experimental results with the application of molecular modeling suggests that the mechanism of chiral discrimination of diastereomeric isoindoles is based on the different conformational rigidity of two diastereomers determined by intramolecular interactions [16]. Electrostatic interactions, hydrogen bonds, and π-interactions play the key role in this process [5, 6, 8, 16, 18].

Despite numerous attempts of applying this approach to chiral analysis of a wide number of amino compounds, the method of OPA precolumn modification is now mainly used for the determination of amino acid enantiomers [19, 20]. This limitation is connected with the fact that diastereomers of the NAC-modified nonfunctionalized amines and amino alcohols are poorly separated [6, 14] unlike the effective fractionation of the corresponding amino acid derivatives.

In this study, we demonstrated the possibility of analyzing stereomers of amino alcohols and nonfunc-

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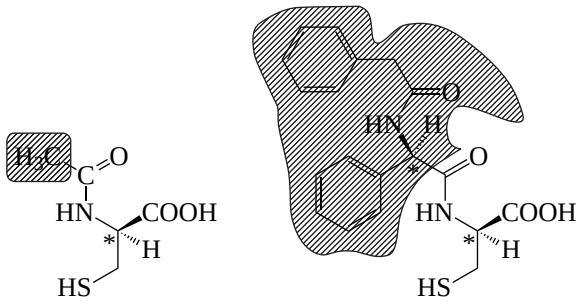
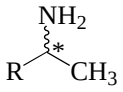
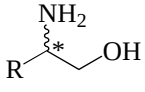
[2] Abbreviations: *Alcaligenes* PA, penicillin acylase from *Alcaligenes faecalis*; NAC, *N*-acetyl-(*S*)-cysteine; NPPC, *N*-phenylacetyl-(*R*)-phenylglycyl-(*S*)-cysteine; OPA, *o*-phthalaldehyde; and rpHPLC, reversed phase HPLC.



Scheme 1. Chiral SH-reagents for the determination of optical purity of primary amines: traditional NAC, *N*-acetyl-(*S*)-cysteine, and NPPC, a new *N*-phenylacetyl-(*R*)-phenylglycyl-(*S*)-cysteine.

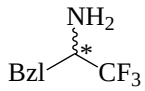




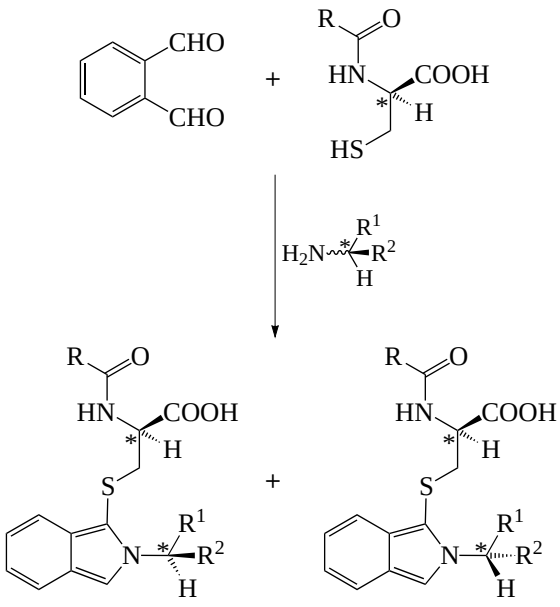
(**I**) 2-Naphtyl–
(**II**) Ph–CH_2–CH_2–
(**III**) Bzl–
(**IV**) Ph–
(**V**) *p*-Cl–Ph–
(**VI**) $(CH_3)_2CH$–$(CH_2)_3$–
(**VII**) CH_3–$(CH_2)_4$–
(**VIII**) CH_3–$(CH_2)_2$–
(**IX**) $(CH_3)_2CH$–
(**X**) $(CH_3)_2CH(OH)$–$(CH_2)_3$–

(**XI**) *p*-Cl–Ph–CH_2–
(**XII**) Ph–CH_2–
(**XIII**) Ph–
(**XIV**) CH_3–CH_2–
(**XV**) CH_3–

(**XVI**)



Scheme 2. Structures of the amines studied.



Scheme 3. Formation of diastereomeric isoindole adducts in the reaction of chiral primary amines with OPA and optically active thiol.

tionalized primary amines by HPLC with the precolumn OPA modification and with the use of NPPC, a new optically active thiol reagent. This method was successfully used both for the determination of enantiomeric excess and the absolute concentration of amine stereomers in various chemical preparations and for monitoring the stereoselective enzymatic processes without fractionation of the analyzed compounds and other components of the reaction system.

RESULTS AND DISCUSSION

Separation of Diastereomeric Isoindoles

NPPC, a new thiol reagent was proposed for the determination of enantiomers of primary amines by the rpHPLC with the precolumn OPA modification. The enantiomerically pure NPPC was synthesized by a chemoenzymatic method based on the regioselective acylation of (*S*)-cysteine amino group catalyzed by *Alcaligenes* PA [21, 22]. The proposed mercaptan and NAC, the classic SH-reagent (Scheme 1), were used for the separation of stereomers of a number of aliphatic and aromatic amines and amino alcohols (Scheme 2). The treatment of primary amines with a mixture of OPA and optically active thiol results in two diastereomeric isoindole adducts (Scheme 3) with a characteristic light absorption maximum at 340 nm. The diastereomers were analyzed by rpHPLC on a C-18 column in isocratic regime using a spectrophotometric detector.

Evidently, the parameters of chromatographic separation are determined by the structure and ionization of adducts resulting under the conditions of analysis. Preliminary experiments demonstrated that the values of capacity coefficient (k'), selectivity coefficient (α, and resolution factor (R) of the isoindole derivatives were improved when pH of mobile phase was shifted from an acidic to neutral value and acetonitrile concentration decreased. This observation is in a good agreement with the published results [10, 16] and is probably due to the dissociation of cysteine carboxyl group. Therefore, we further used a buffer with pH 6.8 as a mobile phase.

As follows from the table, the separation efficiency of the formed diastereomers (α and R parameters) depends on both the nature of amine component itself and the structure of SH-reagent. The resolution selectivity of amino alcohols is significantly higher than that of nonfuctionalized amines, which is explained by the intramolecular hydrogen bond formed between the cysteine carboxyl and the alcohol group [16]. A similar nature of side radicals of the amino component and the thiol reagent (in this case, *N*-acyl moiety of cysteine) favors the diastereomeric resolution. For example, aliphatic amino compounds [amines (**VI**)–(**VIII**) and amino alcohols (**X**), (**XIV**), and (**XV**)] are separated more efficiently with the use of classic reagent NAC, whereas the selectivity of NPPC derivatives is higher for arylamino compounds [amines (**I**), (**II**), and (**V**) and

The determination of stereomers of amino compounds by HPLC with the precolumn OPA modification using NAC and NPPC as optically active SH-reagents

Compound[A]	NAC				NPPC			
	k_1'[B]	α[C]	R[D]	EC, %[E]	k_1'[B]	α[C]	R[D]	EC, %[E]
(I)	83.5	1.00	0	20	113	1.07	1.41	35
(II)	55.9	1.06	0.67	20	95	1.10	1.65	35
(III)	24.1	1.05	1.02	20	47.6	1.00	0	35
(IV)	16.9	1.00	0	20	38.1	1.02	0.41	35
(V)	48.1	1.04	0.95	20	83.1	1.06	0.97	35
(VI)	52.2	1.13	0.80	20	18.4	1.09	0.44	40
(VII)	69.9	1.06	1.02	20	111	1.04	0.45	35
(VIII)	42.6	1.05	0.92	15	53.2	1.00	0	30
(IX)	36.4	1.02	0.36	15	45.9	1.05	0.58	30
(X)	24.5	1.23	4.15	15	23.8	1.00	0	30
(XI)	90.1	1.00	0	15	62.2	1.03	0.42	30
(XII)	31.1	1.00	0	15	26.1	1.13	2.35	30
(XIII)	21.5	1.07	1.41	15	23.2	1.18	2.96	30
(XIV)	30.2	1.23	3.86	10	10.3	1.17	2.72	30
(XV)	13.2	1.19	3.03	10	6.28	1.15	2.00	30
(XVI)	34.1	1.07	1.39	20	62.7	1.08	1.41	35

Note: [A]The corresponding structure is given in scheme 2; [B] $k_1' = t_1' - t_0$, $k_2' = t_2' - t_0$; [C] $\alpha = k_1'/k_2'$; [D] $R = (t_2' - t_1')/(W_1 + W_2)$, where t_0 is a dead time, t_1' and t_2' are the retention times of isomers, and W_1 and W_2 are the half-widths of the peak areas of isomers. [E] Eluent composition is a content of acetonitrile (by volume).

amino alcohols (**XII**) and (**XIII**)]. The most pronounced is the difference in the separation efficiency when the selectivities of NPPC and NAC isoindoles of aromatic amino compounds (**I**), (**IV**), (**XI**), and (**XII**) were compared with those of aliphatic structures (**VIII**) and (**X**).

Thus, the NAC isoindole adducts are characterized by a low diastereoselectivity of absorption, and only some aliphatic amino alcohols can satisfactorily be separated. The use of NPPC improves the analysis of stereomers of amino alcohols and provides for the determination of enantiomers of nonfunctionalized amines. A more efficient resolution of the NPPC-diastereomeric isoindoles can probably be explained by the realization of additional intramolecular interactions with phenylacetyl and phenylglycine residues of the modifier.

The elution order of diastereomeric forms was determined after the modification of individual enantiomers. For both chiral reagents, (*S*)-enantiomers of β-amino alcohols and (*R*)-enantiomers of nonfunctionalized amines (the HO-group changes the attribution of configuration according to the Cahn–Ingold–Prelog nomenclature) are eluted first.

A similar elution order was observed for the stereomers of isoindole derivatives of α-amino acids [2, 6]. The (*R*)-isomer of ε-amino alcohol is eluted before the corresponding (*S*)-isomer in the case of both reagents.

The Determination of Absolute Concentrations of Enantiomers: An Analysis of Reaction Mixture

The interaction of OPA and thiol-containing compounds with amino acids is well studied and used for the quantitative spectrophotometric determination of primary amino groups [23–25]. We could expect that the application of HPLC with precolumn OPA modification would offer the possibility of determination of not only the relative composition or enantiomeric excess, but also the absolute concentration of stereomers of amino compounds as well. It is also of interest, because this method could be used for the quantitative analysis of primary amines and their stereomers in multicomponent systems due to a characteristic absorption maximum of isoindoles at 340 nm.

The dependence of maximum optical density of the formed chromophore adduct on the OPA concentration and concentration of a sulfhydryl reagent in the reaction mixture was studied in order to determine the conditions of the most efficient modification of amino groups. The absolute concentration of primary amino groups can be determined with the use of practically equimolar amount of OPA and three to fourfold excess

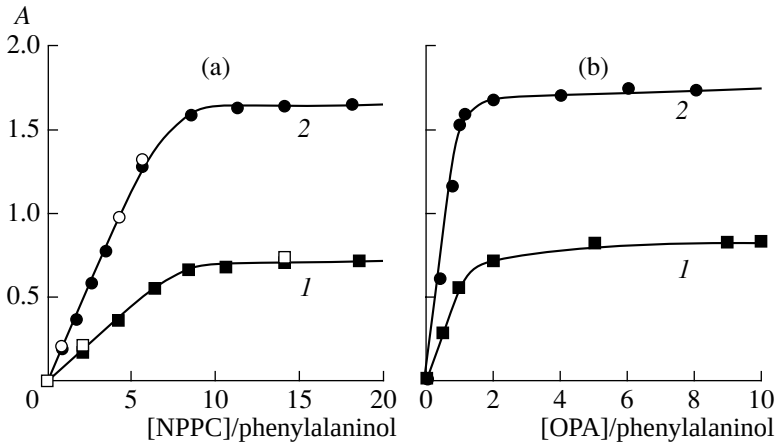


Fig. 1. The dependence of maximal value of optical absorption at 340 nm on the concentration of (a) the thiol reagent and (b) OPA in 0.4 M borate buffer (pH 9.6): (a, curve *1*) 0.1 mM phenylalaninol, (a, curve *2*) 0.25 mM phenylalaninol, (black symbols) 0.3 mM OPA or (open symbols) 3.0 mM OPA; (b, curve *1*) 0.1 M phenylalaninol, (b, curve *2*) 0.25 mM phenylalaninol, and 3 mM NPPC.

of NAC or 8–10-fold excess of NPPC (Fig. 1). The modification of all the studied compounds [except for amine (**XVI**)] took 10–15 min at an approximately 100 μM reagent concentration. The formation of chromophore adduct from amine (**XVI**) proceeded for 3 h possibly due to a low nucleophilicity of its amino group. The molar absorption coefficient of the resulting isoindoles is the same for both stereomers (ε_{340} 6000 ± 100 M^{-1} cm^{-1}) and does not depend on the nature of *N*-acyl moiety of SH-reagent.

This method is useful for the qualitative analysis of chiral amines and amino alcohols due to a high stability of the formed isoindole adducts. The absorption intensity of the samples diluted with eluent remained unchanged within 4–5 h, which is the necessary prerequisite for the qualitative analysis. The quantitative HPLC analysis of stereomers was carried out on the basis of peak areas, which were identical for two diastereomeric forms after modification of racemates of the examined amines. An analysis of standard solutions of individual enantiomers confirmed the absence of racemization in the course of modification of amino groups. The detection limit was 3–5 pmol. The linear dependence of detected signal on the concentration of determined substance for (**IV**), (**XII**),), and (**XIV**) was observed within the concentration range of enantiomers of amines from 20 pmol to 20 nmol (data not given), which indicates ample opportunities of the method.

We used our method for the spectrophotometric monitoring of the absolute concentrations of enantiomers of amino compounds in the course of two stereoselective enzymatic reactions: an enzymatic acylation of racemates of amines and amino alcohols and *Alkaligenes* PA-catalyzed hydrolysis of *N*-acyl derivatives of amines (Fig. 2). Other components of the reaction mixtures did not hinder the analysis. A good agreement was observed between the results of the stereoselective hydrolysis of *N*-phenylacetyl derivative (**XII**) monitored by an ordinary HPLC (detection at 210 nm) and by chiral analysis at 340 nm with the precolumn modification. Note that both analyses were carried out on the same chromatographic column.

Thus, the use of the new NPPC chiral thiol compound improved the HPLC analysis of stereomers of amino alcohols and allowed the enantiomeric analysis of nonfunctionalized amines with the use of the OPA precolumn modification. This method is characterized by a good resolution, sensitivity, and reproducibility of signal and can be applied to the quantitative determination of enantiomers of primary amines in both individual preparations and reaction mixtures.

EXPERIMENTAL

OPA (Koch Light, UK); NAC and phenylacetyl chloride (Sigma, United States); (*R*)- and (*S*)-phenylglycinol, and phenylacetic acid (Aldrich Chemie, Germany); (*S*)-cysteine, (*R*)-, (*S*)- and (±)-1-phenylethylamine, (*R*)- and (*S*)-phenylalaninol, (±)-2-aminopentane, (*R*)- and (*S*)-1-(2-naphtyl)ethylamine, (*R*)- and (*S*)-2-amino-1-propanol, (±)-2-amino-4-phenylbutane, (±)-2-amino-6-methylheptane, and (±)-6-amino-2-methyl-2-heptanol hydrochloride (Fluka, Switzerland); phenylmethylsulfonyl fluoride, methanol and sodium dodecyl sulfate (Merck, Germany); (±)-1-(4-chlorophenyl)ethylamine, (±)-2-heptylamine, (±)-2-aminobutanol, (±)-*p*-chlorphenylalaninol, and (±)-2-amino-3-methylbutane (Acros, Belgium); phenylacetamide (Reakhim, Russia); and acetonitrile (Kriokhrom, Russia) were used in this study. (±)-1-Benzyl-2,2,2-trifluoroethylamine was a kind gift of Professor V.A. Soloshonok (Institute of Oil Chemistry and Bioorganic Chemistry, Kiev, Ukraine). *N*-Phenylacetyl derivatives of amino compounds were synthesized as previously described [21]. (*R*)- and (*S*)-mandelic acid and its

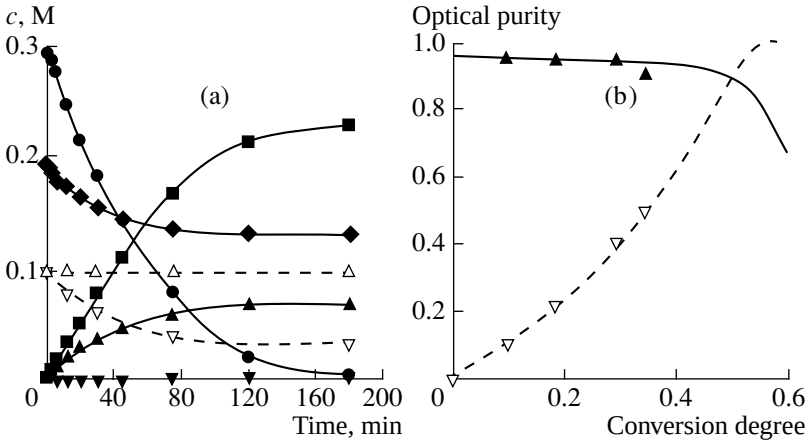


Fig. 2. Acylation of (±)-phenylalaninol with (*R*)-mandelic acid amide catalyzed by *Alcaligenes* PA. (a) Integral curves of changes in the component concentrations of the reaction mixture and (b) the dependence of enantiomeric excess on the conversion degree: (●), (*R*)-mandelic acid amide; (■), (*R*)-mandelic acid; (▲), (*R*)-mandelyl-(*S*)-phenylalaninol; (▼), (*R*)-mandelyl-(*R*)-phenylalaninol; (◆), (±)-phenylalaniol; (△), (*S*)-phenylalaninol; (▽) (*R*)-phenylalaninol. The black symbols designate the results of ordinary HPLC at 210 nm, and the open symbols indicate the data of chiral analysis by HPLC at 340 nm with the precolumn OPA modification. The solid line and the hatched line in Fig. 2b represent the calculated curves obtained by mathematic modeling of the enzymatic reaction with stereo selectivity of 50.

amides, (*R*)-phenylglycine, (*R*)-phenylglycinamide, and an *Alcaligenes* PA preparation were presented by the DSM company (the Netherlands). The concentration of active sites of penicillin acylase was determined according to [26].

HPLC-analysis. Chromatographic system consisted of a Waters M6000 module of eluent supply, a Reodyne 7125 injector with the injection loop of 50-μl volume, a Nucleosil C-18 Chrompack column (Varian, 250 × 4 mm, 5 μm), and a Waters M481 LC detector. The chromatograms were registered on a Multikhrom program-apparatus complex (Ampersand, Russia). Flow rate was 1 ml/min. A mixture of 6 mM phosphate buffer and acetonitrile was used as a mobile phase. Isoindole diastereomers were analyzed at 340 nm with the use of a 10–40 vol % gradient of acetonitrile in 6 mM phosphate buffer (pH 6.8). An HPLC analysis of chemical components was carried out at 210 nm using 6 mM phosphate buffer (pH 3.0) containing 30% (vol) acetonitrile and sodium dodecyl sulfate (0.7 g/l) as a mobile phase. The following retention times were observed: phenylglycine, 5.5 min; *N*-(*R*)-phenylglycyl-(*S*)-cysteine, 11 min; phenylglycine amide, 20 min; phenylacetamide, 4.4 min; and phenylacetic acid, 6.8 min.

The kinetics of indole adduct formation was continuously monitored at 340 nm on a Shimadzu UV-1601 spectrophotometer at 25°C in 0.4 M borate buffer (pH 9.6).

Synthesis of *N*-phenylacetyl-(*R*)-phenylglycyl-(*S*)-cysteine. NPPC was prepared by chemoenzymatic synthesis based on a regioselective acylation of amino group of (*S*)-cysteine by the action of penicillin acylase. At the first stage of the synthesis, *N*-(*R*)-phenylglycyl-(*S*)-cysteine was prepared by the acyl transfer from (*R*)-phenylglycine amide (0.3 M) to the amino group of (*S*)-cysteine (0.2 M) with the use of *Alcaligenes* PA as a catalyst at 25°C and pH 10 of aqueous medium. At the moment of maximal accumulation of the product, the reaction mixture was ajusted to pH 12; the unreacted (*R*)-phenylglycine amide was extracted with ethyl acetate, and the water phase containing *N*-(*R*)-phenylglycyl-(*S*)-cysteine was used an the next stage without any additional purification.

At the second stage, the amino group of *N*-(*R*)-phenylglycyl-(*S*)-cysteine was acylated by phenylacetyl chloride in an aqueous acetone solution at pH 7.5–8.0 and at 0–5°C in an ice bath. After the end of reaction, acetone was evaporated on a rotary evaporator and the product was extracted with ethyl acetate from the acidic solution (pH 3) and recrystallized from aqueous ethanol. The detailed procedure of the synthesis and physicochemical characteristics of NPPC will be given in our next publications.

Precolumn OPA modification of amino groups. Primary amino groups were modified as follows: a methanolic solution of SH-reagent (50 μl) containing thiol (40 mM NAC or 100 mM NPPC) and OPA (20 mM) was added to a 0.5 mM solution of amine (900 μl) in 0.4 M borate buffer (pH 9.6). The reaction mixture was stirred for 15 min, diluted with the chromatographic eluent, centrifuged for 3 min at 1200 rpm, and analyzed. The OPA solution and the thiol solution were stored for a month at +5°C in a dark container.

Quantitative analysis of the reaction mixture. A stereoselective enzymatic reactions of acylation of a racemic amine and a hydrolysis of racemates of the *N*-acylated amino compounds catalyzed by *Alcaligenes* PA were carried out in a thermostatic cell of a 719 S Titrino pH-stat (Metrohm) at 25°C, pH 10 or pH 7.5,

respectively. For the quantitative registration of changes in the component concentrations, an aliquot (50–100 μl) was taken from the reaction mixture, diluted with the eluent (1 ml, pH 3.0) for the complete solution of the reaction components and the stop of enzymatic reaction. One portion of the obtained solution was analyzed by HPLC at 210 nm, and another portion was subjected to the OPA modification (for the determination of concentrations of individual enantiomers of amino compounds) and analyzed on the same chromatographic column at 340 nm.

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