Journal of Chromatography, 476 (1989) 73–82
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1615

AUTOMATED ENANTIOSEPARATION OF AMINO ACIDS BY DERIVATIZATION WITH *o*-PHTHALDIALDEHYDE AND N-ACYLATED CYSTEINES

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SUMMARY

The enantioseparation of standard mixtures composed of protein DL-amino acids was performed by reversed-phase high-performance liquid chromatography of the corresponding diastereomeric isoindolyl derivatives, formed by automated precolumn derivatization with *o*-phthaldialdehyde (OPA) and a series of N-acyl-L-cysteines (Acyl-Cys). A photodiode-array detector, operating at 338 nm, was used for detection. In order to evaluate systematically the influence of the structures of the acyl group in the chiral thiol reagents, a series of novel N-acyl-L-cysteines was synthesized [acyl = *n*-butyryl, isobutyryl (*i*-But), pivaloyl, benzoyl) and the chromatographic behaviour of the diastereomers formed was compared with those of already known reagents, N-acetyl-L-cysteine and N-*tert.*-butyloxycarbonyl-L-cysteine. All Acyl-Cys derivatives of DL-amino acids were resolved. In particular, *i*-But-Cys gave the highest resolutions for most of the amino acid enantiomers in comparison with the other Acyl-Cys. Investigation of yoghurt using OPA–acetyl-Cys demonstrated the applicability of the method to a complex food matrix and the occurrence of D-Asp, D-Glu and D-Ala in this dairy product.

INTRODUCTION

It has been shown that the enantioseparation of mixtures of DL-amino acids can be performed by reversed-phase high-performance liquid chromatography (RP-HPLC) by converting them to the diastereomeric isoindolyl derivatives formed by pre-column derivatization with *o*-phthaldialdehyde (OPA) and chiral thiols, such as N-acetyl-L-cysteine (Ac-Cys)[1–5], N-*tert.*-butyloxycarbonyl-L-cysteine[2] and N-acetyl-D-penicillamine[4]. This successful approach has been extended to the enantioseparation of chiral amino alcohols[4], non-protein α-alkyl-α-amino acids[6,7] and α-hydroxymethyl-α-amino acids[7,8]. An intriguing aspect of this method is that an HPLC instrument dedicated to quantitative amino acid analysis by means of OPA and non-chiral thiols, such as 2-mercaptoethanol[9] or 3-mercaptopropionic acid[10], can also permit the chiral separation of amino acid enantiomers by simple changes of the reagent and the gradient programme. From the structures of the diastereomeric

0021-9673/89/$03.50

isoindolyl derivatives[3], it is also evident that the selection of appropriate chiral thiols is most important for the enantioseparation and that, with N-acyl-L-cysteines (Acyl-Cys), the structures of the acyl groups will influence drastically the elution behaviour of amino acid enantiomers. We were interested in the question of how systematically modified acyl groups in Acyl-Cys will alter and possibly improve the enantioseparation of amino acid standard mixtures containing a large number of DL-amino acids and whether the selection of suitable derivatives and chromatographic conditions will permit the detection and quantification of the D-amino acids assumed to occur in complex matrices such as fermented foods.

EXPERIMENTAL

Instruments

For HPLC an HP 1090 LC system, equipped with a photodiode-array detector and an autosampler with a device for automated precolumn derivatization of amino acids, was used. The workstation consisted of a Series 9000 computer, Model 300, with fixed disk and floppy disk, Model 9153 B, and a plotter, ColorPro Model 7440 A (all from Hewlett-Packard, Waldbronn, F.R.G.).

Chromatography

HPLC was performed using Spherisorb ODS II (3 μm) (Phase Separations, Queensferry, U.K.) as stationary phase, packed in a 125 mm × 4.6 mm I.D. column, connected to a 20 mm × 4.6 mm I.D. precolumn (Novogrom system; Grom, Herrenberg, F.R.G.); the buffer compositions and gradient programmes, are given in the legend to Fig. 1.

Chiral thiol reagents

N-Acetyl-L-cysteine (Ac-Cys) was purchased from Fluka (Buchs, Switzerland) and N-*tert.*-butyloxycarbonyl-L-cysteine (Boc-Cys) from Novabiochem (Läufelfingen, Switzerland). Other Acyl-Cys [*n*-butyryl (*n*-But), isobutyryl (*i*-But), pivaloyl (Piv) and benzoyl (Bz)] were synthesized by Schotten–Baumann bisacylation of L-cystine (Fluka), dissolved in 2 *M* sodium hydroxide solution, by addition of the respective acyl chlorides (1.1 equiv. for each amino group) at 0°C and stirring for 1 h. The solutions were acidified to pH 2.5, the bisacyl-L-cystines were extracted with ethyl acetate and the organic phase was dried with anhydrous sodium sulphate and evaporated to dryness. The oily residues were reduced to the Acyl-Cys by treatment with zinc powder in 2 *M* hydrochloric acid for 1 h[11]. After extraction with ethyl acetate and drying over sodium sulphate, the organic phases were evaporated to dryness and the Acyl-Cys were obtained as solids or oils (for *i*-But-Cys and Bz-Cys). The components were characterized by their mass spectra and had purities of ⩾95% according to thin-layer chromatography [pre-coated silica plates (Merck), solvent system chloroform–methanol–water–acetic acid (65:25:4:3)].

Chemicals and composition of amino acid standards

DL-Amino acids were of analytical-reagent grade and purchased from Sigma (St. Louis, MO, U.S.A.). Standard mixtures were composed of (a) 0.5 m*M* amino acids (*cf.*, Table I) in 0.01 *M* hydrochloric acid, (b) 2 m*M* His and (c) 2 m*M* Asn, Gln and Trp

in 20% aqueous methanol. For storage, (a) and (b) were kept in a freezer and (c) was kept at +4°C. When *i*-But-Cys and *n*-But-Cys were used for enantioseparation, 2 μl of (a), 1 μl of (b) and 0.5 μl of (c) were mixed automatically with the derivatizing reagents (see derivatization procedures). When Ac-Cys, Piv-Cys and Bz-Cys were used, 2 μl of (a) and 1 μl of (b) were mixed, and for Boc-Cys 2 μl of (c) were used.

Acetonitrile was a Baker analyzed HPLC reagent with a 190-nm UV cut-off (J. T. Baker, Deventer, The Netherlands) and sodium acetate trihydrate of *pro analysi* grade was from Merck (Darmstadt, F.R.G.). Water was doubly distilled and eluents were degassed by sonification prior to use.

Derivatization procedures

A volume of 4 μl (5 μl for Bz-Cys) of 0.133 *M* borate buffer, pH 10.4 (Pierce, Rotterdam, The Netherlands), 2 μl of OPA reagent (5 mg of OPA in 1 ml of borate buffer), 2 μl of Acyl-Cys (1 μl of Bz-Cys) reagent and appropriate amounts of standards (see above) were mixed automatically by the mixing device of the autosampler, programmed for six mixing cycles (twelve for *n*-But-Cys and *i*-But-Cys), which required approximately 3 and 6 min, respectively. Acyl-Cys reagents were prepared freshly every day by dissolving the following amounts of Acyl-Cys in borate buffer: Ac-Cys, 8; *i*-But-Cys, 20; *n*-But-Cys, 20; Piv-Cys, 10; Boc-Cys, 11; and Bz-Cys, 22 mg/ml.

For the study with yoghurt, a commercial product from the Dairy Factory of the University of Hohenheim, manufactured by the use of lyophilized starter cultures composed of *Lactobacillus bulgaricus, Lactobacillus acidophilus* and *Streptococcus thermophilus* (Laboratorium Wiesby, Niebüll, F.R.G.), was used. A 15-g amount of yoghurt and 45 ml of 80% aqueous methanol were stirred for 10 min and then centrifuged at 1630 *g*. The supernantant was evaporated *in vacuo* to a volume of *ca.* 10 ml, then 10 ml of a saturated solution of picric acid were added for deproteinization. After centrifugation at 1630 *g*, the supernatant was poured into a separating funnel and defatted twice with 20-ml portions of light petroleum (b.p. 40–60°C)–diethyl ether (1:1, v/v). The aqueous phase was passed through a Dowex 50W-X8 cation exchanger with a bed volume of 5 cm × 1 cm I.D. After washing with water, the amino acids were eluted with *ca.* 30 ml of 2 *M* aqueous amonia. The effluent was evaporated to dryness, the residue was dissolved in 0.5 ml of 0.133 *M* borate buffer and aliquots were subjected to HPLC analysis.

RESULTS AND DISCUSSION

The chiral resolution of standard mixtures, composed of the non-chiral Gly and thirteen DL-amino acids (fourteen when His was included and seventeen when His, Asn, Gln and Trp were included) by the use of various Acyl-Cys are shown in Fig. 1a–f, and the retention times and calculated resolutions of the respective pairs of amino acids are given in Table I. (It is understood in this context and in the following discussion that actually the diastereomeric isoindolyl derivatives formed from amino acids are separated by RP-HPLC.) As can be seen from the chromatograms, in principle all Acyl-Cys reagents employed resulted in the separation of the amino acid enantiomers. When the resolutions resulting from the use of the various reagents are compared, the resolutions of the individual pairs of DL-amino acids and the overall

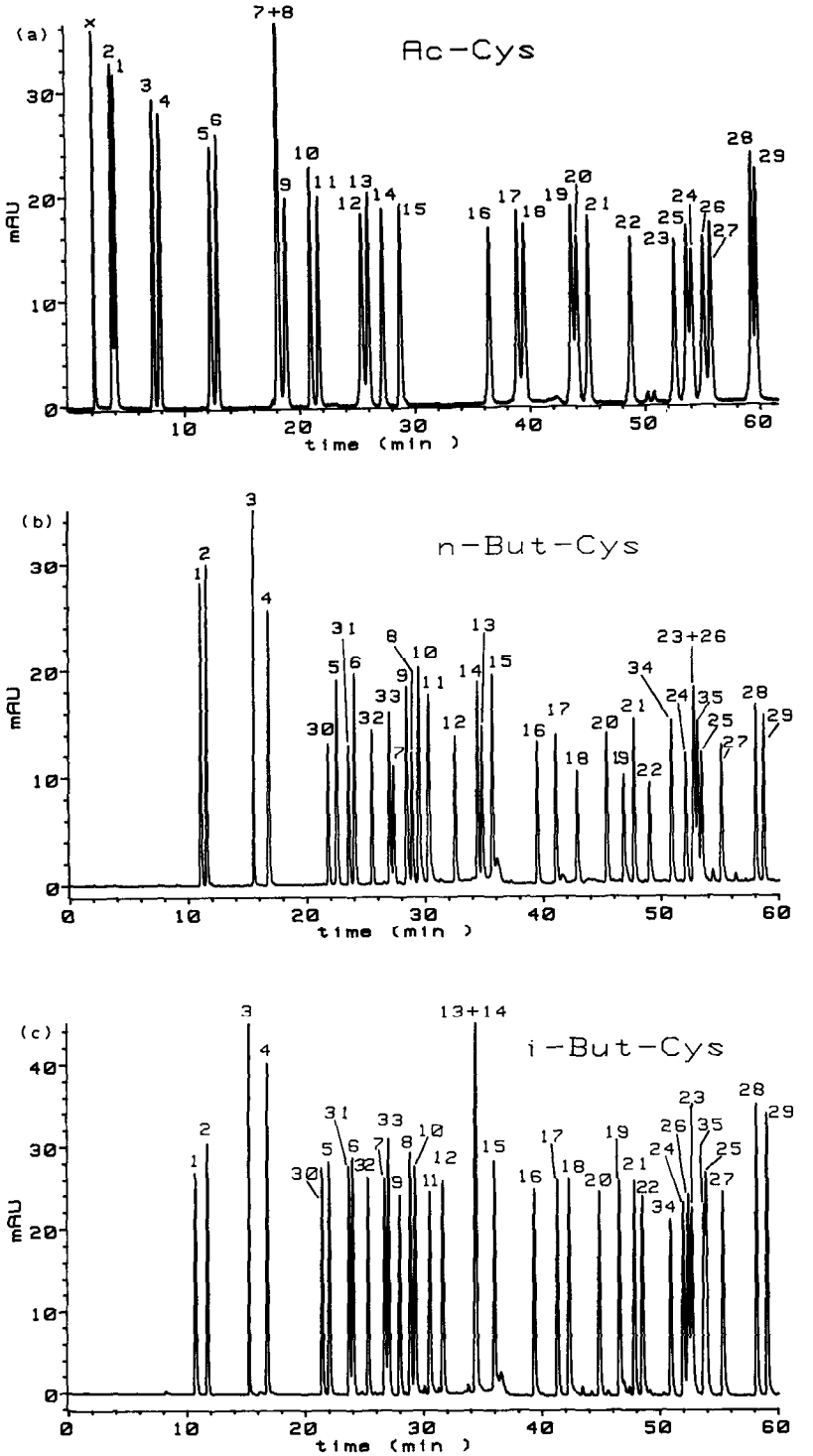
(a)
Ac-Cys
mAU
time (min)
(b)
n-But-Cys
mAU
time (min)
(c)
i-But-Cys
mAU
time (min)

Fig. 1.

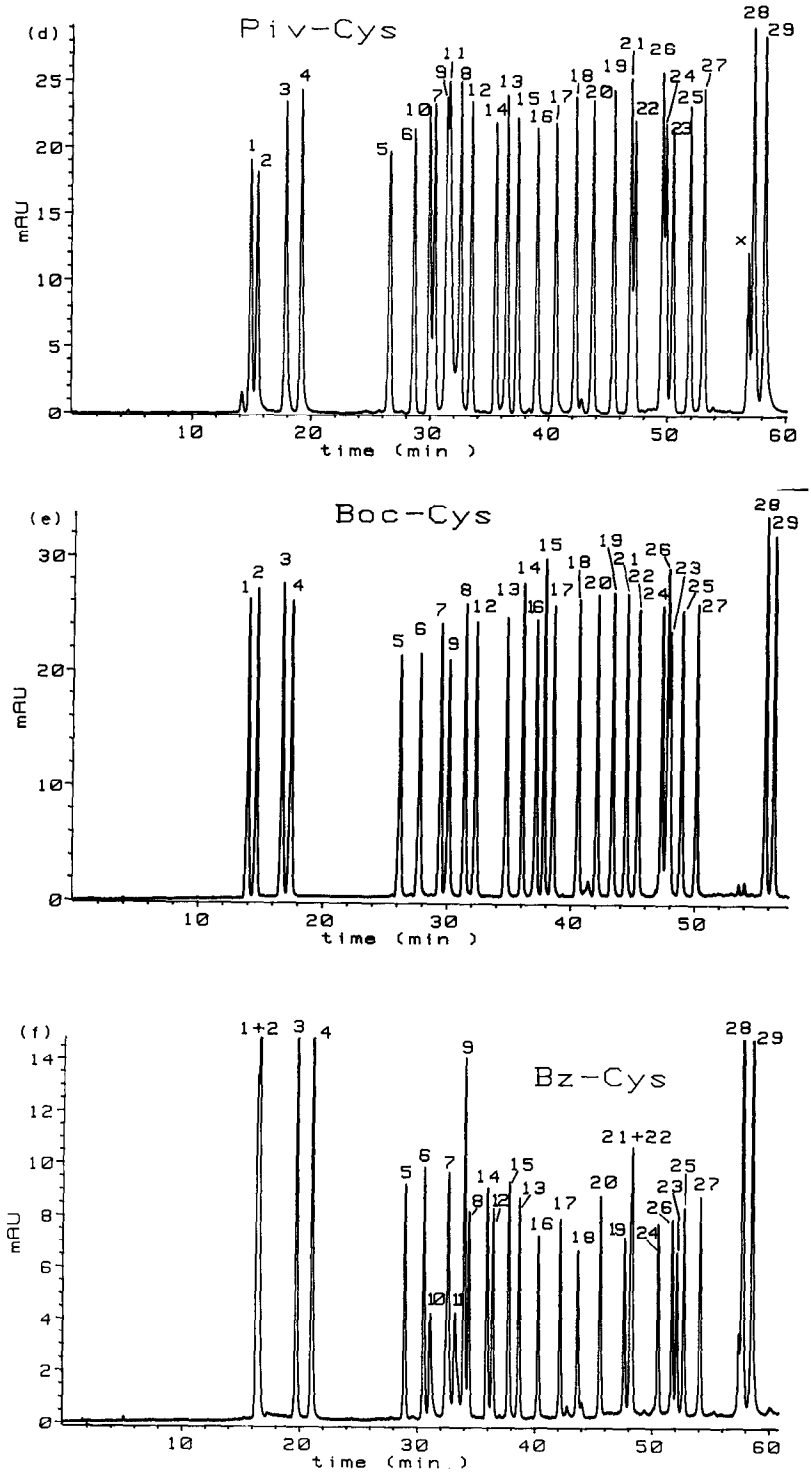

Fig. 1.

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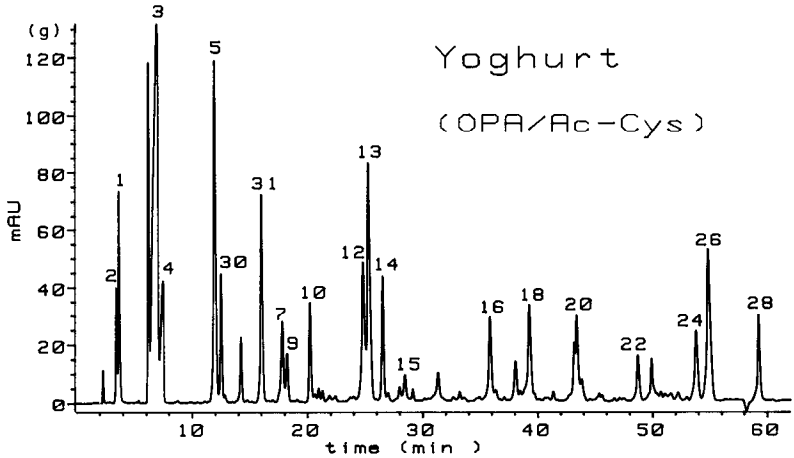

Fig. 1. Enantioseparation of diastereomeric derivatives of (a)–(f) standard mixtures of DL-amino acids and (g) yoghurt after pre-column derivatization with OPA and (a), (g) Ac-Cys, (b) *n*-But-Cys, (c) *i*-But-Cys, (d) Piv-Cys, (e) Boc-Cys and (f) Bz-Cys. Buffer gradients (a)–(g): eluent A, (a), (g) 40 m*M* sodium acetate (pH 6.5); (b) 30 m*M* sodium acetate (pH 6.5); (c) 30 m*M* sodium acetate (pH 6.5); (d) 75 m*M* sodium acetate (pH 7.5); (e) 30 m*M* sodium acetate (pH 7.2); (f) 100 m*M* sodium acetate (pH 7.2); eluent B, (a)–(g), 100% acetonitrile; linear gradient, in 60 min to *x*% B; (a), (g) 18% B; (b) 23.5% B; (c) 23% B; (d) 27.5% B; (e), (f) 29% B; column temperature, (a), (g) 26°C; (b), (c), (f) 25°C; (d) 30°C; (e) 35°C; flow-rate (a)–(g), 0.9 ml/min. pH values of the buffers were adjusted by addition of 1% acetic acid; for other abbreviations, derivatization procedures, and chromatographic conditions, see Experimental. For numbers of amino acids and assignment of the elution order and configuration, see Table I; peaks marked by x originate from reagents; mAU = milli-absorption units.

resolution of complex mixtures of DL-amino acids must be considered. Frequently, optimization of the enatioseparation of individual pairs resulted in poorer separations or peak overlap of other pairs of DL-amino acids in the chromatograms. Further, when the resolutions of amino acids due to the various Acyl-Cys reagents are compared, it must be taken into account that, owing to the different hydrophobicities of the diastereomers formed, the same buffer compositions and gradients cannot be employed. Each of the chromatograms shown in Fig. 1a–f therefore represents the most satisfactory result of about fifteen attempts to optimize the overall resolution of the respective DL-amino acid mixture. In general, it can be said that the more lipophilic the acyl group in Acyl-Cys, the higher the temperature, pH and proportion of the organic modifier and the lower the molarity of the buffer that should be in order to obtain acceptable and comparable elution times. In the present cases, the conditions were adjusted such that the last-eluted amino acid, *i.e.*, Lys, was eluted after *ca.* 60 min. The buffer conditions are given in the legend to Fig. 1.

Fig. 1a–f show the chromatograms of amino acid standard mixtures composed of thirteen (Fig. 1e), fourteen (Fig. 1a, d and f) and seventeen (Fig. 1b and c) pairs of DL-amino acids and, in addition, the non-chiral Gly. Nimura and Kinoshita[3] separated thirteen pairs of DL-amino acids by use of OPA–Ac-Cys, but failed to resolve DL-Lys. They used a combination of three gradient and two isocratic elution programs. Fig. 1a shows the enantioseparaton of thirteen pairs of DL-amino acids, including DL-Lys, with OPA–Ac-Cys using a linear gradient. However, DL-Thr is not resolved

TABLE I

ELUTION ORDER OF ENANTIOMERS OF AMINO ACIDS AFTER PRE-COLUMN DERIVATIZATION WITH OPA/ACYL-L-CYS REAGENTS

Retention times (min) of the first- (t_{R_1}) and second-eluted (t_{R_2}) enantiomers and resolution (R) are given; n.d., not determined.

Amino acid[a,b]	*Reagent*																	
	Ac-Cys[b]			*n-But-Cys*			*i-But-Cys*			*Piv-Cys*			*Boc-Cys*			*Bz-Cys*		
	t_{R_1}	t_{R_2}	R[c]	t_{R_1}	t_{R_2}	R	t_{R_1}	t_{R_2}	R	t_{R_1}	t_{R_2}	R	t_{R_1}	t_{R_2}	R	t_{R_1}	t_{R_2}	R
1,2 LD-Asp	–	–	–	11.1	11.6	2.1	10.7	11.8	3.6	14.9	15.5	1.4	14.0	14.7	2.3	16.3	16.5	0.5
2,1 DL-Asp[b]	3.8	4.1	1.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3,4 LD-Glu	7.4	8.0	1.5	15.6	16.8	5.9	15.3	16.8	7.8	17.9	19.2	3.2	16.7	17.4	2.2	19.7	21.0	4.0
5,6 LD-Ser	12.3	12.9	1.7	22.6	24.1	5.0	22.0	24.0	6.5	26.7	28.7	4.7	26.2	27.8	4.4	28.9	30.5	4.7
7,8 LD-Thr	18.2	18.2	0.0	27.4	28.9	4.9	26.7	28.9	6.8	30.4	32.6	5.3	29.5	31.4	6.0	32.6	34.3	4.5
9 Gly	18.4	–	–	28.5	–	–	28.0	–	–	31.4	–	–	30.1	–	–	34.0	–	–
10,11 LD-His	21.0	21.6	1.9	29.5	30.3	2.1	29.3	30.5	3.4	30.0	31.6	3.4	n.d.	n.d.	–	31.1	33.2	4.1
12,13 LD-Ala	25.4	26.0	1.2	32.6	34.8	6.9	31.6	34.4	7.9	33.5	36.5	7.4	32.3	34.8	7.2	36.4	38.6	6.6
14,15 LD-Arg	27.2	28.8	3.3	34.5	35.7	3.9	34.4	36.0	4.6	35.6	37.4	4.4	36.0	37.8	5.9	35.9	37.7	4.9
16,17 LD-Tyr	36.5	38.9	5.1	39.5	41.1	5.1	39.4	41.3	5.8	39.0	40.6	3.9	37.1	38.5	4.5	40.3	42.1	5.6
18,19 LD-Val	39.5	43.6	8.0	42.9	46.8	11.2	42.3	46.5	11.8	42.3	45.5	8.2	40.6	43.3	8.6	43.6	47.6	12.4
20,21 LD-Met	44.1	45.1	1.9	45.4	47.7	6.9	44.9	47.8	8.4	43.7	46.9	8.0	42.1	44.4	7.7	45.5	48.2	6.9
22,23 LD-Ile	48.8	52.6	7.4	49.0	52.8	10.1	48.5	52.7	11.2	47.3	50.5	8.1	45.4	47.9	8.3	48.2	52.1	10.1
24,25 LD-Phe	–	–	–	52.1	53.5	4.0	52.0	53.9	5.1	49.9	51.9	5.4	47.3	38.9	5.0	50.5	52.7	6.5
25,24 DL-Phe[b]	53.6	54.1	0.8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
26,27 LD-Leu	55.1	55.7	1.2	52.8	55.1	6.1	52.4	55.3	8.0	49.6	53.1	8.7	47.7	50.1	7.5	51.7	54.1	7.3
28,29 LD-Lys	59.3	59.7	0.9	58.1	58.7	2.0	58.2	59.1	2.8	57.3	58.3	2.2	55.7	56.4	2.2	57.7	58.5	2.4
30,31 LD-Asn	12.7	13.7	2.3	21.8	23.6	6.5	21.4	23.7	8.0	25.7	27.5	4.0	25.7	27.2	4.4	27.1	28.3	3.1
32,33 LD-Gln	16.5	18.0	3.2	25.5	27.0	5.2	25.3	27.1	6.0	28.8	30.3	3.4	28.0	29.4	4.0	29.8	31.6	4.5
34,35 LD-Trp	51.5	53.2	2.7	50.9	53.1	6.3	50.9	53.7	8.8	48.7	51.1	5.2	45.4	47.6	6.5	49.3	51.6	5.7

[a] Peak numbers 1–35 refer to the elution order of amino acids in the chromatograms shown in Fig. 1a–g.

[b] Exceptions to the elution order of amino acids (L before D) are Asp and Phe when Ac-Cys is used as reagent; only in these instances is the respective D-amino acid eluted before the L-amino acid.

[c] Resolution R is calculated according to the formula $R = (t_{R_1} - t_{R_2})/(w_1 + w_2)$, where w_1 and w_2 are the peak widths at half-height of the first- and second-eluted peaks of amino acid derivatives. Resolutions of amino acids not shown in the chromatograms were calculated from retention times determined in separate runs.

under these conditions. Employing linear gradient programmes, Buck and Krummen[2] separated five pairs of DL-amino acids using OPA–Ac-Cys, and ten pairs of DL-amino acids using OPA–Boc-Cys. Fig. 1e shows the separation of thirteen pairs of DL-amino acids with OPA–Boc-Cys reagent.

From the chromatograms shown in Fig. 1, it is also obvious that all the Acyl-Cys reagents used are capable of separating mixtures composed of a large number of DL-amino acids. Compared with Ac-Cys and Boc-Lys, Piv-Cys and Bz-Cys offer no advantage when applied to amino acid mixtures under the conditions used, although several pairs of amino acids (His, Val and Phe), derivatized with the latter reagent, show the highest resolutions (*cf.*, Table I).

Remarkably, when the various Acyl-Cys reagents are applied to DL-amino acids the highest resolution is found for DL-Val, with the exception that the use of Piv-Cys gives a slightly higher resolution for DL-Leu (*cf.*, Table I). Further, among the various Acyl-Cys reagents, in most instances *i*-But-Cys effects the highest resolutions for amino acid enantiomers (*cf.*, Table I). It is assumed, therefore, that the isopropyl group, which represents the valyl side-chain and is also part of the isobutyryl group, is responsible for this exceptionally high degree of chiral resolution. This is in agreement with the findings that suitably derivatized L-valine when bonded as a chiral selector to silica gel and used in liquid chromatography[12,13], or when attached to a polysiloxane matrix and used as the stationary phase in gas chromatography[14,15], or when valine is derivatized to so-called ureido phases[16], this amino acid shows a very high degree of chiral recognition. Further, when discussing the chiral resolution of amino acids, one must remember that very often the enantioseparation of a large number of amino acids is not necessary. For example, for proving the optical purity of amino acids, the detection of minor amounts of one enantiomer together in a large excess of the other is essential. In these instances, very high resolutions of the diastereomeric amino acid pairs are advantageous and, in order to obtain high accuracy, the minor enantiomers should be eluted first. In the cases investigated, the L-enantiomers of amino acids were eluted before the D-enantiomers. However, when Ac-Cys was used as the reagent, D-Asp and D-Phe were eluted before the respective L-enantiomers (*cf.*, Table I). As the elution order is reversed when Acyl-L-Cys is replaced with Acyl-D-Cys, the order of emergence of enantiomers is easily reversible by choosing the appropriate chiral reagent.

Application of the method to complex food samples, with Ac-Cys as a commercially available chiral reagent, is exemplified by yoghurt (Fig. 1g) and confirms[17,18] the presence of D-Asp (32.2%), D-Glu (16.4%) and D-Ala (62.0%), calculated with respect to the L-enantiomers. The relative amounts and the kinds of D-amino acids occurring in yoghurt depend on the manufacturing process and starter cultures used. The use of *n*-But-Cys for the determination of D-amino acids in yoghurt and the employment of capillary gas chromatography and the chiral stationary phase Chirasil-Val for the detection of D-amino acids in processed foodstuffs have been reported previously[17,18].

CONCLUSION

From the results it is clear that systematically modified acyl groups in Acyl-Cys, applied together with OPA to DL-amino acids, make it possible to adjust the

hydrophobicities and conformations of the resulting diastereomeric isoindolyl derivatives. Hence it is possible, by selection of appropriate reagents and chromatographic conditions, to optimize the enantioseparation of individual pairs of amino acids and also of complex mixtures consisting of a large number of amino acids, such as those occurring, *e.g.*, in foodstuffs or biomatrices.

It is expected by analogy that much more suitable Acyl-Cys, or N-substituted cysteines in general, will be found for the high-performance enantio separation of chiral amino components. The use of cysteine as a chiral thiol component has the advantage that both enantiomers are available as economic building blocks of high optical purity for derivatization procedures. Moreover, in this instance, the almost unlimited range of N-terminal protecting groups employed by peptide chemists[19] can be considered as candidates for the design of many more N-substituted cysteines suitable for the enantioseparation of amino acids.

The liquid chromatographic approach has the advantage, in contrast to gas chromatography[14], that acid-sensitive amino acids, such as asparagine, glutamine and tryptophan, or those requiring special derivatization conditions for gas chromatography, such as histidine and arginine, can be determined using the routine procedures described above, and that these derivatization procedures for chiral amino acid analyses are fully automated.

A disadvantage of the OPA–thiol approach is that no amino acids with secondary amino groups, such as proline or hydroxyproline, can be determined directly. This major drawback is overcome in the HP 1090 LC analyser (AminoQuant system) used for quantitative, non-chiral amino acid analysis with OPA–2-mercaptopropionic acid, by applying an additional derivatization with 9-fluorenyl chloroformate (Fmoc-Cl)[20]. However, by analogy it is assumed that use of chiral variations of this reagent, *e.g.*, (+)-1-(9-fluorenyl)ethyl chloroformate (Flec-Cl)[21], will overcome this shortcoming.

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