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Chiral determination of amino acids by capillary electrophoresis and laser-induced fluorescence at picomolar concentrations

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Abstract

In this publication we present results on the determination of enantiomers of amino acids at very low concentrations. A fluoresceine-based chiral dye was synthesized to allow the separation of diastereoisomers of D- and L-amino acids. We used capillary electrophoresis with different non-ionic surfactants (Brij). The separation parameters were optimized and separations of D- and L-isovaline, an unusual terrestrial amino acid, were obtained. The sensitivity limits were also determined using a commercial laser-induced fluorescence detector. The quantitation of these amino acids is very important to understand the process of chiral selection on Earth.

Keywords: Enantiomer separation; Diastereomer separation; Buffer composition; Amino acids; Isovaline; Aminoisobutyric acid; Surfactants

1. Introduction

Amino acids are one of the most important molecules in terrestrial life due to their abundance, their role in biological processes and the existence of chiral selection. Only L-amino acids are incorporated into proteins even though chiral amino acids can exist as two chemically equivalent optical isomers (D and L enantiomers). Most of the proposed scenarios for the emergence of terrestrial homochirality require the amplification of an initially small excess of one enantiomer [1]. One way to obtain the first small enantiomeric excess suggested an extra-terrestrial arrival. The determination of amino acids and their enantiomeric ratio in extra-terrestrial samples is thus of prime interest. Much work concerns the determination of the organic matter in carbonaceous chondrites such as the Murchison meteorite [2–8] which are a distinct subclass of stony meteorites. Most of the 70 amino acids (eight terrestrial and more than 50 non-terrestrial) have been identified in Murchison extract [3]. The determination of the enantiomeric ratio of biological amino acids in meteorites is difficult because of the risk of contamination by terrestrial amino acids, and the risk of racemization during entry into the terrestrial atmosphere. The determination of the enantiomeric ratio of non-bio-

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logical amino acids is interesting due to the low risk of contamination and the low risk of racemization by temperature. The α-dialkylamino acids α-amino-isobutyric acid (AIB) and isovaline, which are not included in proteins, are two of the most abundant amino acids present in Murchison [2,3,5]. AIB and isovaline, which are exceedingly rare on Earth, have recently been identified in sediments (the cretaceous/tertiary boundary) associated with the impact of an asteroid or comet with the Earth [9,10]. The determination of these two dialkylamino acids and the determination of the enantiomeric ratio of isovaline is also interesting in the pharmacological field as they have been found in antibiotics [11]. Because the samples are rare, a most sensitive analytical method must be developed in order to reduce the size of the initial sample. The results obtained with capillary electrophoresis with laser-induced fluorescence (LIF) demonstrate that this method is one of the most sensitive as less than 10^{-21} mol has been detected [12,13] using laboratory-made instruments. Recently, Hutt et al. used microfabricated capillary electrophoresis and laser-induced fluorescence detection to analyze extra-terrestrial amino acids [14]. All these results were obtained using laboratory-made instruments. Nouadje et al. obtained similar results using a CE–LIF commercial instrument for the analysis of fluoresceine isothiocyanate-labeled amino acids [15].

Some time ago we reported the resolution of enantiomeric fluoresceine thiocarbamyl D,L-amino acids with CE–LIF, directly with the use of a chiral additive in the electrolyte (β-cyclodextrin) [16,17]. This can also be achieved by an indirect method after derivatization by a pure enantiomeric reagent [12,18,19]. The indirect method is more interesting for the enantiomeric determination of compounds in complex samples [12]. In this paper we report the indirect determination of D,L-isovaline and AIB by CE–LIF. The sensitivity of the detection and the optimization of the separation are reported.

2. Experimental

2.1. Reagents

AIB and L-isovaline were obtained from Acros (Noisy le Grand, France). Brij 35, 56, 58, 52, 72, 76 and 78, sodium tetraborate, sodium hydrogencarbonate, sodium hydrogenocarbonate, fluorescein isothiocyanate isomer I and palladium on activated carbon were purchased from Aldrich (St. Quentin Fallavier, France). ε-Benzyloxycarbonyllysine (*tert*-.butyl ester) was obtained from Bachem (Voisin-le-Bretonneux, France).

Borate buffer 20 m*M* (pH 9) was prepared by dissolving 343 mg of tetraborate in 45 ml of water; carbonate buffer 20 m*M* (pH 9.9) was prepared by dissolving 424 mg of Na_2CO_3 and 336 mg of $NaHCO_3$ in 200 ml of water. Brij is added directly to the buffer at the appropriate concentration.

2.2. Synthesis of AIB and L,D-isovaline derivatives

Chloroethylnitrosourea of ε-benzyloxycarbonyllysine (*tert*.-butyl ester) (CENU-Lys) was prepared (Fig. 1) following a method described elsewhere [20]. A solution of 0.4 mmol of CENU-Lys in acetonitrile (10 ml) is added to 0.8 mmol of AIB or D- (or L-)isovaline in water (10 ml, adjusted to pH 10.5 with 0.1 *M* NaOH) following the protocol described in a previous paper [21]. The benzyloxycarbonyl group is removed by hydrogenolysis with palladium on activated carbon as described by Greene and Wuts [22] before addition of fluorescein isothiocyanate isomer I following the classical protocol [13]. AIB and isovaline fluorescent diastereomers (Fig. 1) were used for all following studies.

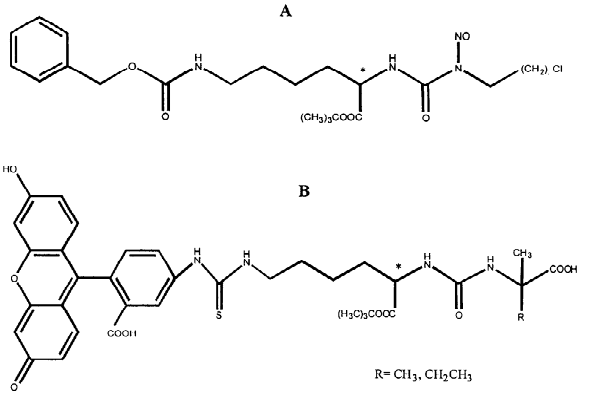

Fig. 1. (A) Chloroethylnitrosourea of ε-benzyloxycarbonyllysine (*tert*.-butyl ester) (CENU-Lys) used for the derivatization of amino acids. (B) Structure of amino acid derivatives for enantiomeric determination with CE–LIF.

2.3. Apparatus

Studies of the effects of Brij and buffer on the separation of the derivatized amino acids were carried out with an HP CE^{3D} (Agilent Technologies, France) system with a fused-silica capillary [64.5 cm (56.5 cm to detector window)×75 μm I.D. at 25°C] (Agilent Technologies). UV detection was at 492 nm.

A CE instrument from Agilent Technologies and its mass spectrometry cassette were used for sensitivity studies. It was coupled to a LIF detector (ZETALIF detector, Picometrics, Ramonville, France). The LIF detector was installed on a support table designed by Picometrics to position the two buffer vials (injection and detection side) at the same level. Fused-silica capillaries [60 cm (55 cm to detector window)×75 μm I.D.] were obtained from Agilent Technologies. New capillaries were first pretreated by flushing with 1 *M* NaOH for 20 min and pure water for 10 min. The samples were introduced under pressure (50 mbar, 10 s). Between runs the capillary was flushed with 0.1 *M* NaOH (2 min), followed by water rinsing for 2 min. These solutions were changed after two to five injections.

3. Results and discussion

3.1. Separation: Brij and buffer effect

The amino acids labeled with fluoresceine homologues, which were studied in this work, have three negative charges due to the fluoresceine nucleus and the carboxylate function. The total charge is −3 under the pH conditions we used (>9).

Amino acid separations are often performed using sodium dodecyl sulfate (SDS) [15–17]. Previously, some separations of fluoresceine thiocarbamyl amino acid enantiomers were obtained using this anionic surfactant and cyclodextrin [17]. Separation of our diastereoisomers was not possible using sodium dodecyl sulfate (results not shown).

Without surfactant, D- and L-isovaline derivatives are co-eluted and partially separated form AIB derivatives (not shown).

In a previous study we demonstrated that the migration order of amino acids labeled with fluoresceine isothiocyanate in capillary zone electrophoresis is explained by their molecular mass [23]. In fact, in this analysis where normal polarity is used and the positive electrode is on the injection side, the observed velocity v_t of a species is:

$$v_t = v_{eo} - v_{ep} \quad (1)$$

where v_{eo} is the velocity of the electroosmotic flow and v_{ep} is the electrophoretic velocity. v_{ep} is the smallest, v_t the highest and the migration time the shortest. Here, AIB is the species which has the lowest molecular mass, while D- or L-isovaline has the highest. This means that AIB has a larger electrophoretic mobility and a longer migration time than isovaline. This is in agreement with our measurements.

As no separation can be achieved with or without SDS we chose non-ionic surfactants. Brij surfactants are high-molecular-mass non-ionic surfactants with low critical micellar concentrations. In an electric field, the velocity of the pseudo-stationary phase is the same as the electroosmotic flow. As a consequence, compounds with negative charge that interact with Brij will have an apparent velocity higher than without Brij.

Fig. 2 shows that the separation of L,D-isovaline and AIB derivatives is possible using capillary electrophoresis and a non-ionic surfactant such as Brij. To show the behavior of the different Brij surfactants in separating the different diastereoisomers, we used the retention factor k' defined as:

$$k' = \frac{v_{ep}}{v_{eo} - v_M} - 1 \quad (2)$$

where v_{ep} is the electrophoretic velocity of the derivatized amino acids in the buffer without Brij, v_{eo} is the electroosmotic velocity and v_M is the migration velocity of the amino acids in the buffer containing Brij. $v_M = (L/t_m)$, with t_m being the migration time of the species with Brij.

Without Brij, in Eq. (2) we have $k' = 0$ and the classical Eq. (1) is obtained. With Brij, the k' values shown in Fig. 3 increase linearly when the concentration of Brij increases because the concentration of the pseudo-stationary phase also increases. These

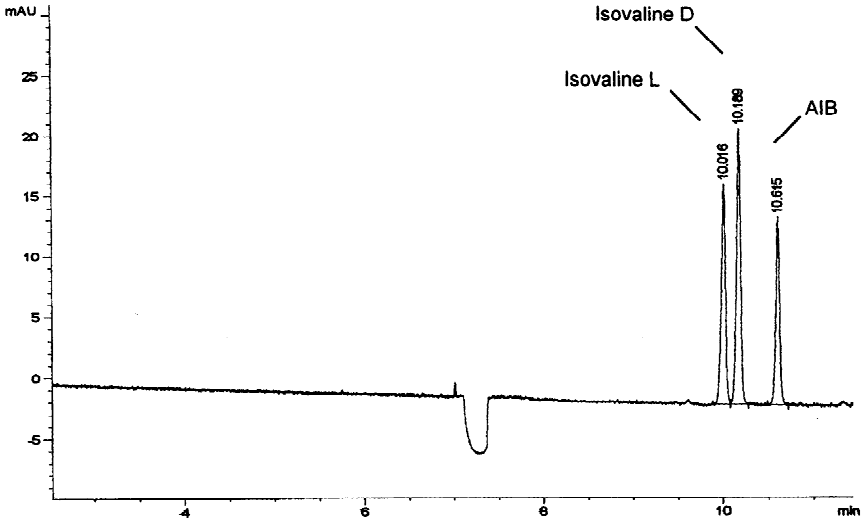

Fig. 2. Separation of L,D-isovaline and AIB derivatives of labeled amino acids. 20 m*M* Borate buffer, 20 m*M* Brij 58, 20 kV, 25°C.

results show that Brij allows separation by the differential distribution of the species between the pseudo-stationary phase and the buffer. The apparent electrophoretic modality ratio for AIB and (*S*)-isovaline without Brij is 1.01 and 1.15 with Brij 58 (20 m*M*). The difference between these ratios again illustrates the existence of an interaction between the compounds and Brij. With Brij, the compounds migrate in the order of decreasing hydrophobicity. Brij does not change the migration order but allows resolution of the diastereomers. We can assume that this separation is possible because the two diastereoisomers do not have the same distribution between the pseudo-stationary phase and the buffer. The distance between the two chiral centers of the isovaline diastereoisomer derivatives is equivalent to two NH–C and two CO–NH bond lengths. The separation, which is presented in Fig. 2, is in agreement with studies on the effect of the distance between two chiral centers on the enantiomeric resolution, which demonstrate that, when the distance is too large (five, six or seven bonds depending on the structure), separation of the diastereoisomers cannot be achieved [24–26]. A urea function between two chiral carbon atoms can increase the separation of the diastereoisomers since it has been shown that diastereoisomeric compounds that have an amide function in their molecular structure exhibit a remarkably high resolution factor [27].

Of the seven different Brij surfactants used, only Brij 35 [$C_{12}H_{35}(OCH_2CH_2)_{23}OH$], 58 [$C_{16}H_{33}(OCH_2CH_2)_{20}OH$] and 78 [$C_{18}H_{37}$-$(OCH_2CH_2)_{20}OH$] allow resolution of the compounds. Brij 52 [$C_{16}H_{33}(OCH_2CH_2)_2OH$] and 56 [$C_{16}H_{33}(OCH_2CH_2)_{10}OH$] are not completely soluble in the electrolyte due to reduction of the polar part $(OCH_2CH_2)_n$ ($n = 20$, 10 and 2 for Brij 58, 56 and 52).

For a defined concentration of Brij, the difference in migration between AIB and isovaline seems to increase when a smaller hydrocarbonated chain Brij is used. In a micellar medium the interaction of compounds depends on the monomer size and the number of aggregates of the monomer. The number of aggregates for Brij 35 is smaller than for Brij 78 (40 versus 70) so we can assume that interaction with Brij 35 is easier than with Brij 78 and this could explain why k' is higher for Brij 35 than for Brij 78 (Fig. 3).

The effects of buffer composition with Brij 58 were studied. The results show that there is no effect of the carbonate and borate buffer concentration on

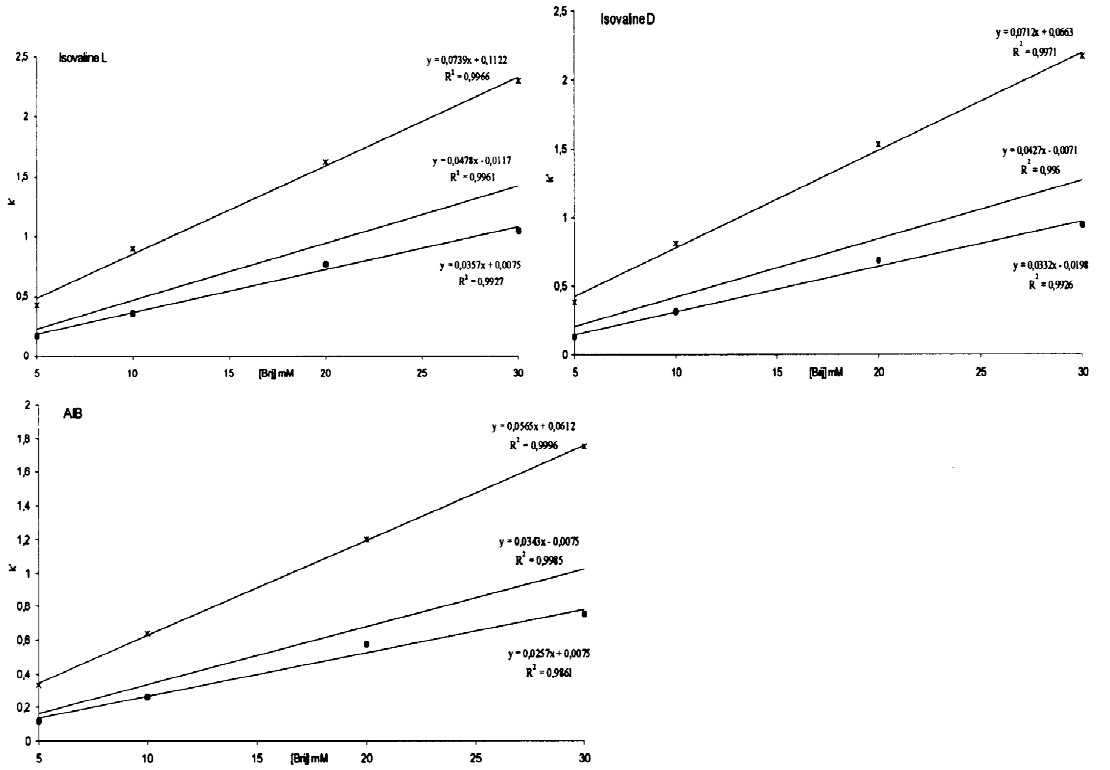

Fig. 3. Effect of Brij (nature and concentration) on the retention factor of the derivatives. Brij 35 (*), Brij 58 (□), Brij 78 (●), tetraborate buffer 20 m*M*, 18 kV, 25°C.

the retention of the three compounds (Fig. 4). There is no significant difference between borate and carbonate buffer.

3.2. Fluorescence detection: derivative properties and sensitivity

The fluorescein moiety is widely used for sensitive detection in LIF [14–17]. This fluorescent moiety is interesting because it is a good fluorescent group and the maximum of the absorbance intensity is in accordance with the most commonly used laser wavelength, i.e. 488 nm (argon ion laser). Its fluorescence quantum yield in basic media is >0.93 and it has a high molar absorptivity, 76 900 M^{-1} cm^{-1} [28].

As shown in Table 1, the derivatives have a maximum absorption near 488 nm, which is the excitation wavelength delivered by an argon laser. The three spectra are similar in the 400–700 nm region, but are different in the 190–300 nm region due to the absorption of the urea function. The difference observed between the absorption of the two diastereoisomers of isovaline illustrates the existence of two different conformations which allow their resolution in our CE system.

The derivatives have a maximum emission at 517 nm in free solution (no Brij, Fig. 5). The characteristics of the fluorescence spectra of the L-isovaline derivatives in the Brij buffer are presented in Table 2. This indicates a slight red shift when the Brij concentration increases and a change of the fluorescence intensity. A possible reason is the interaction of the change of solvent clusters around the fluorescein nucleus when the derivatives interact with the Brij. Similar results were recorded earlier with aromatic anions [25]. With SDS we did not see any change in the fluorescence spectra of the compounds.

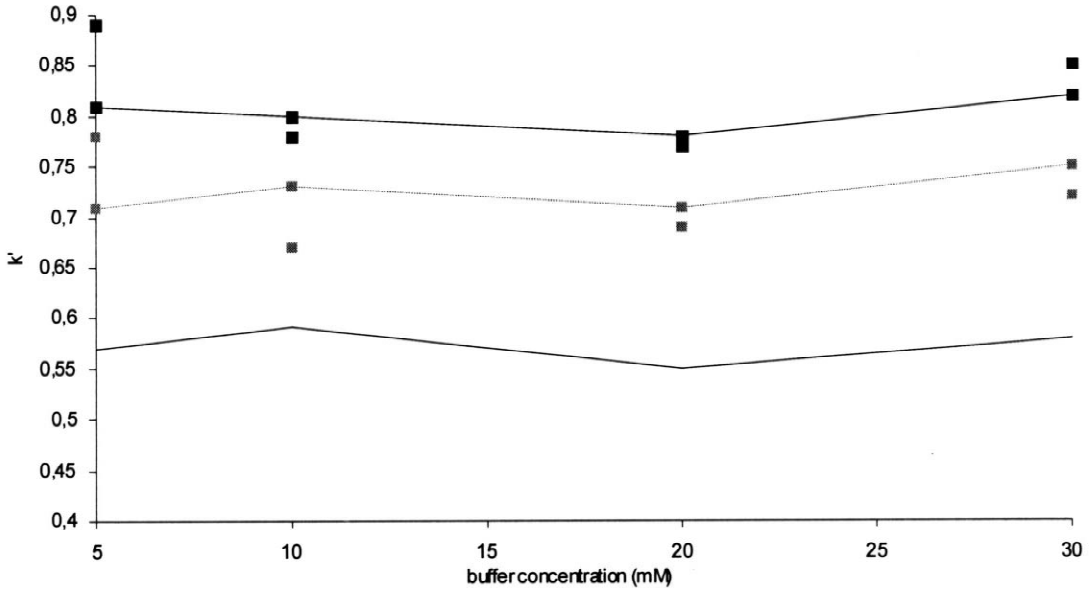

Fig. 4. Effect of buffer (nature and concentration) on L-isovaline (black symbol), D-isovaline (gray) and AIB (empty). Borate buffer (no line) and carbonate buffer (line).

This is in agreement with our CE results. To the best of our knowledge, this is the first time that the fluorescence behavior of a fluorescein nucleus with Brij has been reported.

The need for a diamino chiral compound for the synthesis of the derivatives led us to use lysine even though it has been shown that the sensitivity ($8.6 \cdot 10^{-11}$ *M*) of detection with the fluorescein lysine thiourea derivative is one of the worst [13]. With our derivatives, the limit of detection (in borate buffer and Brij 58, 20 m*M*) with a signal-to-noise ratio of 3 is 10.2 p*M* for (*S*)-isovaline 12.2 p*M* for (*R*)-isovaline and 29.6 p*M* for AIB. The detection is linear between 3 n*M* and 10 p*M*; the regression equation is $y = ax + b$, where x is the concentration of derivatives (*M*) and y the peak area/s is $y = 3 \cdot 10^9 x - 0.0231$ for (*S*)-isovaline, $y = 9 \cdot 10^8 x - 0.011$ for (*R*)-isovaline and $y = 2 \cdot 10^9 x - 0.0519$ for AIB.

These limits of detection are lower than the limits of detection obtained by Cheng and Dovichi [13] with the fluorescein–lysine derivative (90 p*M*). We can explain this difference by the quantum yield of the derivatives and also by the efficiency of the detector. The use of carbonate buffer instead of borate buffer does not change the limit of detection. For a reproducible quantification of amino acids and

Table 1
Absorption wavelength in the ultraviolet and absorbance (*A*) measured on a solution of derivatives at $5.33 \cdot 10^{-6}$ *M* [(*S*)-isovaline], $5.66 \cdot 10^{-6}$ *M* [(*R*)-isovaline] and $5.2 \cdot 10^{-6}$ *M* (AIB) in carbonate buffer (pH 10.3, 5 m*M*)

Compound	$\lambda_{max\ 1}$ (nm)/*A* (Au)	$\lambda_{max\ 2}$ (nm)/*A* (Au)	$\lambda_{max\ 3}$ (nm)/*A* (Au)
(*S*)-Isovaline derivatives	492.4/0.319	233/0.284	199/0.201
(*R*)-Isovaline derivatives	492/0.288	239.4/0.152	
AIB derivatives	492.5/0.416	239.4/0.288	203.2/0.238

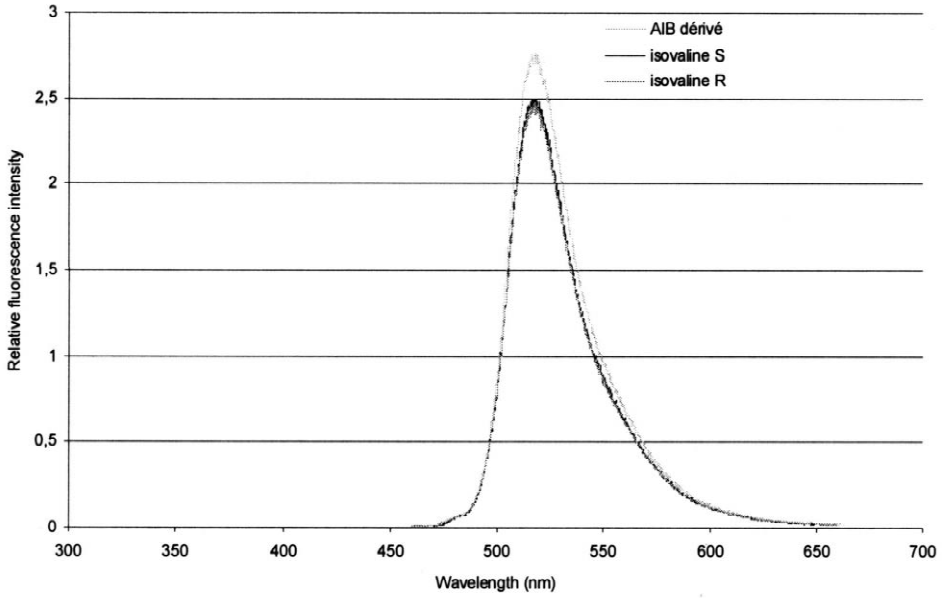

Fig. 5. Fluorescence spectrum of amino acid derivatives measured in carbonate buffer 5 m*M* (pH 10.3), with excitation at 480 nm (2 nm bypass band in excitation, 0.5 nm bypass band in emission, photodetector Hamamatsu R 936).

Table 2
Effect of the concentration of Brij 58 on the fluorescence of L-isovaline derivatives

Brij 58 (m*M*)	λ_{max} (nm)	Relative peak height (at λ_{max})
0	517	17.4
5	519	16.3
10	520	15.2
20	521	14.8
30	521	14.6

their enantiomeric ratio, injecting less than 6000 molecules into the column is not recommended for statistical reasons.

4. Conclusion

In conclusion, we have shown that the derivatives allow a good resolution of the diastereoisomers of D- and L-isovaline and AIB using a Brij-containing buffer. The limit of detection is lower than the limits presented in the literature. This sensitive detection opens up new possibilities in the analysis of D,L-amino acids and will be ideally suited for the search of amino acids in very small samples such as micrometeorites (30 μg) and Martian samples.

Acknowledgements

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