

## Trace analysis in chiral separation of selected amino enantiomers<sup>1</sup>

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### Abstract

A Crownpack CR(+) column was used for the highly sensitive quantitation of amino acid enantiomers. The use of a mobile phase of exclusively aqueous perchloric acid enabled the use of low-wavelength UV detection (200 nm) before on-line derivatization with *o*-phthalaldehyde for fluorimetric detection. The use of simultaneous UV and fluorimetric detection permitted the simultaneous quantification of the impurity and its parent compound. The method was used for the evaluation of some commercially available amino acid standards. Enantiomeric impurities as low as 0.001% (10 ppm) can be determined in some cases. High precision for the determination of trace levels of D-amino acids in the presence of large amounts of corresponding L-enantiomer is demonstrated.

**Keywords:** Amino acids; High performance liquid chromatography; OPA derivatization; Optical purity determination; Trace analysis

### 1. Introduction

Amino acids from a large group of compounds of pharmaceutical and biochemical interest. The stereoisomers of amino acids differ in biological activity, and their configuration and optical purity are very important in many fields. In order to study the effects of radiosterilization upon optical activity, this laboratory required a method that was both simple and inexpensive for rapid deter-

mination of the optical purity of D- and L-amino acids when one enantiomer is present in a large excess of the other.

Amino acids in their native form are generally poor chromophores, and do not possess electrochemical activity. This means that for analytical purposes they must first be chemically modified (derivatized) to enhance detectability. As has been shown in recent years, the method of choice for the determination of amino acids is high performance liquid chromatography (HPLC) with fluorimetric detection [1–21], which requires pre- or post-column derivatization in order to achieve high sensitivity. The reactivity of all common

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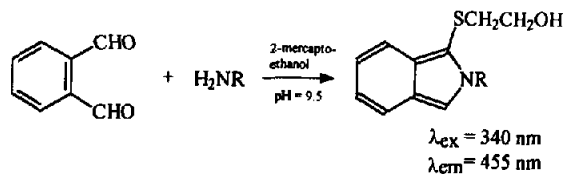


Fig. 1. Reaction scheme of OPA with a primary amine.

derivatization reagents is directed towards the amino group(s) in the amino acid structure. The most common reagents are 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [1-3], dansyl chloride (DNS-Cl) [4-8], phenylisothiocyanate (PITC) [9-11], o-phthalaldehyde-mercaptoethanol (OPA-ME) [12-16] and 9-fluorenylmethyl chloroformate (FMOC-Cl) [17-21]. Among the fluorogenic reagents, OPA plays the most significant role. The major advantage of OPA is that it does

not fluoresce intrinsically, and its drawbacks are its chemical specificity towards primary amines and the relative instability of the resulting fluorophores [22]. The reaction scheme of OPA, as well as the structure of the fluorophore produced, are presented in Fig. 1. The above methods have been successfully used for the chiral and achiral separation of a number of amino acids, and each has distinct advantages and disadvantages. However, none permitted the simultaneous quantification of the enantiomeric impurity and its parent compound. Moreover, the methods using a pre-column derivatization, although often giving excellent results, required an additional validation because it is necessary to establish that the derivatization reaction does not induce any racemization of the chiral centre(s).

This paper reports a simple and sensitive

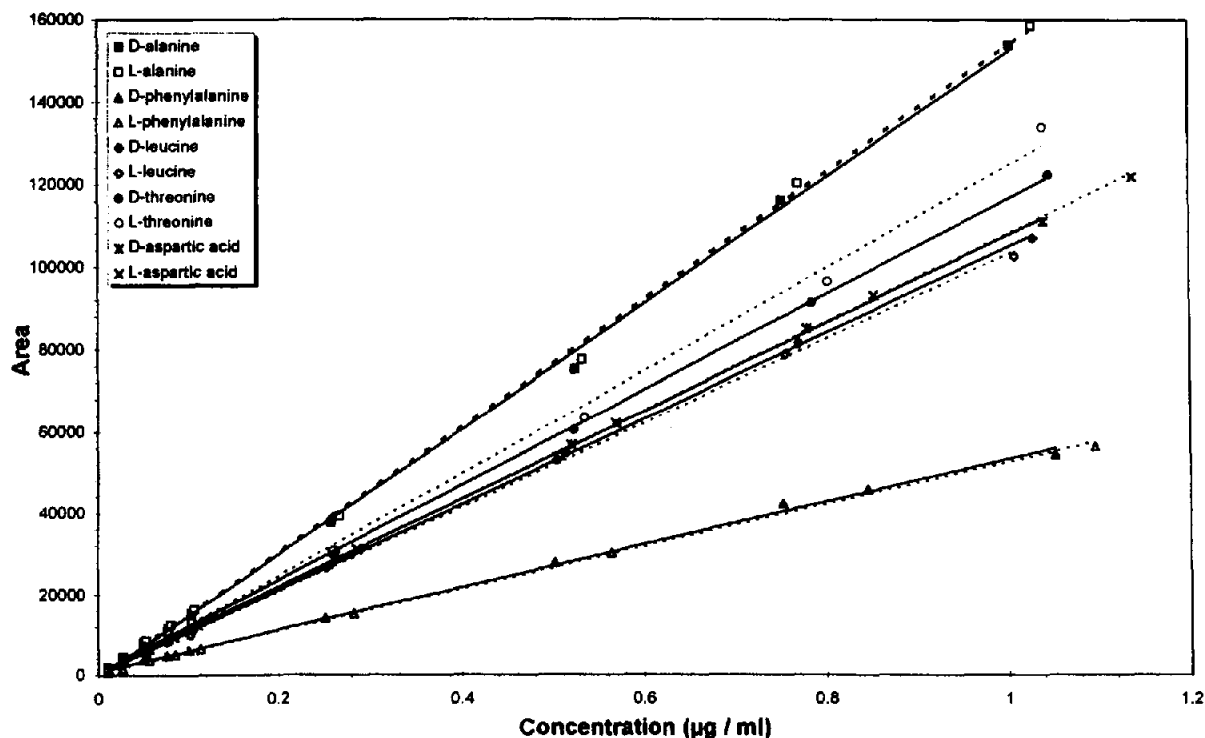


Fig. 2. Calibration curves obtained with the fluorimetric detection for the amino acids studied.

method for trace analysis in chiral separation of amino acid enantiomers. The enantiomeric separation was obtained on a Crownpack Cr(+) (Daicel) chiral stationary phase (CSP). This phase uses a chiral crown ether moiety as a chiral selector, and discrimination between enantiomers relies on the formation of diastereoisomeric inclusion complexes between the ammonium ion moiety of the amino acid and the chiral crown ether entity of the stationary phase. This kind of chiral HPLC column can resolve not only amino acids, but also many compounds bearing a primary amine near the chiral centre [23]. The use of a mobile phase of exclusively aqueous  $\text{HClO}_4$  enabled a low-wavelength UV detection (200 nm) prior to an on-line derivatization with OPA-ME for fluorimetric detection.

## 2. Experimental

### 2.1. Apparatus

The HPLC system consisted of a Spectra Physics SP8810 for the mobile phase, an injection valve, a column and a Spectra 100 UV detector set at 200 nm. A Waters 510 pump then delivered the derivatisation reagent to a tee connector added to the UV detector effluent and the mixture was transferred to a stainless-steel coil connected to a Merck-Hitachi F1050 fluorescence detector set at an excitation wavelength of 340 nm and an emission wavelength of 450 nm. Chromatograms were recorded using a PC-based Borwin 1.20 data acquisition system. The column used for the separation was a 150 mm  $\times$  4 mm i.d. Daicel Crown-

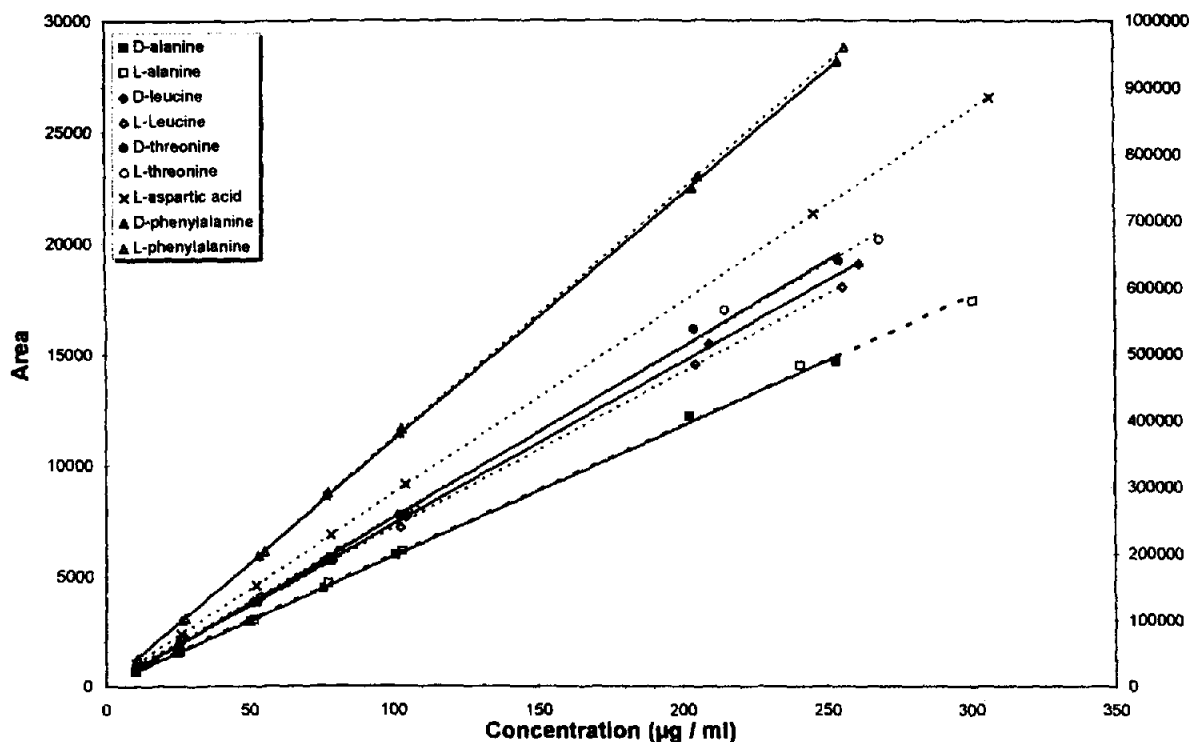


Fig. 3. Calibration curves obtained with the UV detection for the amino acids studied. The scale on the right is only used for the phenylalanine, which possesses a good chromophore, and thus gives a more intense spectrophotometric response.

pack Cr(+) ( $5 \mu\text{m}$ ). Mobile phase elution was performed isocratically using a filtered ( $0.45 \mu\text{m}$ ), degassed aqueous solution of perchloric acid diluted to obtain the required pH. No organic modifier was included in the mobile phase. Flow rate and pH depend on the amino acid studied, and are reported in Tables 2 and 3 for each compound analysed. Temperature control of the column was effected by the use of a cooling jacket containing isopropanol. The helical coil, serving as mixing chamber for the mobile phase and the derivatization reagent, was made by bending standard stainless-steel HPLC tubing ( $0.01$  in i.d.), and was maintained at ambient temperature. The length of the coil was adapted so as to give a void volume of  $1 \text{ ml}$  (reaction time of about  $1 \text{ min}$  in most cases), enabling the reaction of OPA and

the amino compounds to go to completion [22,24].

## 2.2. Chemicals

Amino acids were purchased from Sigma, and used without further purification. HPLC-grade-water was obtained via a Milli-Q water system. All other chemicals were of analytical-grade or better and were used as received.

## 2.3. Procedure

The derivatization reagent was prepared by mixing  $300 \text{ ml}$  of  $0.4 \text{ M}$  borate buffer pH 10.5 with  $600 \mu\text{l}$  of OPA ethanolic solution ( $1 \text{ g}$  of OPA in  $10 \text{ ml}$  absolute ethanol) and  $150 \mu\text{l}$  of mercaptoethanol. It was freshly prepared each

Table 1  
Results from the calibration curves of the amino acids studied

Compound	Detection mode	No. of experimental data points	Regression	$R^2$	Std. dev. of slope	MAD (ng)	MAQ (ng)
L-Alanine	Fluo.	36	$152635.84x + 175.63$	1.0000	295.63	0.1	0.4
D-Alanine	Fluo.	36	$149138.70x + 342.25$	1.0000	325.90	0.1	0.4
L-Phenylalanine	Fluo.	27	$54520.71x + 157.42$	1.0000	248.51	0.2	0.9
D-Phenylalanine	Fluo.	27	$58007.44x + 107.32$	1.0000	472.50	0.5	1.6
L-Aspartic acid	Fluo.	36	$108929.97x + 261.12$	1.0000	133.92	0.05	0.2
D-Aspartic acid	Fluo.	36	$110079.00x + 338.54$	1.0000	232.85	0.05	0.4
L-Threonine	Fluo.	36	$122405.62x + 455.54$	1.0000	338.91	0.1	0.5
D-Threonine	Fluo.	36	$118829.26x + 320.16$	1.0000	226.67	0.05	0.3
L-leucine	Fluo.	36	$102487.15x + 106.58$	0.9998	122.44	0.05	0.2
D-Leucine	Fluo.	36	$106225.16x + 115.52$	1.0000	140.21	0.05	0.2
L-Alanine	UV	28	$59.12x + 40.72$	0.9995	41.87	30	30
D-Alanine	UV	28	$58.46x + 64.49$	0.9992	29.85	10	80
L-Aspartic acid	UV	21	$65.88x + 106.86$	0.9985	44.15	5	75
L-Threonine	UV	28	$75.20x + 148.90$	0.9981	57.25	5	110
D-Threonine	UV	38	$75.38x + 62.46$	0.9994	56.60	30	130
L-Leucine	UV	25	$71.73x + 104.88$	0.9982	48.35	10	100
D-Leucine	UV	25	$72.61x + 86.40$	0.9992	35.37	5	75

Table 2  
Chromatographic data from the UV detection

Compound	$k'_d$	$k'_i$	$\alpha$	$R_s$	Chromatographic conditions
Alanine	0.72	2.20	3.06	4.78	HClO <sub>4</sub> , pH 1.5, temperature set at 5°C, flow rate 0.5 ml min <sup>-1</sup>
Phenylalanine	5.89	8.57	1.46	4.20	HClO <sub>4</sub> , pH 2.0, temperature set at 15°C, flow rate 0.8 ml min <sup>-1</sup>
Leucine	2.15	3.65	1.70	4.47	HClO <sub>4</sub> , pH 2.0, temperature set at 25°C, flow rate 0.5 ml min <sup>-1</sup>
Threonine	0.39	1.42	3.64	3.76	HClO <sub>4</sub> , pH 1.5, temperature set at 0°C, flow rate 0.4 ml min <sup>-1</sup>
Aspartic acid	Not determined	2.29	Not determined	Not determined	HClO <sub>4</sub> , pH 1.5, temperature set at 0°C, flow rate 0.4 ml min <sup>-1</sup>

day from stock solution. The reagent was delivered to the system at a constant flow rate of 0.5 ml min<sup>-1</sup>.

### 3. Results and discussion

For this study, five amino acids as their D- and L-enantiomers were selected. They were chosen to represent the general chromatographic behaviour displayed by the amino acids. They were: alanine (hydrophobic), leucine (highly hydrophobic), phenylalanine (aromatic), threonine (hydrophilic) and aspartic acid (highly hydrophilic). The composition of the derivatization reagent, as given in Section 2, has been optimised (data not shown) so as to give a maximum fluorimetric response.

#### 3.1. Order of elution

The elution order was checked by the injection of pure enantiomers. The D-enantiomers were eluted prior to the L-enantiomers, which is an advantage because the L-enantiomers are the dominant components in most biological samples. It has been shown that when traces are eluted before the major enantiomer, the quantitative determination of enantiomeric composition becomes more accurate and precise, and trace detectability can be improved tremendously [25].

#### 3.2. Sensitivity of the method

The lack of a suitable chromophore in many amino acids limits the sensitivity of direct spectrophotometric methods. Here, however, this poor response in the UV detection is used to determine the enantiomer present in large excess, giving in one step the results for both the optical impurity and its parent compound. The results from the calibration curves of several amino acids are presented in Fig. 2 (fluorimetric detection), Fig. 3 (UV detection), and Table 1. The calibrations were obtained by diluting the corresponding amino acid of the highest purity available to the required concentration. As can be seen, the use of a combined UV and fluorimetric detection gives a

Table 3  
Chromatographic data from the fluorimetric detection

Compound	$k'_d$	$k'_l$	$\alpha$	$R_s$	Chromatographic conditions
Alanine	0.52	1.64	3.15	4.40	HClO <sub>4</sub> , pH 1.5, temperature set at 5°C, flow rate 0.5 ml min <sup>-1</sup>
Phenylalanine	4.16	6.10	1.47	4.23	HClO <sub>4</sub> , pH 2.0, temperature set at 15°C, flow rate 0.8 ml min <sup>-1</sup>
Leucine	1.70	2.88	1.69	4.24	HClO <sub>4</sub> , pH 2.0, temperature set at 25°C, flow rate 0.5 ml min <sup>-1</sup>
Threonine	0.29	1.06	3.66	3.10	HClO <sub>4</sub> , pH 1.5, temperature set at 0°C, flow rate 0.4 ml min <sup>-1</sup>
Aspartic acid	0.70	1.61	2.29	3.65	HClO <sub>4</sub> , pH 1.5, temperature set at 0°C, flow rate 0.4 ml min <sup>-1</sup>

Table 4  
Optical purity of some commercial samples of D- and L-amino acids. Chromatographic conditions as in Tables 2 and 3

Compound	Quality	Batch	Concentration of opposite enantiomer (ppm)	Optical purity (%)	RSD (n = 4)
L-Leucine	Purified	118F00571	15662	98.434	0.47
L-Alanine	Sigma ultra	72H08471	56	99.994	1.79
D-Alanine	Sigma grade	108F0324	103	99.990	3.45
L-Phenylalanine	Sigma grade	71H0993	390	99.961	0.77
L-Aspartic acid	Sigma grade	12H0020	363	99.964	0.84
L-Aspartic acid	Sigma ultra	64H03321	21	99.998	2.75
L-Threonine	Sigma ultra	43HO4091	Not detected	–	–

linear response range covering at least 1–100 000, enabling the quantification of impurities down to 0.001% of the main compound. The minimum amounts detected (MAD) and quantitated (MAQ) were estimated by the use of the standard deviation of the slope from the calibration curves (Sb), with the following formulae [26]:

$$\text{MAD} = 3 \text{ Sb}$$

$$\text{MAQ} = 10 \text{ Sb}$$

As can be seen from Table 1, the average MAD is expected to be in most cases around 0.1 ng injected, and the MAQ should be in the 0.5–1.0 ng range.

### 3.3. Selectivity of the method

The chromatographic data for all amino acids investigated are given in Tables 2 and 3 for the UV and fluorimetric detection respectively. As can be seen from the Tables, the use of the mixing chamber did not modify the selectivity ( $\alpha$ ) of the system, but the resolutions ( $R_s$ ) observed for the fluorimetric detection are somewhat lower than

those obtained from the data of the UV detection, due to a slight increase in peak width resulting from the increased void volume between the two detectors. From the parameters commonly used to control the separation (organic modifiers, pH and temperature), only the temperature seems to be of significant use. Organic modifiers should be avoided because most solvents, such as methanol or acetonitrile, have a relatively high cut-off value, which seriously impairs the low-wavelength UV detection. Moreover, a mixture of these solvents with perchloric acid can result in explosion or fire. For a separation to occur, it is necessary to carry out chromatography in a highly acidic medium 10<sup>-1</sup>–10<sup>-2</sup> M HClO<sub>4</sub>, not only to ensure the presence of the ammonium ion on the amino acid, but also to protonate the  $\alpha$ -carboxylic acid group, whose electronegativity would otherwise prevent the formation of the inclusion complex with the CSP. So, even if the column is stable in the pH range 1–9, a typical range for the mobile phase is from 1–2. For trace analysis, where a high resolution between the enantiomers is needed [27], most separation will be achieved at pH 1.5, severely limiting the use of pH as a useful

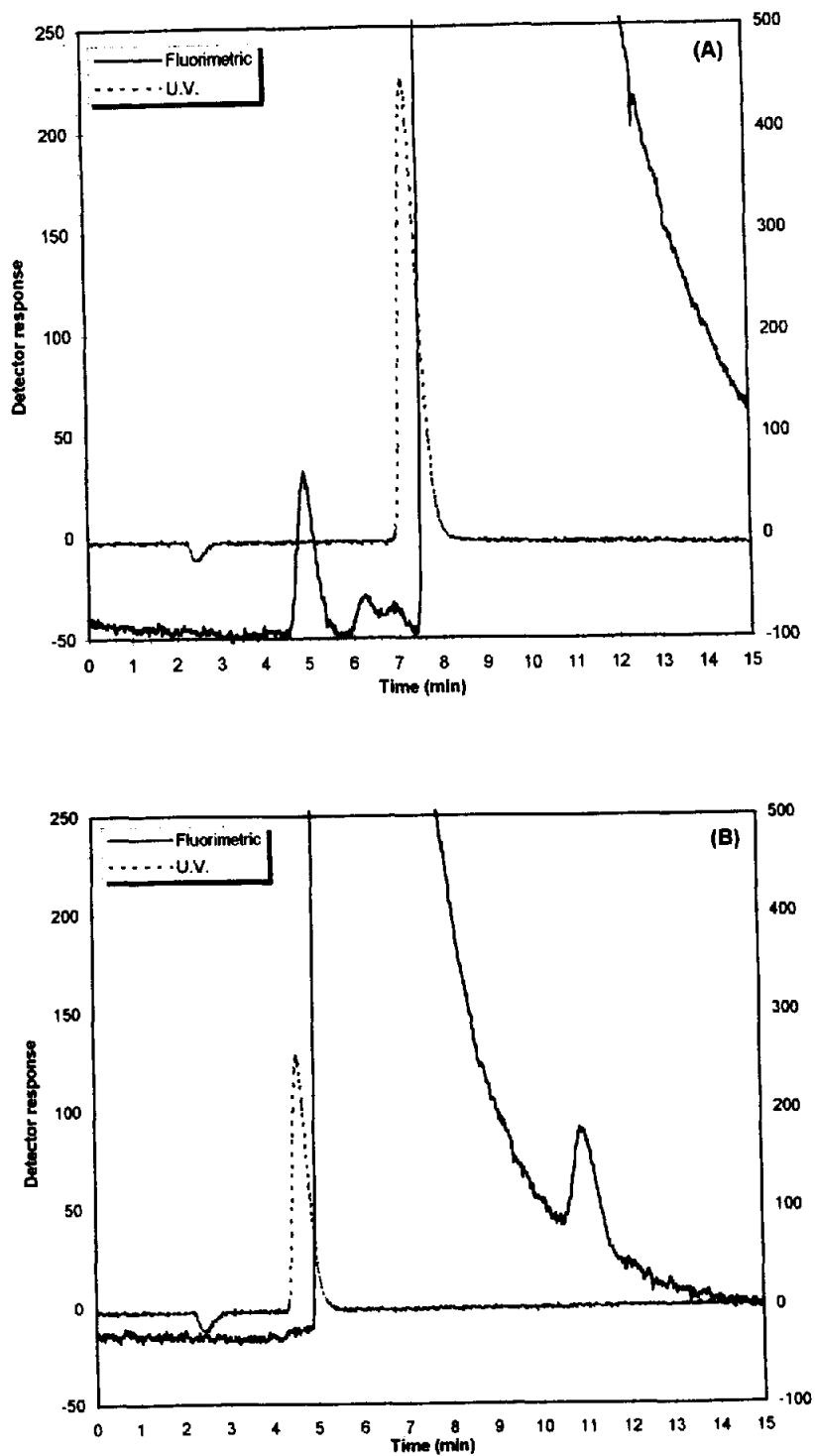


Fig. 4. Example of chromatogram used for the evaluation of the enantiomeric purity of (A) L-alanine, Sigma ultra and (B) D-alanine, Sigma grade. Eluent: aqueous HClO<sub>4</sub>, pH 1.5, temperature set at 5°C. The scales on the right-hand side of the chromatograms are used for the UV detection.

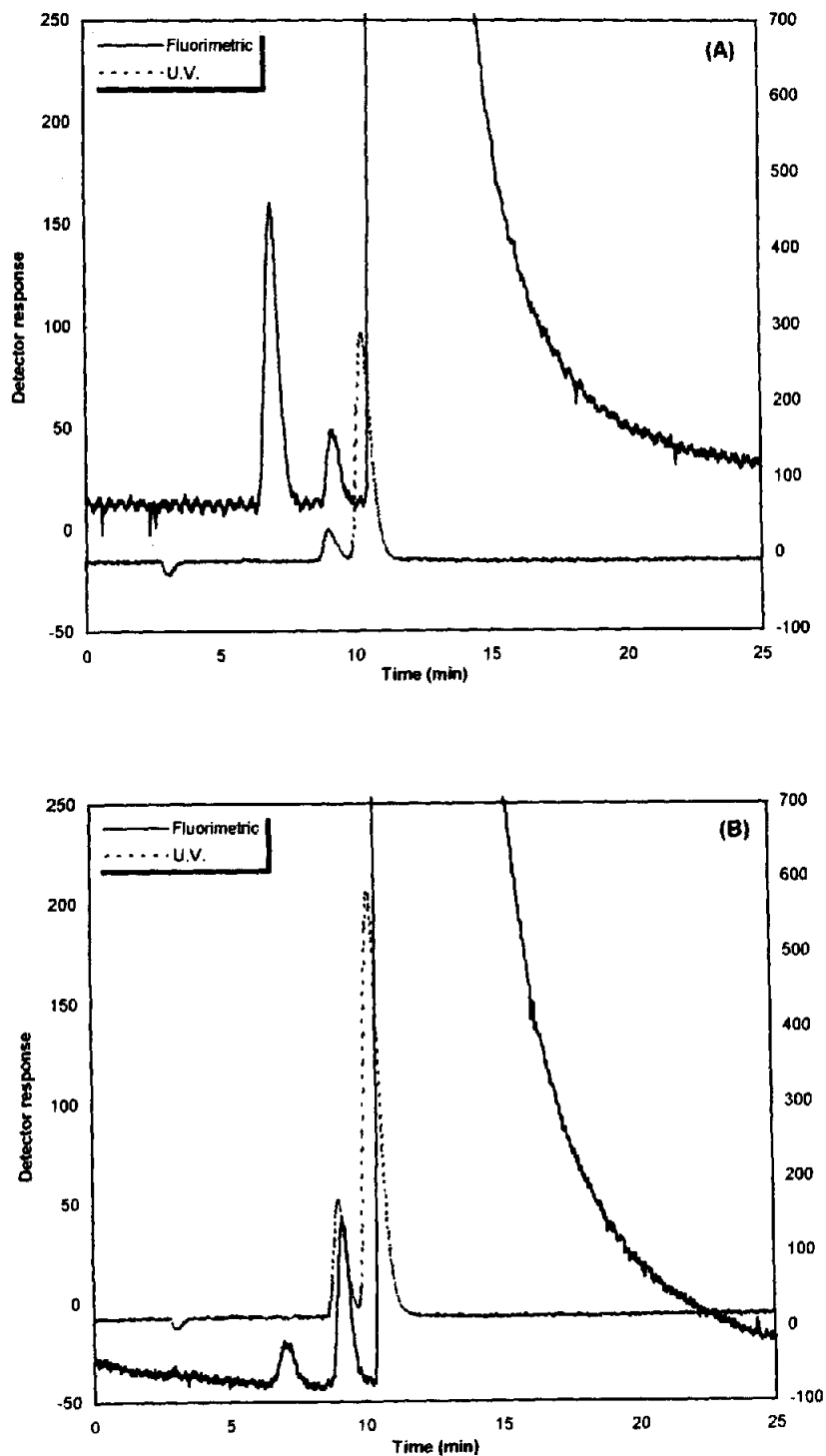


Fig. 5. Example of chromatogram used to evaluate the enantiomeric purity of (A) L-aspartic acid, sigma grade and (B) L-aspartic acid, sigma ultra. Eluent: aqueous  $\text{HClO}_4$ , pH 1.5, temperature set at  $0^\circ\text{C}$ . The scales on the right-hand side of the chromatograms are used for the UV detection.



tool for the control of the separation. The enantio-recognition mechanism of the CSP is dependent on thermodynamic parameters, which can be easily accessed by the separation factor  $\alpha$  [28,29], as the difference in the molar Gibbs energies ( $-\Delta_{\pm}(\Delta G)$ ) of the two enantiomers is related to  $\alpha$  by

$$-\Delta_{\pm}(\Delta G) = RT \ln \alpha \quad (1)$$

where  $T$  is the absolute temperature and  $R$  is the gas constant ( $8.3143 \text{ J K}^{-1} \text{ M}^{-1}$ ). From Eq. (1) it can be seen that an increase in temperature will cause a decrease in the enantioselectivity. Temperature is the best way to control the selectivity of the separation.

### 3.4. Practical applications

This method was used for the determination of enantiomeric contamination in a number of commercial amino acids. The results are summarised in Table 4, and examples of chromatograms obtained during the analysis are given in Figs. 4 and 5. The chromatographic conditions are the same as those specified in Tables 2 and 3. Some level of impurity was found in all samples analysed, except for the L-threonine, where no enantiomeric contamination could be detected. As can be seen from Table 4, the method enables trace determination of optical purity for both L- and D-enantiomers.

Quantitative analysis of contaminating D-enantiomers in L-amino acids gave a very high precision, with RSD values of 2.75% (L-aspartic acid, Sigma ultra, 21 ppm  $n=4$ ), 1.79% (L-alanine, Sigma ultra, 56 ppm,  $n=4$ ), and 0.84% (L-aspartic acid Sigma grade, 363 ppm,  $n=4$ ). For the L-enantiomer as the trace compound, the situation is complicated by the tailing from the enantiomerically rich component, resulting in quantitative determinations with lower precision. This is shown in Fig. 4B. As an example, the results from the analysis from the D-alanine gave an RSD value of 3.45% ( $n=4$ ) for a contamination level of 103 ppm of L-alanine.

## 4. Conclusions

Even if great emphasis was put on the analysis of some amino acids, the method described could certainly be extended for trace determination of optical purity of both L- and D-enantiomers of all amino acids containing a primary amine. Quantitative analysis gave high sensitivity and precision for the presence of the D-enantiomer and the L-compound. For traces of the L-enantiomer in the D-compound the situation is a little bit more complicated because of the tailing of the main component. However, good results can be obtained by the use of either a large resolution or a Daicel Crownpace Cr(-) column, which possesses a reversed enantioselectivity and thus will provide a reversed elution order.

As this technique provides a short analysis time (in most cases  $<20$  min), it may be a good candidate for routine analysis.

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