Anal Bioanal Chem
DOI 10.1007/s00216-014-7961-y

RESEARCH PAPER

D- and L-amino acids in Antarctic lakes: assessment of a very sensitive HPLC-MS method

Elena Barbaro · Roberta Zangrando · Marco Vecchiato · Clara Turetta · Carlo Barbante · Andrea Gambaro

Received: 27 March 2014 / Revised: 19 May 2014 / Accepted: 6 June 2014

Abstract Amino acids represent a fraction of organic matter in marine and freshwater ecosystems, and a source of carbon, nitrogen and energy. L-Amino acids are the most common enantiomers in nature because these chiral forms are used during the biosynthesis of proteins and peptide. To the contrary, the occurrence of D-amino acids is usually linked to the presence of bacteria. We investigated the distribution of L- and D-amino acids in the lacustrine environment of Terra Nova Bay, Antarctica, in order to define their natural composition in this area and to individuate a possible relationship with primary production. A simultaneous chromatographic separation of 40 L- and D-amino acids was performed using a chiral stationary phase based on teicoplainin aglycone (CHIROBIOTIC TAG). The chromatographic separation was coupled to two different mass spectrometers—an LTQ-Orbitrap XL (Thermo Fisher Scientific) and an API 4000 (ABSciex)—in order to investigate their quantitative performance. High-performance liquid chromatography coupled with mass spectrometry methods were evaluated through the estimation of their linear ranges, repeatability, accuracy and detection and quantification limits. The high-resolution mass spectrometer LTQ-Orbitrap XL presented detection limits between 0.4 and 7 $\mu g L^{-1}$, while the triple quadrupole mass spectrometer API 4000 achieved the best detection limits reported in the literature for the quantification of amino acids (between 4 and 200 $ng L^{-1}$). The most sensitive method, HPLC-API 4000, was applied to lake water samples.

Keywords Amino acids · Chiral separation · Antarctica · HPLC-MS/MS · HPLC-orbitrap · Lakes

Introduction

Chirality is an important feature of many compounds, as biologically active molecules for which it attributes different biological proprieties or influences the enrichment or the stability in the environment [1]. Most amino acids contain an asymmetric centre, and the analysis of L- or D-enantiomers is very important in several research fields, such as food chemistry [2], geochronology [3] and exploration of other planets [4]. The homochirality of life on Earth occurs because L-amino acids are the only enantiomers used during the biosynthesis of proteins and peptides [5]. To the contrary, the occurrence of certain D-amino acids in cell walls can be attributed to bacterial origin [6]. Some studies demonstrated that D-enantiomers can also be produced by racemisation, indicating an age effect [7–10].

Amino acids are a common source of carbon, nitrogen and energy in both marine and freshwater ecosystems. They operate as important intermediates in the flux of carbon, although their concentrations are usually very low and represent only a small fraction of dissolved organic carbon (DOC) [11].

Berman and Bronk [11] described how amino acids are used as nitrogen sources not only by bacteria but also by numerous phytoplankton species. These compounds are

Electronic supplementary material The online version of this article (doi:10.1007/s00216-014-7961-y) contains supplementary material, which is available to authorized users.

E. Barbaro (✉) · A. Gambaro
Department of Environmental Sciences, Informatics and Statistics, University of Venice, Ca' Foscari, Calle Larga Santa Marta 2137, 30123 Venice, Italy
e-mail: barbaro@unive.it

E. Barbaro · R. Zangrando · M. Vecchiato · C. Turetta · C. Barbante · A. Gambaro
Institute for the Dynamics of Environmental Processes CNR, Dorsoduro 2137, 30123 Venice, Italy

M. Vecchiato
Department of Physical Sciences, Earth and Environment, University of Siena, Strada Laterina, 8, 53100 Siena, Italy

Published online: 25 June 2014

released from living phytoplankton, either directly or by cellular lysis of senescent algae [12]. Amino acids can also be generated by the solubilisation of organic material, aerosol particles or transparent proteinaceous particles [13]. Terrestrial plants or the soil's organic matter can also be important contributors to the production of amino acids [14].

One of the main aims of our study was to quantify enantiomeric forms of several amino acids in lacustrine water collected in Terra Nova Bay, Antarctica.

Four samples were collected in three sites: Inexpressible Island (lake 10), Edmonson Point (lakes 14 and 15A) and Tarn Flat (lake 20). The sampling was repeated two times during the austral summer to evaluate possible changes in the composition or concentration of amino acids during the defrost process.

Antarctica represents an excellent natural laboratory to estimate the natural presence, concentration and variability of organic or inorganic compounds due to its distance from anthropogenic emissions [15]. Antarctica is the largest cold desert on Earth [16], except for a few weeks during the austral summer, when waters from melting ice and snow flow into several small ponds and lakes.

Recently, Antarctic lacustrine ecosystems have been investigated to determine major, minor or trace element distributions [17–24], while organic compounds were less extensively studied [16, 25–27]. Antarctic lake waters collected in the McMurdo Dry Valleys were studied in-depth, yielding information on the relationship between water chemistry and microorganisms [17, 18], paleoclimatology [28], chemical alteration and sedimentation [29, 30]. The region of Terra Nova Bay has been studied since 1985, year of the beginning of the Italian National Research Program in Antarctica (P.N.R.A.), but very few investigations [19, 20, 22, 26] addressed the geochemistry of its lacustrine ecosystems. The area's inorganic chemistry was investigated [19, 20, 22, 26], but this is the first study to characterise it by defining its amino acid distributions and to individuate a relationship with primary production.

Another important aim of our research was to develop a simultaneous method to quantify trace concentrations of L- and D-amino acids using a hyphenated technique high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) in lake water samples.

Enantiomeric separation is usually performed by conventional reversed-phase liquid chromatography, which requires the formation of diastereoisomeric derivates before chromatographic separation with a chiral reagent [31, 32]. However, this approach presents several disadvantages linked to derivatisation procedures, such as derivate instability, the inadequate enantiopurity of some reagents or long preparation times. A derivatisation procedure is necessary also when gas chromatography is used because the derivate should increase the analyte's volatility [33].

Other possible solutions to separate two enantiomers include using chiral ligand-exchange chromatography [34] or a chiral stationary phase based on α-cyclodextrin [35], crown ether [36] or teicoplanin [37, 38]. The detector most frequently used to analyse underivatised amino acids is the amperometric/electrochemical detector [39], which has low specificity. Amino acids are ionisable, thermolabile and polar compounds that can be analysed by the most selective electrospray ionisation (ESI) mass spectrometry. Reversed-phase chromatography used on derivatised amino acids can be coupled with mass spectrometry [32], whereas normal phase chromatography, with hexane and heptane as mobile phases, is generally applied to chiral stationary phases to separate underivatised amino acids, and is incompatible with ESI source. The stationary phase based on teicoplanin [40] is able to separate underivatised amino acids primarily in the reversed-phase mode, which makes it compatible with ESI-mass spectrometry.

The main aim of this work was to develop one single chromatographic 35-min run to enantiomerically separate 40 underivatised amino acids using a stationary phase. By coupling with two different mass spectrometers—a high-resolution LTQ-Orbitrap XL mass spectrometer and a triple quadrupole API 4000—we were able to investigate the quantitative performance of two recently developed HPLC-MS methods to determine L-and D-amino acids in environmental samples at trace levels. The best sensitivity was achieved by coupling HPLC with a triple quadrupole. This is also the first study where an high-resolution mass spectrometry (HRMS) method was improved for simultaneous qualitative and quantitative analyses. The application of the latter technique is one of the new trends in environmental analysis to individuate *untargeted* compounds that cannot be detected by low-resolution MS.

Experimental section

Reagents and standard solutions

Ultra-grade methanol (MeOH) was purchased from Romil LTD (Cambridge, UK), while Ultrapure water (18.2 MΩ, 0.01 TOC) was produced using a Purelab Ultra System (Elga, High Wycombe, UK). Formic acid (≥98 %) eluent additive for HPLC system was obtained from Fluka (Sigma Aldrich®, Buchs, Switzerland) and hydrochloric acid (HCl) 37 % ACS was supplied by Carlo Erba.

Each amino acid standard solution [D- and L-alanine (D-/L-Ala), D- and L-arginine (D-/L-Arg), D- and L-asparagine (D-/L-Asn), D- and L-aspartic acid (D-/L-Asp), D- and L-glutamic acid (D-/L-Glu), glycine (Gly), D- and L-glutamine (D-/L-Gln), D- and L-hydroxyproline (D-/L-Hyp), D- and L-histidine (D-/L-Hys), D- and L-isoleucine (D-/L-Ile), D- and L-leucine (D-/L-

Leu), D- and L-lysine (D-/L-Lys), D- and L-methionine (D-/L-Met), D- and L-ornithine (D/L-Orn), D- and L-phenylalanine (D-/L-Phe), L-proline (L-Pro), D- and L-serine (D-/L-Ser), D- and L-threonine (D-/L-Thr), D- and L-tyrosine (D-/L-Tyr), D- and L-tryptophan (D-/L-Trp), and D- and L-valine(D-/L-Val)] was prepared by solid standard (purity ≥98 %), and diluted in HCl 0.1 M. The solid standards were purchased from Sigma Aldrich®.

Isotopically labelled ^{13}C amino acids (L-[$^{13}C_3$] alanine (Ala*), L-[$^{13}C_4$] aspartic acid (Asp*), L-[$^{13}C_5$] glutamic acid and L-[$^{13}C_6$] arginine (Arg*); purity of 98 %) were obtained from Sigma Aldrich while L-[$^{13}C_1$] leucine (Leu*), L-[$^{13}C_1$] phenylalanine (Phe*), L-[$^{13}C_1$] proline (Pro*) and L-[$^{13}C_1$] valine (Val*) (purity ≥98 %)) were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA).

Liquid chromatography–mass spectrometry systems

Underivatised amino acids were separated into L- and D-enantiomers using a CHIROBIOTIC TAG column (2.1×250 mm, Advanced Separation Technologies Inc. USA). The column temperature was maintained at 25 °C, and flow rate was 200 μL min^{-1}. Mobile phase A was 0.1 % formic acid, and B was methanol containing 0.1 % formic acid. At the beginning, an isocratic step with 30 % mobile phase B was used to elute the majority of amino acids in 15 min. The linear gradient started with 30 % mobile phase B and reached 100 % over 5 min. To wash the column, the 100 % concentration of eluent B was maintained for 5 min, while the equilibration step was carried out for five additional minutes. The injection volume was 10 μL.

The analysis of underivatised amino acids was performed using two different HPLC-MS systems.

An UltiMate 3000 system (Thermo Fisher Scientific. Sunnyvale, CA, USA) with a quaternary pump, vacuum degasser, autosampler and thermostated column compartment was coupled with a LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany) using a heated electrospray interface operated in positive ionisation mode.

The tune method was optimised by directly infusing a 1-mg L^{-1} amino acid standard water solution into the mass spectrometer's ion source. The tune method was based on the following parameters: spray voltage, 4 kV; temperature, 350 °C; capillary voltage, 5 V; tube lens, 40 V; sheath gas, 50 arb units; and auxiliary gas, 15 arb units. All mass spectra were acquired in profile mode using the Orbitrap mass analyser at a mass resolving power of 60,000 (FWHM, at *m/z* 400; 0.5 s scan cycle time) withthe diisooctil-phthalate at *m/z* 391.2845 as lock mass. Accurate mass measurements of $[M+H]^+$ of the amino acids were carried out by scanning from 60 to 400*m/z*. The ion injection time was 50 ms with the automatic gain control (AGC, corresponding to the number of changes transferred from the front-stage ion trap to the orbitrap analyser) target set at 1×10^5.

Data were processed using Xcalibur 2.1.0 (Thermo Fisher Scientific, San Jose, CA, USA) and Thermo LCquant 2.6.1 software. The extracted ion chromatograms (XICs) were generated by extracting a small range (±10 ppm), centred on the exact *m/z* of each analyte. The elementary composition of each amino acid, the theoretical monoisotopic *m/z* of each $[M+H]^+$ and the measured mass accuracy (ppm) are summarised in Table 1.

An Agilent 1100 Series HPLC Systems (Waldbronn, Germany; with a binary pump, vacuum degasser, autosampler and thermostated column compartment) was coupled with an API 4000 Triple Quadrupole Mass Spectrometer (Applied Biosystem/MSD SCIEX, Concord, Ontario, Canada) using a TurboV electrospray source that operated in positive mode.

The mass spectrometer's parameters were optimised as follows: temperature, 500 °C; ion spray voltage, 5,450 eV; GS1, 22 psi; GS2, 70 psi; curtain gas, 30 psi; collision gas (CAD), 4 psi; and entrance potential, 10 V. Data were collected in multiple reaction monitoring (MRM) mode. The first quadrupole (Q1) selected the precursor ion, while the third quadrupole selected the product ion. Both Q1 and Q3 were set at unit resolution with peak width of 0.7±0.1 amu at 50 % of maximum peak height. Two transitions were considered for each analyte: The most intense one was applied to the quantification, while the second was used to confirm the presence of amino acids.

In order to achieve the highest possible sensitivity, declustering potential, collision energy and collision cell exit potential were optimised. Optimisation was performed using direct infusions of 1 mg L^{-1} of individual standard of amino acids. The monitored transition and instrumental parameters for each compound are summarised in Table 1. Analyst Software version 1.5.2 (Applied Biosystems, MDS SCIEX Instruments) was used for the identification and quantification of the target compounds.

Study site

The sampling was carried out during the 2011–2012 austral summer field activity in Antarctica in the framework of the "Progetto Nazionale di Ricerche in Antartide" (PNRA).

Antarctic lacustrine waters were collected in four different lakes located at Inexpressible Island (lake 10), Edmonson Point (lakes 14 and 15A) and Tarn Flat (lake 20). The lakes considered in this study are frozen during the winter but completely ice free during a few weeks in the austral summer. Snow and ice melt are the only water input for these small and shallow lakes. The geographic characteristics of the sampling points and the sampling features are presented in the Electronic Supplementary Material (ESM) Table S1.

Table 1 Summary of the theoretical mass used to construct an XIC for each analyte with 10 ppm as tolerance from Orbitrap full scan and each MRM transition used by the API 4000

Amino acid	Elemental composition	Theoretical mass $[M+H]^+$	Mass accuracy (ppm)[a]	MRM transitions	DP/CE/CXP
D-/L-Ala	$C_3H_7NO_2$	90.0550	2.2	90>44	32/17/7
D-/L-Arg	$C_6H_{14}N_4O_2$	175.119	0.2	175>70 (116)	53/33/13 (53/21/21)
D-/L-Asp	$C_4H_7NO_4$	134.0448	0.1	134>74 (88)	34/21/13 (34/15/13)
D-/L-Asn	$C_4H_8N_2O_3$	133.0608	0.7	133>74 (87)	35/22/7 (35/13/8)
D-/L-Gln	$C_5H_{10}N_2O_3$	147.0764	−0.6	147>130 (84)	38/15/11 (38/22/7)
D-/L-Glu	$C_5H_9NO_4$	148.0604	0.3	148>84 (130)	40/24/16 (40/14/23)
Gly	$C_2H_5NO_2$	76.0393	2.6	76>76 (30)	33/6/11 (33/17/4)
D-/L-Hyp	$C_5H_9NO_3$	132.0655	−0.1	132>68 (86)	47/31/6 (47/21/7)
D-/L-Hys	$C_6H_9N_3O_2$	156.0768	0.3	156>110 (83)	46/20/20 (46/36/15)
D-/L-Leu/Ile	$C_6H_{13}NO_2$	132.1019	0.8	132>86 (69)	41/15/16 (41/26/13)
D-/L-Lys	$C_6H_{14}N_2O_2$	147.1128	2.8	147>84 (130)	32/24/15 (32/15/23)
D-/L-Met	$C_5H_{11}NO_2S$	150.0583	−0.2	150>104 (133)	39/15/16 (39/15/23)
D-/L-Orn	$C_5H_{12}N_2O_2$	133.0972	0.6	133>70 (116)	45/24/11 (45/12/15)
D-/L-Phe	$C_9H_{11}NO_2$	166.0863	1.6	166>120 (103)	43/18/22 (43/38/19)
L-Pro	$C_5H_9NO_2$	116.0706	−0.6	116>70	42/24/13
D-/L-Ser	$C_3H_7NO_3$	106.0499	0.1	106/60 (88)	34/17/10 (34/15/16)
D-/L-Thr	$C_4H_9NO_3$	120.0655	1.5	120/103 (77)	78/26/18 (78/36/14)
D-/L-Trp	$C_{11}H_{12}N_2O_2$	205.0972	−0.3	205>188 (146)	53/15/17 (53/24/13)
D-/L-Tyr	$C_9H_{11}NO_3$	182.0812	−0.5	182>165 (136)	43/15/15 (43/21/12)
D-/L-Val	$C_5H_{11}NO_2$	118.0863	2.2	118>72 (55)	35/17/13 (35/30/10)
L-[$^{13}C_3$] Ala	$C_3H_7NO_2$	93.0650	0.4	93>46	37/19/8
L-[$^{13}C_6$] Arg	$[^{13}C_6]H_{14}N_4O_2$	181.1391	−0.2	181>74 (121)	55/21/7 (55/21/11)
L-[$^{13}C_4$] Asp	$[^{13}C_4]H_7NO_4$	138.0588	3.6	138/76 (91)	42/22/7 (42/15/10)
L-[$^{13}C_5$] Glu	$[^{13}C_5]H_9NO_4$	153.0772	0.5	153>135 (106)	39/13/12 (39/19/10)
L-[$^{13}C_1$] Leu	$[^{13}C_1]C_5H_{13}NO_2$	–	–	133>86 (44)	45/16/7 (45/31/8)
L-[$^{13}C_1$] Phe	$[^{13}C_1]C_8H_{11}NO_2$	–	–	167>121 (104)	51/19/11 (51/38/10)
L-[$^{13}C_1$] Pro	$[^{13}C_1]C_4H_9NO_2$	–	–	117>70 (68)	40/24/6 (40/41/6)
L-[$^{13}C_1$] Val	$[^{13}C_1]C_4H_{11}NO_2$	–	–	119>72 (55)	45/18/6 (45/29/5)

For each MRM transition we reported the values of declustering potential (DP), collision energy (CE) and cell exit potential (CXP). The parenthetical values advert to the qualifier ions

[a] Measured from the 100 ng mL^{-1} standard solution using to generate the calibration curves

Each lake has its unique characteristics in terms of origin, depth and the nature of its surrounding area.

Inexpressible Island is situated in front of a coastal polynya of Terra Nova Bay, which is free from sea-ice cover all year round. This marine area is characterised by intense and persistent katabatic winds. These cold and dense air masses moving from the Antarctic plateau to the sea promote the formation of the polynya. Lake 10 extends over 4,000 m^2, with a maximum depth of about 2 m and limited accumulation of mats above surface sediments.

The two lakes at Edmonson Point (14 and 15A) are located near the active volcano of Mount Melbourne, and this site extends over ca. 6 km^2 with hills, knolls and moraines separated by small valleys and by a beach. The area's ground surface consists of coarse volcanic materials such as scoria, pumice, tuff and lava. The availability of freshwater and nutrients, derived by marine aerosol and by nesting seabirds, encourages the development of vegetal communities [41]. The main phototrophs in inland freshwaters are cyanobacteria [42], but there are many other organisms such as diatoms, bacteria, yeasts, protozoans, rotifers, nematodes and tardigrades [43]. The sampling was conducted in the small lake 14, which covers a surface area of about 400 m^2 and has a maximum depth of about 1 m. The dimension of this lake was drastically reduced over the last decade, as demonstrated by Bargagli et al. [44], who reported a surface area of 3,500 m^2 in January 2006. The other sampled site was lake 15A, with a surface area of 2,000 m^2 and a depth of 1 m.

The Tarn Flat area is located between the Larsen and Reeves Glaciers, and alternates between wide hills and depressions. Tarn Flat Lake (lake 20) lies in one of these depressions (−70 m). It has a surface area of about 17,700 m^2 and a

maximum depth of 4 m. The main input of this lake is the melt water of glaciers, while persistent winds prevent snow accumulation.

Each lake was sampled at two different dates (ESM Table S1) to evaluate the changes in amino acid composition that may have occurred during the defrost process.

Samples collection and processing

Lacustrine waters were collected into two 2-L glass bottles, pre-cleaned with water and methanol.

In the laboratory of the "Mario Zucchelli Station" (Antarctica), the water sample contained in the first bottle was processed in a clean environment under a Class-100 laminar flow bench-hood. Water was filtered using pre-ignited (400 °C for 4 h) filters (Whatman GF/F), and the filtered water was frozen at −20 °C to await analysis. This filtrated water was used to determine amino acid concentration in the dissolved fraction, after spiking with internal standards solution (average concentrations about 40 μg L^{-1}).

An aliquot of collected water (500 mL) was filtered using mixed cellulose ester membrane filters (MCE) (0.2 μm, Ø 0.47 mm. Whatman, Dassel, Germany). Nutrient analyses [PO_4^{3-}, NO_3^-, NO_2^-, NH_4^+ and $Si(OH)_4$] were carried out on the filtrate of this aliquot using American Public Health Association (APHA) standard method 4500 with appropriate colorimetric kits. The MCE filters were extracted with acetone/water solution (9:1), and chlorophyll *a* was determined. The absorption was measured spectrophotometrically at 665 nm [45].

The second lacustrine water bottle was immediately frozen at −20 °C. The filtration step was carried out following the defrost process and an ultrasonically sonification (for 30 min) in order to release amino acids by cell lysis. The total concentration of amino acids was determined after spiking with internal standards solution (average concentrations, 40 μg L^{-1}).

The concentration of amino acids in the particulate fraction was calculated by subtracting of total concentration and that in dissolved fraction.

Results and discussion

Chromatographic separation

After optimising the parameters of the orbitrap-MS as detailed in the "Experimental section", the chromatographic method was performed in order to simultaneously distinguish several L- and D-amino acids in aqueous samples without derivatising them.

Several methods were applied to the enantiomeric separation of amino acids using conventional reversed-phase columns after the formation of diastereoisomers with chiral reagents [31, 32].

Berthod et al. [46] reported that the stationary phase based on teicoplanin aglycone, called CHIROBIOTIC TAG, is characterised by higher selectivity and resolution for the majority of amino acids than other teicoplanin stationary phases. In their works, the composition of the mobile phase was changed to obtain the best chromatographic performance (enantioselectivity and resolution) for each couple of enantiomers.

In our study, this stationary phase was chosen to simultaneously separate several L- and D-amino acids [46].

CHIROBIOTIC TAG is one of the most appropriate columns to analyse amino acids with a hyphenated system HPLC-MS and without any previous derivatisation because it permits to separate optical isomers using volatile mobile phase with a high percentage of organic solvent, preserving the spray stability [38, 47, 48].

The diverse chemical features of amino acids require mobile phases with different compositions for neutral, basic or acid compounds, in order to obtaining the best selectivity and resolution.

Our aim was to develop a unique HPLC-MS method to quantify trace concentrations of 40 L- and D-amino acids for environmental applications in remote areas.

Enantiomeric separations with CHIROBIOTIC TAG column is usually improved by an alcoholic mobile phase [40].

Four different starting compositions of the mobile phase (10, 20, 30 and 40 % of eluent B) were tested considering retention time, peak width for each amino acids, enantioselectivity and resolution for each L- and D- optical isomer (ESM Tables S3 and S3).

The chromatographic run performed in this study consisted in a first 15-min isocratic step, where most amino acids were eluted, followed by a gradient step to 100 % methanol in order to elute some D-amino acids and to clean the column from organic compounds. This step permits to regenerate the column and to achieve great stability and reproducibility in our chromatographic separation.

In general, we observed that the peak width of D-amino acids were larger than those of L-compounds because the stationary phase of CHIROBIOTIC TAG column has more affinity with D-amino acids, whose retention time is always higher than in L-isomers [40].

An isocratic elution with 10 or 20 % of eluent B was carried out to co-elute L- and D-glutamine and L- and D-histidine, while 30 % of eluent B permitted to obtain an enantioselectivity >1 for each analyte (ESM Table S3).

We demonstrated that the stationary phase based on teicoplanin aglycone cannot separate positional isomers such

as leucine and isoleucine, which are separated using the stationary phase of CHIROBIOTIC T column [37].

A starting composition of 40 % of eluent B produced the best enantioselectivity and resolution for the majority of amino acids considered in this study, but it generated a broadening effect on D-arginine and D-lysine peaks, as reported in ESM Table S3.

For these reasons, the starting composition of 30 % of eluent B was preferred for our chromatographic separation (Fig. 1 and ESM Fig. S1).

Quality control

The accuracy of the analysis is often affected by undetected matrix components, which co-elute with the target compounds and enhance or suppress ion intensity in the HPLC/MS interface [49, 50].

In this study, we demonstrated that such matrix effect (ME) can be considered by using the internal standard through a quantitative study, as suggested by Matuszewski et al. [51]. For these evaluations, we used the HPLC-MS/MS method, which demonstrated the best sensitivity (as reported below).

We calculated the ME for each amino acid by dividing the signal response of the standard present in the matrix by the response of the standard prepared in ultrapure water, and by expressing the result as a percentage. When no ME is observed, the value is 100 %; a value >100 % indicates ionisation enhancement, while a value <100 % indicates ionisation suppression. In ESM Table S4, we can observe ionisation suppression for several amino acids when no internal standard is used (ME %−IS). A ME with internal standard (ME %+IS) for each amino acid was also by dividing the signal ratio of amino acids and internal standard in the matrix by the signal ratio of the target compound and internal standard in ultrapure water. As shown in ESM Table S4, the internal standard method reduces the ME: Values close to 100 % (no ME) were obtained for all amino acids considered in our study, except for D-Ser, where a higher suppression remained.

In order to ensure that our analytical method could be applied to real samples, we needed to evaluate its accuracy, i.e., the degree of closeness of the determined value to the known "true" value. It is expressed as an error percentage, calculated as $(Q-T)/T\times 100$, where Q is the determined value and T is the "true value".

In our experiments, we considered a real matrix (lake 20 of 20 December) and calculated the concentration of each amino acid using the addition standard method with a range of average concentrations until to 100 μg L^{-1} (ppb).

The true values in the lake matrix were considered as the known addition of standard amino acids minus the values present in the matrix.

We calculated the error percentage using the external calibration curve without internal standard, obtained by diluting the standard in ultrapure water [52]. The ionisation suppression, verified by quantifying the ME, compromised the quantification, as demonstrated by the error percentage (E% ECC)>±10 % for the majority of amino acids (ESM Table S4).

A matrix-matched calibration permits to correct the incidence of the ME [52], yielding an error percentage (E% ICC)≤±10 % for all the investigated amino acids and

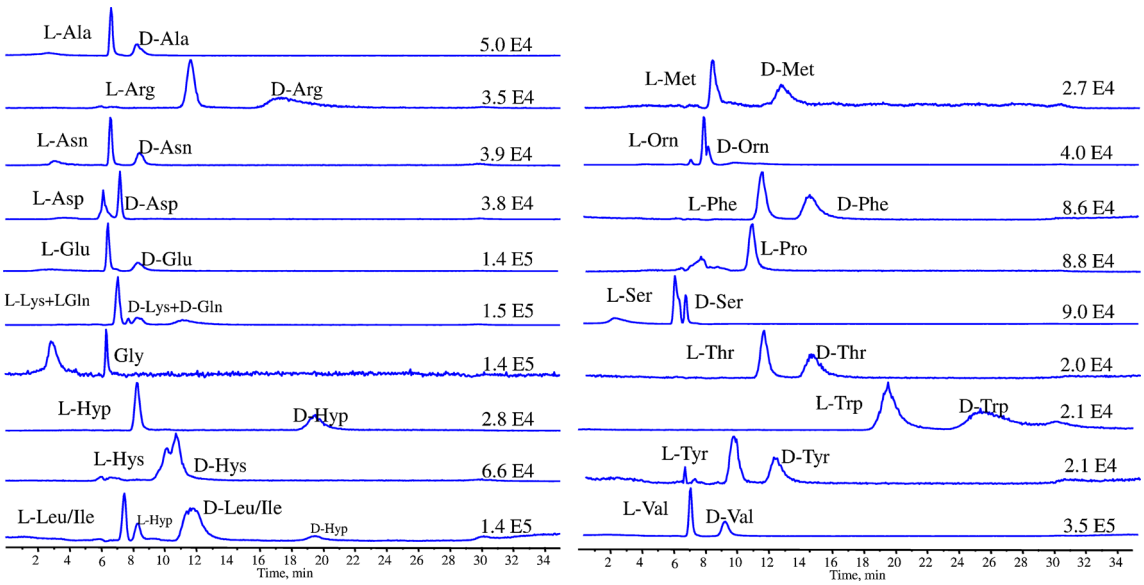

Fig. 1 Chromatograms of single amino acids using a 2.1×250 mm CHIROBIOTICTAG column coupled with API 4000 mass spectrometer. Each ion chromatogram is related to the quantifier ions used in the MRM method for a standard solution of amino acids with an average concentration of 10 ng mL^{-1}

demonstrating that this quantification method can be applied to the analysis of real samples.

The use of a calibration curve with an internal standard prepared in ultrapure water permits to standardise the procedure for lacustrine water samples and requires no matrix to quantify the samples. The reduction of ME allowed that the quantification with the internal standard method produce an error percentage (E% IS)≤±10 % for the all amino acids except for D-Ser, D-Orn, L- and D-Trp for which this method is not applicable (ESM Table S4).

Comparison of quantitative performance between LTQ-Orbitrap XL full scan and API 4000 MRM detection

In order to compare the quantification performances achieved by the full scan method of the LTQ-Orbitrap XL mass spectrometer and by the MRM method of the API 4000 (triple quadrupole), the same chromatographic conditions reported in the "Experimental section" were applied to the separation of amino acid enantiomers. The two methods were evaluated in terms of linear range, repeatability and detection limits.

The internal standard method by isotope dilution was used to quantify the amino acids and to compare the compound peak area with the ^{13}C-labelled isotopomers. Some ^{13}C-mono-labelled amino acids (L-[$^{13}C_1$] Leu, L-[$^{13}C_1$] Phe, L-[$^{13}C_1$] Pro and L-[$^{13}C_1$] Val) could not be used with the orbitrap analyser because, using the full scan method, the internal standard quantification was heavily interfered with by the natural abundance of native amino acids present in the sample. For this reason, these ^{13}C-labelled isotopomers were used only with the triple quadrupole analyser. A ^{13}C-labelled amino acid with similar chemical features was considered in the quantification when the specific ^{13}C-labelled amino acid was not available. The specific ^{13}C-labelled amino acids used for each quantification both with the orbitrap and triple quadrupole analysers are reported in Table 2.

The linearity of the calibration curves for the quantitative determination of single amino acids with internal standard was evaluated using a series of standard solutions prepared in ultrapure water at average concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 50, 75, 100, 200, 300 and 500 μg L^{-1} and a constant internal standards concentration (ranging between 40 and 60 μg L^{-1}). By considering the ratio between the concentration of native and labelled amino acids and the ratio between the relative peak areas, we obtained linearity values of $R^2 \geq 0.99$for both analysers. The coefficients of variation (CV) were calculated at five average concentrations (0.01, 0.1, 1, 10 and 100 μg L^{-1}) using the triple quadrupole analyser and at three average concentrations (1, 10 and 100 μg L^{-1}) using the orbitrap analyser in order to evaluate instrumental repeatability. The CV values were always lower than 10 % for both analysers.

The high mass accuracy obtained with full scan HRMS can effectively separate the target compound from the background ions or compounds that have the same nominal mass but a different exact mass. We verified these features in our analysis of Gln and Lys. The analysis conducted with MRM showed that these amino acids have the same precursor ion (147 *m/z* with at unit resolution) and the same product ions (130 and 84 *m/z*). The use of the triple quadrupole to quantify these compounds was rejected because no chromatographic separation was performed. Different exact masses (Table 1) of Gln and Lys enabled a quantification using the full scan HRMS method.

The main difference between the two methods regards their instrumental limits of detection and quantification (LOD and LOQ), calculated according to Bliesner [53], who defined LOD and LOQ as three and ten times the signal-to-noise ratio of the known absolute amounts of the analysed target compound in a standard solution. The LODs obtained with the orbitrap analyser ranged between 0.4 (L-Phe and L-Thr) and 7 μg L^{-1} (D-Arg and D-Orn), while the LOQs ranged between 1 (L-Phe and L-Thr) and 24 μg L^{-1} (D-Orn). The triple quadrupole yielded LOD values between 0.004 (L-Glu, L-Ser and L-Val) and 0.2 μg L^{-1} (L- and D-Orn), while LOQs yielded values between 0.01 (L-Glu, L-Ser and L-Val) and 3 μg L^{-1} (D-Met) (Table 2).

Due to the higher LOD values, the linear response obtained with the orbitrap analyser was three orders of magnitude; with the triple quadrupole analyser, it was five or six orders of magnitude.

Petritis et al. [39] conducted a very interesting comparison between liquid chromatographic detectors. They demonstrated that MS tandem spectrometry is the most sensitive technique to quantify amino acids, with LOD ranging between 0.08 and 8 μg L^{-1}. Another important detection advantage of mass spectrometry is its high specificity, although co-elution of several amino acids can occur. Petritis et al. [39] noted that their LOD values were affected by analyte ionisation in the aqueous mobile phase, which decreased the ion extraction. Their values were higher than the LODs obtained using hydrophilic interaction liquid chromatography (HILIC) [54], where acetonitrile/water (75:25) was used as mobile phase.

In contrast, the LOD values obtained in our study were very similar to those yielded by non-chiral HILIC separation with the same MS tandem spectrometer [55, 56] thanks to the use of a heated ionisation source. To our knowledge, the MS tandem proposed in this paper to determine the single enantiomers of 20 amino acids is the most sensitive method reported in the literature.

Although the HRMS method yielded higher LOD values than those found using the triple quadrupole, the good quantitative performance of HRMS method was demonstrated, and this method can be applied to samples where the

Table 2 Comparison between the quantitative performances of the HPLC-LTQ-Orbitrap XL and the HPLC-API 4000 to analyse amino acid enantiomers

	HPLC-LTQ Orbitrap XL method							HPLC-API 4000 method						
	IS	R^2	Linear range (μg L^{-1})			LOD (μg L^{-1})	LOQ (μg L^{-1})	IS	R^2	Linear range (μg L^{-1})			LOD (μg L^{-1})	LOQ (μg L^{-1})
L-Ala	Ala*	0.999	9	–	557	1	4	Ala*	0.999	0.02	–	871	0.02	0.05
D-Ala	Ala*	0.999	9	–	575	5	16	Ala*	0.991	0.09	–	899	0.4	1
L-Arg	Arg*	0.999	7	–	447	1	5	Arg*	0.997	0.01	–	279	0.005	0.02
D-Arg	Arg*	0.999	13	–	426	7	22	Arg*	0.997	1	–	266	0.1	0.3
L-Asn	Arg*	0.999	6	–	400	2	5	Val*	0.992	0.01	–	625	0.01	0.03
D-Asn	Arg*	0.999	14	–	446	4	12	Val*	0.983	0.01	–	696	0.08	0.2
L-Asp	Asp*	0.992	7	–	436	3	11	Asp*	0.998	0.1	–	681	0.08	0.3
D-Asp	Asp*	0.985	8	–	512	4	14	Asp*	0.992	0.2	–	320	0.07	0.2
L-Gln	Arg*	0.996	8	–	520	1	3	–	–	–	–	–	–	–
D-Gln	Arg*	0.997	6	–	364	2	7	–	–	–	–	–	–	–
L-Glu	Glu*	0.998	10	–	610	1	3	Glu*	0.998	0.02	–	954	0.004	0.01
D-Glu	Glu*	0.998	13	–	404	2	8	Glu*	0.991	0.1	–	631	0.05	0.2
Gly	Ala*	0.986	10	–	160	3	10	Ala*	0.999	0.1	–	1000	0.08	0.3
L-Hyp	Ala*	0.999	8	–	520	3	9	Pro*	0.986	0.02	–	813	0.007	0.02
D-Hyp	Ala*	0.987	6	–	364	1	4	Pro*	0.984	0.1	–	569	0.08	0.2
L-Hys	Arg*	0.995	6	–	407	1	5	Arg*	0.996	0.1	–	127	0.005	0.02
D-Hys	Arg*	0.999	8	–	485	2	8	Arg*	0.994	0.2	–	152	0.01	0.04
L-Leu/Ile	Arg*	0.988	12	–	790	1	3	Leu*	0.990	0.02	–	494	0.01	0.04
D-Leu/Ile	Arg*	0.999	13	–	851	6	20	Leu*	0.996	0.03	–	798	0.006	0.02
L-Lys	Glu*	0.993	6	–	382	1	2	–	–	–	–	–	–	–
D-Lys	Glu*	0.993	13	–	406	5	17	–	–	–	–	–	–	–
L-Met	Arg*	0.984	9	–	553	1	2	Leu*	0.989	0.09	–	864	0.07	0.2
D-Met	Arg*	0.998	12	–	377	4	12	Leu*	0.998	0.12	–	589	0.8	2
L-Orn	Arg*	0.987	6	–	406	1	5	Arg*	0.994	0.01	–	254	0.2	0.6
D-Orn	Arg*	0.999	9	–	588	7	24	Arg*	0.953	0.2	–	367	0.2	0.5
L-Phe	Arg*	0.999	6	–	386	0.4	1	Phe*	0.995	0.06	–	121	0.01	0.03
D-Phe	Arg*	0.999	6	–	383	1	4	Phe*	0.992	0.1	–	120	0.04	0.1
L-Pro	Arg*	0.998	8	–	482	6	22	Pro*	0.997	0.02	–	753	0.008	0.02
L-Ser	Arg*	0.998	10	–	637	1	4	Arg*	0.990	0.02	–	398	0.004	0.01
D-Ser	Arg*	0.993	6	–	400	3	10	Arg*	0.994	0.6	–	250	0.1	0.4
L-Thr	Arg*	0.998	7	–	450	0.4	1	Arg*	0.997	0.01	–	148	0.005	0.02
D-Thr	Arg*	0.992	7	–	428	1	2	Arg*	0.997	0.07	–	134	0.009	0.03
L-Trp	Arg*	0.996	7	–	440	1	4	Phe*	0.992	0.06	–	125	0.01	0.04
D-Trp	Arg*	0.999	19	–	1200	1	5	Phe*	0.992	0.6	–	130	0.1	0.04
L-Tyr	Arg*	0.998	6	–	398	1	4	Arg*	0.994	0.1	–	138	0.01	0.05
D-Tyr	Arg*	0.999	13	–	417	6	21	Arg*	0.996	0.2	–	188	0.01	0.4
L-Val	Ala*	0.998	8	–	538	3	9	Val*	0.991	0.02	–	168	0.004	0.01
D-Val	Ala*	0.997	7	–	426	5	17	Val*	0.994	0.2	–	125	0.06	0.2

concentrations of amino acids is higher respect to that of samples from a remote area.

In contrast with the MRM method, one of the advantages of full scan HRMS is that the data can be mined at a later time without any need of re-injecting the samples, so obtaining an *untarget* analysis. The main trend in environmental research is currently to discover other possible markers and the HRMS permits to obtain such specificity thanks to mass resolution and mass accuracy.

This is the first study that considers this technique for amino acids analysis, and the method we developed provides good quantitative and qualitative performance.

Amino acids in Antarctic freshwater samples

The internal standard method used to determine amino acid enantiomers with the hyphenated technique HPLC-orbitrap MS was applied to the freshwater samples collected in four Antarctic lakes. The analyte concentration was always below the LODs, showing that a more sensitive method was necessary. The application of the instrumental method to the HPLC-API 4000 system produced the expected results, with amino acid concentration above LOD.

The four samples of lacustrine water (lakes 10, 14, 15A and 20) collected during the XXVII Italian Antarctic Campaign (2011–2012) presented total amino acid concentrations ranging between 10 μg C L^{-1} (0.2 μM) and 110 μg C L^{-1} (2.3 μM) during the first sampling (December 2011); those values varied between 6 μg C L^{-1} (99 nM) and 472 μg C L^{-1} (8.5 μM) during the second sampling (January 2012), when the defrost process was completed. An increase in amino acid concentration was generally observed for lakes 10 and 14, while lakes 20 and 15A indicated possible dilution during the defrost process.

These values were very similar to those individuated in several European lakes[14, 57–64], where the concentrations of free amino acids ranged between 2 and 800 nM, but considerably lower than those of Organic Lake (East Antarctica) (48–268 μM) reported by Gibson et al. [25]. To our knowledge, no other research was conducted to individuate amino acids in Antarctic lacustrine water.

The composition and relative abundance of amino acids were very variable in the different lakes investigated, due to their different location, depth and to the nature of their freshwater.

In lake 10, situated on Inexpressible Island, the total concentration in free amino acids varied between 207 nM (22 December 2011) and 643 nM (07 January 2012), as reported in Fig. 2. This increase in concentration was probably linked to an increment of fluorescence, which is a proxy of primary productivity. The concentration of chlorophyll *a* was below LOD in the first sample, while a concentration of 0.6 mg L^{-1} was detected in the second sample. Moreover, in the sample collected in January, the amino acids were principally distributed in the particulate fraction, confirming the presence of biological material in freshwater. In this lake, we found concentrations above LODs for L-Ala, L-Arg, L-Glu, Gly, L-Leu/Ile, L-Phe, L-Pro, L-Tyr and L-Val (Fig. 2). The most abundant amino acids in this lake were Gly (20–22 %), L-Glu (11–21 %) and L-Leu/Ile (23–9 %). The absence of D-amino acids was probably due to a negligible presence of bacteria.

At Edmonson Point, two different lakes (14 and 15A) were sampled, but the composition and abundance of amino acids were completely different.

Primary growth was evident in lake 14, where values of chlorophyll *a* increased from 5 to 16 mg L^{-1}. High concentrations of nutrients (ESM Table S5), also due to the presence of penguins in the area, offered a good substratum to algal bloom. Primary productivity heavily influenced the abundance and composition of amino acids (Fig. 2), and concentrations above the LOD (110 and 472 μg C L^{-1}) were detected for the following amino acids: L-Ala, D-Ala, L-Arg, L-Asp, D-Asp, L-Glu, Gly, L-Leu/Ile, L-Met, L-Orn, L-Phe, L-Pro, L-Thr, L-Tyr, and L-Val. A homogenous distribution was observed, with percentages between 7 % (L-Tyr, 20 December) and 18 % (D-Ala, 20 December), while lower concentrations were verified for D-Asp, Gly, L-Met, L-Orn and L-Pro. The presence of bacteria was confirmed by relevant concentrations of D-Ala and D-Asp: These compounds are released by the biodegradation of peptidoglycan membranes [65].

At Edmonson Point, lake 15A showed lower concentrations of amino acids (Fig. 2) than lake 14, and a small decrease in total amino acid concentrations from 19 μg C L^{-1} (20 December) to 15 μg C L^{-1} (07 January). A possible cause could be the presence of primary production in December (concentration of chlorophyll *a* of 0.9 mg L^{-1}), while the value of chlorophyll *a* was below LOD in the second sample. The decrease in concentration can also be linked to dilution during defrost or to the stripping of biological material with the formation of the outlet stream. Lake 15A was characterised by the presence of the following amino acids: L-Ala, L-Arg, L-Asp, D-Asp, L-Glu, Gly, L-Leu/Ile, L-Phe, L-Pro, L-Thr, L-Tyr and L-Val (Fig. 2). The presence of bacteria was confirmed by the quantified concentration of D-Asp.

Lake 20 situated in Tarn Flat had concentrations of amino acids very similar to those of lake 15A, with values ranging between 15 μg C L^{-1} during sampling in December and of 6 μg C L^{-1}in January. However, only 11 amino acids were detected (L-Ala, L-Arg, L-Asp, L-Glu, L-Leu/Ile, $MetSO_2$, L-Phe, L-Pro, L-Thr, L-Tyr and L-Val) (Fig. 2). L-Leu was one of the most abundant compounds in this hollow area (15 % in December and 20 % in January), while the concentration of D-amino acids above LODs suggests a negligible presence of bacteria.

The average concentrations of amino acids in lakes 15A and 20 were about half than those detected in lake 10 (Fig. 2). These values suggest that sea spray influences the water chemistry in lake 10. This marine influence, also verified by Abollino et al. [19], varies as a function of the distance from the sea and of geomorphological conditions, such as the presence of surface melt water or shallow level ground water. Lakes 10 and 15A (ESM Table S1) are situated at the same distance from the sea, but the input of low salinity water from fresh water by the snow and glaciers in lake

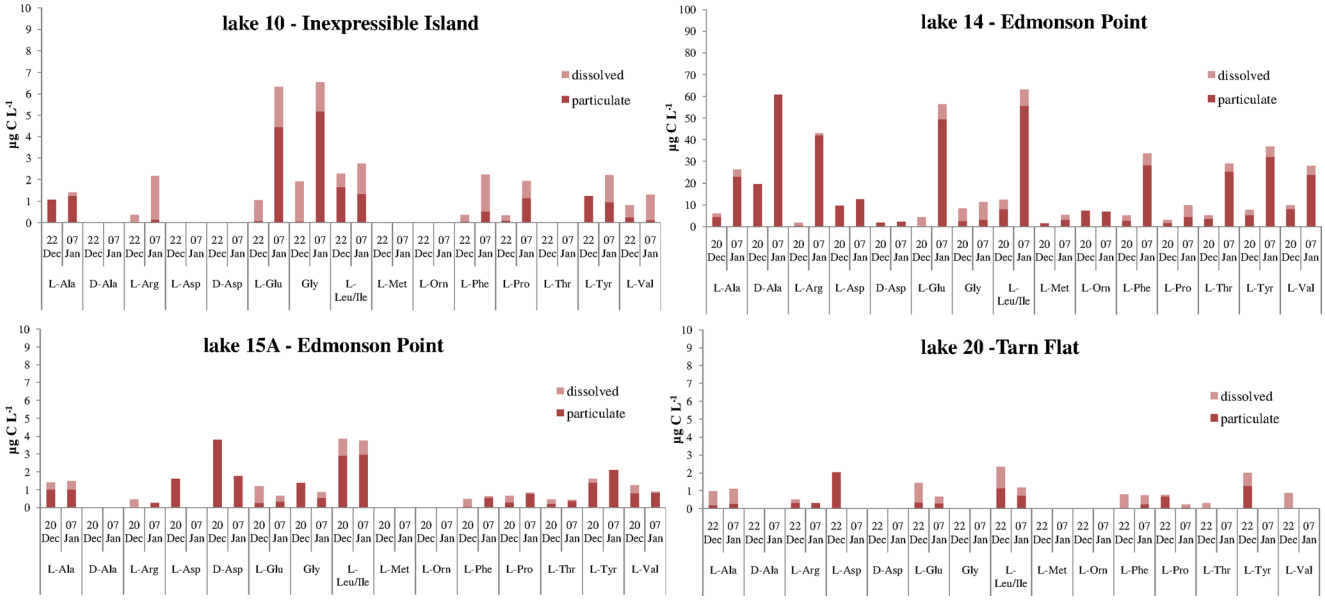

Fig. 2 Distribution of D- and L-amino acids in four Antarctic lakes

15A causes the dilution of amino acids, as demonstrated by the salinity value (ESM Table S4).

Conclusions

We developed a unique simultaneous chiral separation with chiral stationary phase based on teicoplaninaglycone (CHIROBIOTIC TAG) to determine 40 L- and D-amino acids using a HPLC-MS system. To our knowledge, this is the first method that permits to simultaneously separate several amino acids in 35 min. The coupling with an orbitrap MS, we obtained an innovative high-resolution method to determine the single enantiomers of amino acids with detection limits between 0.4 and 7 µg L^{-1}. The most sensitive method was performed using the coupling with the triple quadrupole API 4000, obtaining LODs values between 4 and 200 ng L^{-1}. To our knowledge, this is the most sensitive method to quantify single enantiomeric forms of amino acids. While the triple quadrupole analyser achieves excellent sensitivity, the orbitrap analyser allows also an *untargeted* analysis.

In order to demonstrate the applicability of this method to the environmental field, the most sensitive HPLC-MS methods developed in this study were applied to eight water samples collected in four Antarctic lakes, in order to characterise their amino acid distribution and to investigate a possible relationship with primary production.

Four different lakes were sampled at different times. Lake 14, located at Edmonson Point, had the highest concentration in amino acids. These concentrations were associated to primary growth, which increased between the first and second sampling. In this lake, the presence of D-amino acids suggested the growth of bacteria. The main differences between this lake and the others were its dimensions and the presence of penguins as N sources. Climate changes drastically reduced the lake's area in the last decade, and probably, this have carried out to a concentration of nutrients with a primary growth.

In the others sites, the concentrations of amino acids were lower than in lake 14, due to negligible or very low primary growth. We demonstrated that the sea influence on lake 10 at Inexpressible Island produced a small increase in amino acid concentrations.

We can conclude that amino acids can be used as markers of primary production, but that monitoring of these compounds during the next sampling periods will be necessary to control the influence of climate changes on biological production.

Acknowledgements This work was financially supported by the Italian Programma Nazionale di Ricerche in Antartide (PNRA) through the project "Caratterizzazione Biogeochimica dei laghi sub-glaciali antartici (CaBiLA)" (2009/A2.02). The research was also funded by the National Research Council of Italy (CNR) and by the Early Human Impact ERC Advance Grant of the European Commission's VII Framework Programme, grant number 267696, contribution no. 13. The authors thank ELGA LabWater for providing the PURE-LAB Option-R and Ultra Analytic, which produced the ultrapure water used in these experiments.

We would also like to thank Dr. Daniela Almansi for the revision of our manuscript. In conclusion, we wish to thank Dr. S. Illuminati (Polytechnic University of Marche – Ancona, Italy) for her help and cooperation during the sampling activities in Antarctica.

References

1. Hegeman WJM, Laane RWPM (2002) Enantiomeric enrichment of chiral pesticides in the environment. In: Ware GW, Nigg H, Doerge DR (eds) Reviews of environmental contamination and toxicology. Springer, New York
2. Friedman M (1991) Formation, nutritional value, and safety of D-amino acids. Nutr Toxicol Consequences Food Process 289:447–481
3. Fitznar HP, Lobbes JM, Kattner G (1999) Determination of enantiomeric amino acids with high-performance liquid chromatography and pre-column derivatisation with o-phthaldialdehyde and N-isobutyrylcysteine in seawater and fossil samples (mollusks). J Chromatogr A832:123–132
4. Cronin JR, Pizzarello S (1999) Amino acid enantiomer excesses in meteorites: origin and significance. Life Sci Exobiol 23:293–299
5. Cronin JR, Pizzarello S (1997) Enantiomeric excesses in meteoritic amino acids. Science 275:951–955
6. Voet D, Voet JG (199) Biochemistry. John Wiley and Sons, New York
7. Bada JL (1984) In vivo racemization in mammalian proteins. Methods Enzymol 106:98–115
8. Mahaney WC, Rutter NW (1989) Amino acid D-L ratio distributions in 2 late quaternary soils in the afroalpine zone of Mount - Kenya, East-Africa. Catena 16:205–214
9. Ingrosso D, Perna AF (1998) D-amino acids in aging erythrocytes. EXS 85:119–141
10. Amelung W (2003) Nitrogen biomarkers and their fate in soil. J Plant Nutr Soil 166:677–686
11. Berman T, Bronk DA (2003) Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. Aquat Microb Ecol 31:279–305
12. Bronk DA, Glibert PM, Ward BB (1994) Nitrogen uptake, dissolved organic nitrogen release, and new production. Science 265:1843–1846
13. Berman T, Viner-Mozzini Y (2001) Abundance and characteristics of polysaccharide and proteinaceous particles in Lake Kinneret. Aquat Microb Ecol 24:255–264
14. Jonsson A, Strom L, Aberg J (2007) Composition and variations in the occurrence of dissolved free simple organic compounds of an unproductive lake ecosystem in northern Sweden. Biogeochemistry 82:153–163
15. Bargagli R (2008) Environmental contamination in Antarctic ecosystems. Sci Total Environ 400(2008):212–226
16. Bargagli R (2005) Antarctic ecosystems. Environmental contamination, climate change and human impact. Springer, Berlin
17. Green WJ, Ferdelman TG, Gardner TJ, Varner LC, Angle MP (1986) The residence times of 8 trace metals in a closed basin Antarctic lake. Lake Hoare. Hydrobiologia 134:249–255
18. Green WJ, Angle MP, Chave KE (1988) The geochemistry of Antarctic streams and their role in the evolution of four lakes of the McMurdo Dry Valleys. Geochim Cosmochim Acta 52:1265–1274
19. Abollino O, Aceto M, Buoso S, Gasparon M, Green WJ, Malandrino M, Mentasti E (2003) Distribution of major, minor and trace elements in lake environments of Antarctica. AntarctSci 16:277–291
20. Abollino O, Malandrino M, Zelano I, Giacomino A, Buoso S, Mentasti E (2012) Characterization of the element content in lacustrine ecosystems in Terra Nova Bay, Antarctica. Microchem J105:142–151
21. Borghini F, Bargagli R (2004) Changes of major ion concentrations in melting snow and terrestrial waters from northern Victoria Land, Antarctica. Antarct Sci 16:107–115
22. Borghini F, Colacevich A, Caruso T, Bargagli R (2008) Temporal variation in the water chemistry of northern Victoria Land lakes (Antarctica). Aquat Sci 70:134–141
23. Gragnani R, Torcini S (1992) Major, minor and trace element distributions in surface water in Terra Nova Bay, Antarctica. Sci Total Environ 125:289–303
24. Wharton RA, Lyons WB, Marais DJD (1993) Stable isotopic biogeochemistry of carbon and nitrogen in a perennially ice-covered Antarctic lake. Chem Geol 107:159–172
25. Gibson JAE, Qiang XL, Franzmann PD, Garrick RC, Burton HR (1994) Volatile fatty and dissolved free amino acids in Organic Lake, Vestfold Hills, East Antarctica. Pol Biol 14: 545–550
26. Borghini F, Colacevich A, Bargagli R (2007) Water geochemistry and sedimentary pigments in northern Victoria Land lakes, Antarctica. Pol Biol 30:1173–1182
27. Borghini F, Colacevich A, Bargagli R (2010) A study of autotrophic communities in two Victoria Land lakes (Continental Antarctica) using photosynthetic pigments. J Limnol 69:333–340
28. Doran PT, Wharton RA, Lyons WB (1994) Paleolimnology of the McMurdo Dry Valleys, Antarctica. J Paleolimnol 10:85–114
29. Nedell SS, Andersen DW, Squyres SW, Love FG (1987) Sedimentation in ice-covered Lake Hoare, Antarctica. Sedimentology 34:1093–1106
30. De Carlo EH, Green WJ (2002) Rare earth elements in the water column of Lake Vanda, McMurdo Dry Valleys, Antarctica. Geochim Cosmochim Acta 66:1323–1333
31. Marfey P (1984) Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsb Res Commun 49:591–596
32. Buck RH, Krummen K (1987) High-performance liquid chromatographic determination of enantiomeric amino acids and amino alcohols after derivatization with o-phthaldialdehyde and various chiral mercaptans: Application to peptide hydrolysates. J Chromatogr 387: 255–265
33. Dolowy M, Pyka A (2014) Application of TLC, HPLC and GC methods to the study of amino acid and peptide enantiomers: a review. Biomed Chromatogr 28:84–101
34. Remelli M, Fornasari P, Pulidori F (1997) Study of retention, efficiency and selectivity in chiral ligand-exchange chromatography with a dynamically coated stationary phase. J Chromatogr A 761: 79–89
35. Armstrong DW, Yang X, Han SM, Menges RA (1987) Direct liquid chromatographic separation of racemates with an alpha-cyclodextrin bonded phase. Anal Chem 59:2594–2596
36. Gimenez F, Soursac M, Farinotti R (1997) Enantiomeric separation of five amino acids by high-performance liquid chromatography on a chiral crown ether column. Chirality 9:150–152
37. Petritis K, Valleix A, Elfakir C, Dreux M (2001) Simultaneous analysis of underivatized chiral amino acids by liquid chromatography-ionspray tandem mass spectrometry using a teicoplanin chiral stationary phase. J Chromatogr A 913:331–340
38. Schlauch M, Frahm AW (2000) Enantiomeric and diastereomeric high-performance liquid chromatographic separation of cyclic beta-substituted alpha-amino acids on a teicoplanin chiral stationary phase. J Chromatogr A 868:197–207
39. Petritis K, Elfakir C, Dreux M (2002) A comparative study of commercial liquid chromatographic detectors for the analysis of underivatized amino acids. J Chromatogr A 961:9–21

40. ASTEC, Advantaged Separations Technology Inc. (2004) Chirobiotic handbook, a guide to using macrocyclic glycopeptide bonded phases for chiral LC separations
41. Bargagli R, Smith RIL, Martella L, Monaci F, Sanchez-Hernandez JC, Ugolini FC (1999) Solution geochemistry and behaviour of major and trace elements during summer in a moss community at Edmonson Point, Victoria Land, Antarctica. Antarct Sci 11:3–12
42. Andreoli C, Scarabel L, Spini S, Grassi C (1992) The picoplankton in Antarctic lakes of Northern Victoria Land during summer 1989-1990. Pol Biol 11:575–582
43. Fumanti B, Cavacini P, Alfinito S (1997) Benthic algal mats of some lakes of Inexpressible Island (northern Victoria Land, Antarctica). Pol Biol 17:25–30
44. Bargagli R, Monaci F, Bucci C (2007) Environmental biogeochemistry of mercury in Antarctic ecosystems. Soil Biol Biochem 39:352–360
45. American Public Health Association (1998) Standard method for the examination of water and wastewater, American Public Health Association, Washington, DC.
46. Berthod A, Chen XH, Kullman JP, Armstrong DW, Gasparrini F, D'Acquarica I, Villani C, Carotti A (2000) Role of the carbohydrate moieties in chiral recognition on teicoplanin-based LC stationary phases. Anal Chem 72:1767–1780
47. Peter A, Torok G, Armstrong DW, Toth G, Tourwe D (1998) Effect of temperature on retention of enantiomers of beta-methyl amino acids on a teicoplanin chiral stationary phase. J Chromatogr A 828:177–190
48. Tesarova E, Bosakova Z, Pecakova V (1999) Comparison of enantioselective separation of N-tert.-butyloxycarbonyl amino acids and their non-blocked analogues on teicoplanin-based chiral stationary phase. J Chromatogr A 838:121–129
49. Kebarle P, Tang L (1993) From ions in solution to ions in the gas phase—the mechanism of electrospray mass spectrometry. Anal Chem 65:972A–986A
50. King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T (2000) Mechanistic investigation of ionization suppression in electrospray ionization. J Am Soc Mass Spectrom 11:942–950
51. Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 75:3019–3030
52. Boyd RK, Basic C, Bethem RA (2008) Front matter, in trace quantitative analysis by mass spectrometry. John Wiley & Sons, Ltd, Chichester, UK. doi: 10.1002/9780470727140.fmatter
53. Bliesner DM (2006) Validating chromatographic methods a practicalguide. John Wiley & Sons, Inc, Hoboken
54. Petritis K, Brussaux S, Guenu S, Elfakir C, Dreux M (2002) Ion-pair reversed-phase liquid chromatography-electrospray mass spectrometry for the analysis of underivatized small peptides. J ChromatogrA 957:173–185
55. Barbaro E, Zangrando R, Moret I, Barbante C, Cescon P, Gambaro A (2011) Free amino acids in atmospheric particulate matter of Venice, Italy. Atmos Environ 45:5050–5057
56. Zangrando R, Piazza R, Cairns WRL, Izzo FC, Vianello A, Zendri E, Gambaro A (2010) Quantitative determination of un-derivatised amino acids in artistic mural paintings using high-performance liquid chromatography/electrospray ionization triple quadrupole mass spectrometry. Anal Chim Acta 675:1–7
57. Jorgensen NOG, Sondergaard M (1984) Are dissolved free amino acids free? MicrobEcol 10:301–316
58. Jorgensen NOG (1986) Fluxes of free amino acids in three Danish lakes. Freshw Biol 16:255–268
59. Jorgensen NOG (1987) Free amino acids in lakes: concentrations and assimilation rates in relation to phytoplankton and bacterial production. Limnol Oceanogr 32:97–111
60. Riemann B, Jorgensen NOG, Lampert W, Fuhrman JA (1986) Zooplankton induced changes in dissolved free amino acids and in production rates of freshwater bacteria. MicrobEcol 12:247–258
61. Simon M (1985) Specific uptake rates of amino acids by attached and free living bacteria in a mesotrophic lake. Appl Environ Microbiol 49:1254–1259
62. Munster U (1991) Extracellular enzyme activity in eutrophic and poly-humic lakes. In: Microbial enzymes in aquatic environments. Spinger Verlag, New York, pp 96–122
63. Munster U (1992) Microbial extracellular enzyme activities in HUMEX lake Skjervatjern. Environ Internatl 18:637–647
64. Munster U (1992) Microbial extracellular enzyme activities and biopolymer processing in two polyhumic lakes. Arch Hydrobiol Ergebn Limnol 37:21–32
65. Wedyan MA, Preston MR (2008) The coupling of surface seawater organic nitrogen and the marine aerosol as inferred from enantiomer-specific amino acid analysis. Atmos Environ 42:8698–8705