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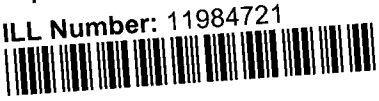

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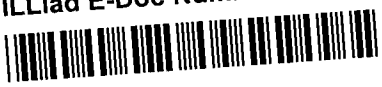

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JOURNAL OF CHROMATOGRAPHY A

Short Communication

Reversed-phase high-performance liquid chromatography of the stereoisomers of some sweetener peptides with a helical nickel(II) chelate in the mobile phase

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Abstract

The use of a chiral mobile phase additive in the form of the helically distorted, square-planar, chiral nickel(II) chelate *dl*-[4,4'-(1-methyl-2-propylethane-1,2-diyldiimino)bis(pent-3-en-2-onato)]nickel(II) was investigated for the resolution of optical isomers of dipeptide-type sweeteners, *viz.*, aspartame, alitame and antiaspartame, and some of their decomposition products, *e.g.*, diketopiperazines. The chiral discrimination mechanism for the solutes was elucidated. The proposed chiral RP-HPLC system was applied to the stereoselective determination of aspartame impurities in samples of its commercial dietetic and pharmaceutical formulations.

1. Introduction

The sweetness of non-nutritive dipeptide-type sweeteners (see Fig. 1), *e.g.*, N-aspartylphenylalanine methyl ester (aspartame, **1**), aspartyl-N-(tetramethyl-3-thietanyl)alaninamide (alitame, **2**) and N-acetylphenylalanyllysine (antiaspartame, **3**), is strictly related to only the single

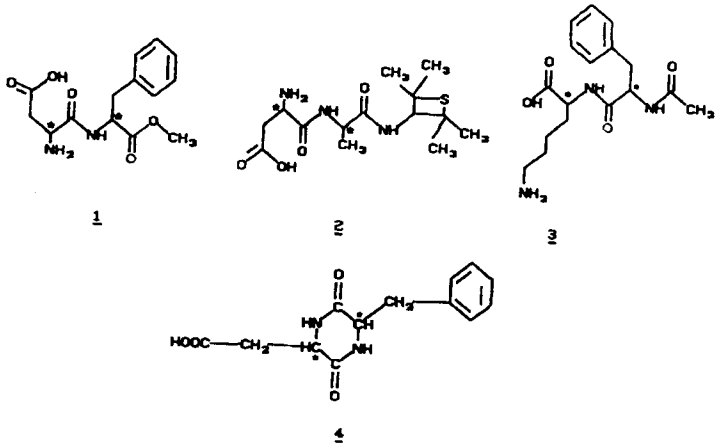

Fig. 1. Structural formulae of the compounds studied: **1** = α-L,L-aspartame; **2** = α-L,D-alitame; **3** = α-L,L-antiaspartame; **4** = diketopiperazine (DKP).

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stereoisomeric and conformationally stable form that enables them to adopt the particular three-point arrangements of chemical groups with respect to the human sweet taste receptors [1]. However, there is lack of reports concerning any possible racemization of the individual intense sweet isomers of synthetic dipeptide-type sweeteners, *viz.*, α-L,L-aspartame [1], α-L,D-alitame [2] and α-L,L-antiaspartame [3], during syntheses or decomposition processes which can lead to the formation of one or more of the three other undesirable, non-sweet stereoisomers, *e.g.*, D,D, D,L- and L,D- for aspartame. Moreover, until now our knowledge about metabolic disorders caused by the consumption of such useless forms of these sweeteners or their β-conformers (identified in stored aspartame-sweetened products [4,5]) has been limited [6,7].

Using high-performance liquid chromatographic (HPLC) methods, stereoselective analyses of unmodified [4,8,9] or protected [10] aspartame isomers have been achieved on achiral [8] and chiral chromatographic columns containing immobilized α-chymotrypsin [9], a crown ether coated on a polymeric support [4] and cellulose tris-3,5-dimethylphenylcarbamate coated on a silica gel surface [10].

For the partial separation of protected aspartame diasteromers, thin-layer chromatography (TLC) using octadecylsilica layers impregnated with (2*S*,4*R*,2′*RS*)-N-(2′-hydroxy-*n*-dodecyl)-4-hydroxyproline and copper (II) ion has also been reported [10]. Some years ago Cooke *et al.* [11] and Lindner *et al.* [12] described the baseline enantioselective separation of dansyldipeptides by reversed-phase (RP) HPLC using chiral metal chelates, *i.e.*, (4-dodecyldiethylenetriamine)zinc(II) and (L-2-isopropyl-4-octyldiethylenetriamine)nickel(II), as chiral mobile phase additives. The results indicated that with a properly designed chiral selector in the form of a transition metal chelate, superior HPLC resolution of free diastereomeric dipeptides could be achieved with high selectivity comparable to the efficiency of HPLC systems employing one of the chiral stationary phases [4,9,10] previously mentioned.

In this study, a direct stereoselective separation of structurally related sweeteners, *i.e.*, underivatized aspartame, alitame and antiaspartame, was developed, employing RP-HPLC on an octadecylsilane stationary phase and a chiral eluent containing the recently synthesized, helically distorted nickel(II) Schiff base chelate *dl*-[4,4′-(1-methyl-2-propylethane-1,2-diyldiimino)bis(pent-3-en-2-onato)]nickel(II) [13,14], which has already been successfully applied to the HPLC assay of diastereomers and enantiomers of aliphatic mono- and diamines [15,16] and amino alcohols [17]. The separation efficiency of the proposed method was also demonstrated by the analysis of aspartame decomposition products, especially the separation of the stereoisomers of cyclic dipeptides [diketopiperazines (DKPs)].

2. Experimental

2.1. Reagents and samples

The crystalline chelate *dl*-[4,4′-(1-methyl-2-propylethane-1,2-diyldiimino)bis(pent-3-en-2-onato)]nickel(II) [Ni(MPA); see Fig. 2], applied as the chiral additive to the mobile phase in RP-HPLC experiments, was prepared as described previously [13,14] with a tetradentate Schiff base derived from the condensation *in situ* (in a 2:1 molar ratio) of (*S*,*S*)-2,3-diaminohexane with 2,4-pentanedione, and nickel(II) acetate pentahydrate in acetone solution. The identity and the helically distorted structure of the synthesized Ni(MPA) chelate was checked by spectral methods [13,14].

All stereoisomeric samples of α-aspartame and

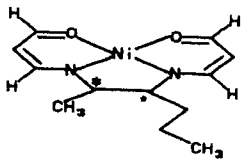

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Fig. 2. Structure of Ni(MPA) chelate used as the mobile phase additive in the developed HPLC system. The chiral centres are indicated by asterisks (some hydrogen atoms have been omitted for clarity).

α-alitame and their β-conformers were obtained as a gift from NutraSweet (Skokie, IL, USA) and Pfizer (Sandwich, Kent, UK), respectively. The α-antiaspartame samples and all stereoisomers of α- and β-aspartylphenyl (Asp–Phe) dipeptide and pure D- and L-enantiomers of phenylalanine (Phe) were supplied by Sigma (St. Louis, MO, USA). L-Phenylalanine methyl ester was obtained from Aldrich (Milwaukee, WI, USA). All stereoisomers of cyclic Asp–Phe [5-benzyl-3,6-dioxo-2-piperazineacetic acid; diketopiperazine (DKP)] were supplied by NutraSweet. Individual solutions (100 μ*M*) of stereoisomers of the compounds studied were prepared in doubly distilled water and 10-μl volumes were injected directly into the HPLC column.

Commercially available samples of sweetener formulations, containing aspartame as the main ingredient, were supplied from five different Polish manufacturers. An amount of the powdered sample equivalent to about 50 mg of aspartame was extracted with 25 ml of acetonitrile–20 mmol/l potassium phosphate buffer (85:15, v/v) (pH 3.5). An aliquot (1.0 ml) of the extracted solution was added to 2.5 ml of the internal standard (3-hydroxybenzoic acid) solution (0.1 mg/ml) and the final mixture was diluted to 10 ml with the solution used previously in the extraction stage.

2.2. Chromatographic conditions

RP-HPLC analyses were performed on an LDC–Milton Roy Model 3000 high-performance liquid chromatograph equipped with a multiwavelength UV detector operating at 210 nm and at a sensitivity of 0.05 AUFS. An octadecylsilane reversed-phase Polsil ODS column (25 × 0.4 cm I.D.; $d_p = 7$ μm) (ZOCh, Lublin, Poland) was used. The mobile phase, consisting of acetonitrile (85 ml), 20 mmol/l potassium phosphate buffer (15 ml) and dissolved 2 mmol/l of Ni(MPA) chelate, adjusted to pH 3.5 with 0.1 mol/l potassium hydroxide, was filtered through a 0.45-μm membrane filter and degassed ultrasonically under vacuum before use. The flow-rate was 0.8 ml/min. Prior to sample injection the chromatographic column was equilibrated with 22.5 ml of freshly prepared mobile phase. All measurements were made at 25°C and repeated four times to calculate the capacity factor (k') values.

2.3. Molecular model calculations

The molecular modelling program PCMODEL (Serena Software, Bloomington, IN, USA), implemented on an IBM 386/SX microcomputer, was applied for creation of the proposed structure of associates formed between solutes and Ni(MPA) during chiral recognition in the developed HPLC system.

3. Results and discussion

3.1. Characterization of Ni(MPA)

In the synthesized Ni(MPA) chelate (see Fig. 2), the central nickel(II) ion is surrounded by the coordinated imine and carbonyl groups defining a nearly square-planar surface [18]. However, the non-equivalence of alkyl substitution of the two stereogenic centres, localized in the ethylene bridge connecting imine nitrogen atoms, by methyl and *n*-propyl groups, leads to the formation of the two conformational isomers of the Ni(MPA) molecule [13,14,18]. The steric and energetic factors imply greater stability and population of the (λ–Λ)-isomer in which the more bulky *n*-propyl substituent occupies the axial position with respect to the plane formed by nitrogen and oxygen donor atoms [17,18]. This situation leads to an antiperiplanar configuration and a *trans* orientation of alkyl substituents on the chiral centres, stabilizing the helical distortion of the Ni(MPA) molecule [13,14]; this also means non-planarity of the six-membered chelate rings in the Ni(MPA) molecule [13,18]. The axial position of the *n*-propyl substituent also causes steric hindrance around the coordinatively unsaturated central nickel(II) ion acting as the Lewis acid site [18]. Most of the partial negative charge in the Ni(MPA) chelate is localized on the oxygen carbonyl atoms. However, the surface area of Ni(MPA) is mostly

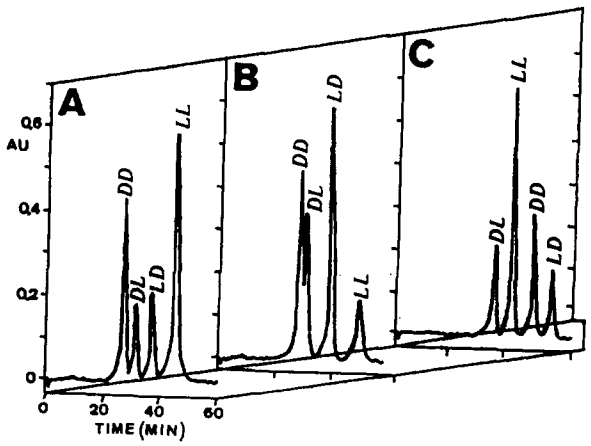

Fig. 3. Stereoselective separation of (A) aspartame, (B) alitame and (C) antiaspartame isomers by RP-HPLC with Ni(MPA) chelate as chiral additive in the mobile phase (see Experimental).

non-polar, *viz.*, it occupied nearly 212 Å^2 of the 283 Å^2 total surface area of the chelate. As was postulated previously based on measured adsorption isotherm data [16], the Ni(MPA) chelate can be partly sorbed on the octadecylsilane stationary phase in a chromatographic column with an axial *n*-propyl substituent by involving specific hydrophobic interactions.

3.2. Retention of sweetener stereoisomers

Typical chromatograms of stereoisomer mixtures of each sweetener studied with the developed chiral mobile phase are shown in Fig. 3. Table 1 given a detailed comparison of capacity factors and parameters of the separation efficiency calculated for the respective diastereoisomers and enantiomers of individual sweeteners. The data in Fig. 3 and Table 1 show that the retention orders of aspartame and alitame isomers in the proposed chiral HPLC system are identical. One can suggest that the configuration of the analogous aspartyl moiety present in the aspartame and alitame molecules is the main factor controlling the diastereomeric retention order for these solutes. However, replacing the N-terminal residue of esterified phenylalanine, as in aspartame, with a non-aromatic substituent in the form of tetramethyl-3-thietanylalaninamide, as in alitame (see Fig. 1), leads to a non-baseline resolution ($R_s = 0.7$) of the pair of D,D/D,L-diastereomers of the latter sweetener.

Table 1
Values of capacity factors (k'), separation factors (α) and resolution factors (R_s) of isomers of sweeteners obtained with the proposed chiral RP-HPLC system

Sweetener	Stereoisomer	k'	α	R_s
α-Aspartame	D,D[a]	2.7		
	D,L	3.2	1.20[b]	1.4[b]
	L,D	3.9	1.23	2.0
	L,L	5.1	1.31	2.5
α-Alitame	D,D[a]	2.9		
	D,L	3.1	1.07	0.7
	L,D	4.3	1.38	2.5
	L,L	5.7	1.35	2.8
α-Antiaspartame	D,D[c]	3.7		
	L,L	4.5	1.21	2.3
	D,L	5.5	1.22	2.8
	L,D	6.3	1.15	2.3

Mobile phase flow-rate, 0.8 ml/min; $t_0 = 7.2$ min.
[a] First letter assigns the Asp configuration in the sweetener molecule.
[b] Calculated for successive pairs of peaks.
[c] First letter assigns the Lys configuration in the sweetener molecule.

Comparing the retentions of aspartame and antiaspartame isomers, which contain an analogous phenylalanine part in their molecules but differ in the N- and O-terminal residues (see Fig. 1), large retention and selectivity increases associated with the drastic change in the elution sequence of the antiaspartame isomers can be observed. On the chromatograms of antiaspartame isomers the peaks of the enantiomer pairs D,D/L,L and D,L/L,D were registered in close proximity, whereas on the aspartame chromatograms the diastereomer pairs D,D/D,L and L,D/L,L eluted one after the other. Probably, the proposed chiral RP-HPLC system with Ni(MPA) in the mobile phase is more selective during the separation of phenylalanine-containing dipeptides when the highly hydrophobic fragment (*e.g.*, lysine instead of a methyl group) is introduced at the O-terminus of such solutes.

For sweeteners containing peptide and amide bonds, *i.e.*, alitame and antiaspartame, the observed increase in their retention and selectivity accompanied by a reversed vicinity of their stereoisomeric pairs can be partly explained by the increasing flatness of the antiaspartame molecule caused by the phenyl substituent connected to the second chiral centre located between the flat amide bonds of this sweetener. Probably this factor also has a great impact on the differences in the observed retentions and separation efficiencies of antiaspartame and aspartame isomers, where the flatness increase is caused by the presence of a terminal methyl amide group instead of a methyl ester group, respectively.

3.3. Chiral discrimination mechanism

The formation of unstable associates with 1:1 stoichiometry between the square-planar nickel(II) chelates and electron-donor compounds, including di- and tripeptides, was intensively studied as a model of enzyme active site reactivity [18]. The equatorial position of the dipeptide molecule in relation to the planar nickel(II) chelate forced by its helical structure was suggested on the basis of results of *ab initio* molecular calculations [19].

The observed stereospecific separation and retention order of stereoisomers of dipeptide-type sweeteners in the proposed HPLC system can be explained by the occurrence of association processes on the basis of the "three-point-interactions" rule of Dalgliesh [20]. Molecular models of Ni(MPA)–dipeptide associates (see Fig. 4) indicate that chiral discrimination of

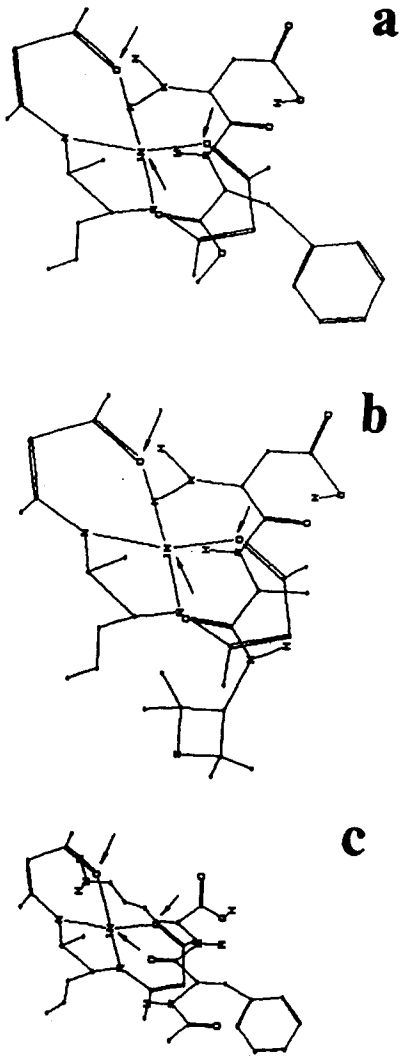

Fig. 4. Views of proposed structures of diastereomeric associates formed between Ni(MPA) and (a) α-L,L-aspartame, (b) α-L,L-alitame and (c) α-L,D-antiaspartame in the developed chiral HPLC system (some hydrogen atoms have been omitted for clarity). Arrows indicate positions of the possible interaction sites.

aspartame (Fig. 4a) an alitame (Fig. 4b) stereoisomers in the developed HPLC system occurs predominantly by electrostatic interactions of the carbonyl group in the vicinity of the chiral carbon atom localized in the phenylalanine moiety of the solute and the central nickel(II) ion in the Ni(MPA) chelate molecule. At a second interaction point, weak hydrogen bond formation between the amide hydrogen of the solute and one of the coordinated carbonyl groups in the chelate molecule can be postulated. With the applied acidic pH of the mobile phase the primary aspartyl amino group of such dipeptide-type sweeteners is protonated [21], leading to electrostatic interactions with the second coordinated carbonyl group of the Ni(MPA) chelate. Hence in this way the third interaction site is formed.

For the L,L-isomer of aspartame or alitame, all three proposed interactions points are "saturated", increasing the relative stability of the associates and thus leading to their stronger retention (see Table 1). For the D,L-isomer the protonated aspartyl amino group in the aspartame or alitame molecule is situated above the peptide bond plane so its distance from the coordinated carbonyl groups of Ni(MPA) is increased, hindering the possible electrostatic interactions, diminishing the stability of the associate and thus reducing the observed HPLC retention of such a solute. The active role of the aspartyl primary amine group in controlling the chiral recognition mechanism of free aspartame isomers in their temperature-gradient HPLC separation with a chiral crown ether stationary phase was also stressed by Motellier and Wainer [4].

For antiaspartame stereoisomers the configuration of the lysine chain with charged primary amino group is essential for the observed enantioselectivity of their separation using the RP-HPLC system described here. However, in this case probably the configuration of the phenylalanine moiety also partly influences the stability of the formed associates (see Fig. 4c) by diminution of interactions between the carbonyl group in the peptide bond of antiaspartame and the coordinatively unsaturated nickel(II) ion in the Ni(MPA) chelate molecule.

3.4. Retention of aspartame decomposition products

The process of aspartame decomposition under acidic conditions leads to the formation of the stereoisomers of species such as the cyclic 5-benzyl-3,6-dioxo-2-piperazineacetic acid [diketopiperazine (DKP); **4** in Fig. 1], aspartylphenylalanine (Asp–Phe), phenylalanine methyl ester (Phe methyl ester), free phenylalanine (Phe) and β-conformers of aspartame and Asp–Phe dipeptide [1,21]. To test the usefulness of the developed HPLC system for the direct, one-run stereoselective separation of the aspartame degradation products the L,L-isomer of α-aspartame was dissolved in acidified water (pH 3) and stored at ambient temperature. The composition of the solution was determined chromatographically after 72 h under HPLC conditions identical with those used previously for the sweetener isomer separation studies. The results of this investigation are illustrated in Table 2 and Fig. 5A.

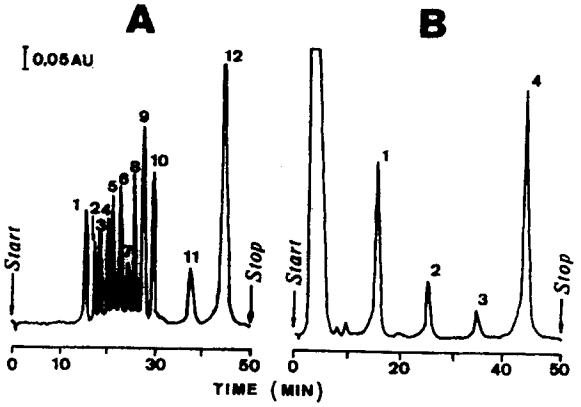

Fig. 5. Chromatograms showing the separation of (A) α-L,L-aspartame decomposition products and (B) α-L,L-aspartame impurities in a commercial product (third sample in Table 3) with the developed HPLC system with Ni(MPA) as chiral additive to the mobile phase. Peaks: (A) 1 = D-phenylalanine; 2 = L-phenylalanine; 3 = D-phenylalanine methyl ester; 4 = L-phenylalanine methyl ester; 5 = D,D-aspartylphenylalanine; 6 = D,L-aspartylphenylalanine; 7 = L,D-aspartylphenylalanine; 8 = L,L-aspartylphenylalanine; 9 = L,L + D,D-diketopiperazine (DKP); 10 = L,D + D,L-diketopiperazine (DKP); 11 = β-L,L-aspartame; 12 = α-L,L-aspartame; (B) 1 = 3-hydroxybenzoic acid (internal standard); 2 = L,L + D,D-diketopiperazine (DKP); 3 = α-L,D-aspartame; 4 = α-L,L-aspartame.

Table 2
Values of capacity factors (k'), separation factors (α) and resolution factors (R_s) of aspartame decomposition products obtained with the proposed chiral RP-HPLC system

Product	Stereoisomer	k'	α	R_s
Phe	D	1.2		
			1.20[a]	2.5[a]
	L	1.4		
Phe methyl ester	D	1.5		
			1.10	1.6
	L	1.6		
Asp–Phe	D,D	1.7		
			1.17	2.2
	D,L	2.0		
			1.05	1.5
	L,D	2.1		
			1.09	2.3
	L,L	2.3		
DKP	L,L	2.5		
			1.00	0.0
	D,D	2.5		
			1.04	1.6
	L,D	2.6		
			1.00	0.0
	D,L	2.6		

Mobile phase flow-rate, 0.8 ml/min; $t_0 = 7.2$ min.
[a] Calculated for successive pairs of peaks.

Using the proposed RP-HPLC system with Ni(MPA) chelate as a chiral additive to the mobile phase, all the diastereomers and enantiomers of Asp–Phe dipeptide, Phe amino acid and Phe methyl ester were satisfactorily resolved with high separation factors (α values). For Asp–Phe dipeptide the chromatographic system is rather diastereoselective because its D,D/D,L- and L,D/L,L-diastereomers eluted as successive peaks (see Table 2).

However, for DKP stereoisomers, two of its enantiomeric pairs, L,L/D,D and L,D/D,L, were not separated ($\alpha = 1$). Again, only the diastereoselectivity of the proposed chiral RP-HPLC system was manifested ($\alpha = 1.04$). It can be assumed that the apical association of the cyclic DKP isomer molecule with the Ni(MPA) chelate through single attractive interactions involve one of the DKP carbonyl groups and the coordinated nickel(II) ion. The enantiospecificity of such a "one-point interaction" chiral recognition mechanism [17,22] is significantly diminished compared with the "three-point interaction" mechanism postulated in this work for the separation of dipeptide-type sweetener stereoisomer mixtures using the chiral RP-HPLC approach.

The peak of β-L,L-aspartame with capacity factor $k' = 4.2$ was recognized on the chromatogram of α-L,L-aspartame degradation products (see Fig. 5A) by injection of an appropriate standard solution. Probably owing to the weaker association with Ni(MPA) chelate in the mobile phase, β-L,L-aspartame is eluted before α-L,L-aspartame with a high value of the separation factor α of the conformers of *ca.* 1.20.

3.5. Analysis of commercial aspartame formulations

Commercial preparations of aspartame were analysed using the developed RP-HPLC method

Table 3
Amounts of impurities in aspartame commercial formulations as determined with the proposed chiral RP-HPLC method

Sample	Aspartame[a,b] (%)				DKP[b,c] (%)	
	L,L	L,D	D,D	D,L	L,L/D,D	L,D/D,L
Dietetic	98.5	–[d]	–	–	0.28	–
Dietetic	96.4	–	–	–	0.32	–
Dietetic	97.8	1.2[b]	–	–	0.15	–
Dietetic	95.9	–	–	–	0.63	–
Pharmaceutical[e]	99.1	–	–	–	0.73	–

[a] The results are averages of four determinations (R.S.D. < 3%) and are expressed as a percentage of the claimed content of α-L,L-aspartame.
[b] In aspartame formulations the D,D- and D,L-isomers were not detected. Also not detected was L,D/D,L in the DKP sample.
[c] The results are expressed as values relative to the α-L,L-aspartame content.
[d] Dashes indicate not detected.
[e] Other ingredient: mannitol.

with a chiral eluent containing Ni(MPA) chelate for the determination of the potential presence of aspartame isomers or synthesis impurities (see Fig. 5B). These compounds were identified on the basis of their peak retention times and peak-height ratios at 210, 215 and 230 nm. The results in Table 3 indicate that all the commercial solid samples of aspartame (stored for 1 year at ambient temperature and humidity) analysed contained DKP in the form of an L,L/D,D-isomer mixture (eluted as a single peak) as a minor contaminant. In one sample of commercial aspartame the presence of traces of α-L,D-aspartame, which is non-active to human sweet taste receptors, was determined but this can probably be attributed in part to initiation of racemization processes of α-L,L-aspartame [23] owing to the low dielectric constant ($\varepsilon = 42.7$) of the applied extraction solution (see Experimental).

4. Conclusions

The RP-HPLC method with a chiral mobile phase containing a helically distorted Ni(MPA) chelate allows the selective determination of non-derivatized diastereomers and enantiomers of most common dipeptide-type sweeteners, permitting the effective monitoring of their un-desirable racemization processes in special kinds of dietary formulations.

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