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Improved analytical methods for determination of nitrogenous stress metabolites occurring in *Limonium* species

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Abstract

Efficient and reliable high-performance liquid chromatographic procedures have been developed for metabolic analyses of amino acids, polyamines and betaines in *Limonium* species. The adaptive significance of accumulated low-molecular-mass nitrogenous compounds in dry or salt environments is under study. HPLC profiles of dansylated water-soluble polyamines revealed 1,3-diaminopropane and tyramine as the most abundant amines in the species under study whereas common aliphatic di- and polyamines (i.e., putrescine, spermidine and spermine) were poorly represented as their free forms. Nevertheless acylated conjugates of putrescine, 1,3-diaminopropane and spermidine were also characterized, especially in *L. vulgare*, a halophytic salt marshes species. Direct derivatization of amino acids of the crude aqueous extracts with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate allowed efficient determinations of most proteinic amino acids as well as non-proteinic ones such as ornithine, γ-aminobutyric acid and β-alanine also related to polyamine metabolism. Analysis of betaines was improved, especially for β-alanine betaine, which is quite uncommon in higher plants whose metabolic routes from β-alanine are poorly understood. β-Alanine betaine was first converted to acrylic acid through trimethylamine β-elimination under alkaline treatment of the crude extracts and then quantified by HPLC. Thus *Limonium* species ranged from high β-alanine betaine accumulators, to low accumulators and finally to a third group where it was not detected, since β-alanine betaine was replaced by glycine betaine as shown here by ^{1}H-NMR investigations. Metabolic links between these nitrogenous solutes and the adaptive significance of such adjustments are discussed. Special emphasis is directed towards the possible involvement of aliphatic polyamine oxidative catabolism, which seems to be effective in these species, to precursor recycling for compatible solute biosynthesis. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Limonium; Amino acids; Polyamines; Betaines; Alanine betaine; Amines

1. Introduction

The economic impact of soil salinity is becoming an important issue in the agricultural systems all over the world by influencing the production and availability of crop plants [1]. Therefore a challenge to plant scientists is to genetically improve crops for salt resistance or tolerance. The mechanisms of stress sensitivity or tolerance are difficult to apprehend, because of the multiple genes involved, a lack of models and the inability to discriminate developmental processes clearly from stress responses [2].

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According to Cheeseman [3] the mechanisms of salt tolerance could involve the control of Na^+ (and/or Cl^-) uptake, and its translocation and compartmentation in plants, the metabolic adjustment to the ionic composition of the internal milieu and the changes in water activity. Particularly important to the understanding of the responses of higher plants to salt stress is their ability to divert carbohydrates and nitrogenous compounds from precursors involved in biomass production towards those involved in solute accumulation which allow osmotic adjustment and turgor maintenance. Most higher plants, when grown under saline conditions, accumulate low-molecular-mass organic solutes which are assumed to act as compatible osmoprotectants. The solutes concerned include carbohydrates and related polyols, free amino acids, trimethylated quaternary ammonium or dimethylated sulfonium compounds [4]. Despite a large number of studies, direct evidences is still lacking which shows any adaptive value of such solutes in the tissues of higher plants experiencing water stress. The polyamine levels in stressed plant tissues are also known to be modified in comparison to their basal amount in non stressed ones [5, and references therein]. However the polyamines constitute a class of plant growth regulators [6]. In rice, one of the mechanisms contributing to saline resistance has been ascribed to the relatively high increases of polyamines versus diamines. Alternatively, salt sensitivity could be due to large increase of diamines and an inability to maintain high levels of polyamines [7]. It is now quite clear that the polyamine titre is regulated at both the anabolic and the catabolic levels; this is despite the paucity of data dealing with the changes induced in the catabolic processes of polyamines in response to abiotic stresses and the fate and function of the catabolic products.

The *Plumbaginaceae*, a highly stress tolerant family, contains species well adapted to a wide range of harsh environments among which the *Limonium* species are found to accumulate a wide range of low-molecular-mass nitrogenous compounds including proline, pipecolic acid, β-alanine, γ-aminobutyric acid and quaternary ammonium compounds (QACs), like β-alanine betaine and glycine betaine [8,9]. In contrast, little is known about the polyamines produced by these species or the changes in their pattern of distribution [10]. The flexibility of their metabolism in relation to salinity, if any exists, has not been described. Moreover, catabolic products derived from the oxidative deamination of common aliphatic polyamines such as putrescine, spermidine and spermine could lead to the production of some precursors needed for biosynthesis of either proline (via γ-aminobutyric acid) or β-alanine betaine (via β-alanine), the typical compatible solutes of the halophytic *Plumbaginaceae*. The present work describes reliable and accurate chromatographic procedures for the analysis of low-molecular-mass soluble nitrogenous compounds responsive to salt stress in *Limonium* species. A special emphasis is directed towards the polyamines considered as both metabolic and growth modulators and intermediary metabolites from which salt regulated catabolic products could be recycled towards the synthesis of compatible solutes such as amino acid and betaines.

2. Experimental

2.1. Plant samples

Plants of *Limonium vulgare* were collected from a coastal salt marsh (Lancieux, France), transplanted and grown for two years in the botanical garden of the University of Rennes (France) without any supply of sodium chloride. Well developed plants of *L. latifolium, L. tataricum* and *L. gmelini* of ornamental interest were purchased from the nursery gardener Lepage (Les Ponts-de-Cé, France). Plants of *L. perezii* and *L. peregrinum* of ornamental interest were micropropagated through meristem cultures obtained from the CTIH (Antibes, France). Plants of *L. dumosum* and *L. sinuatum* were obtained from seeds purchased from the seed trader Schupisser (Cannes, France). Except for *L. vulgare*, plants were grown in pots filled with 70% compost supplemented with 30% blond peat/brown peat under controlled conditions in a growth cabinet (14 h light, 10 h dark, temperature 24°C day and 18°C night, light intensity 50 μmol m^{-2} s^{-1} at leaf level, relative humidity 75 and 90% during day and night, respectively). Plants were fertilized with Hoagland medium [11] twice a week.

2.2. Isolation of polyamines, amino acids and betaines

After fresh mass determination the leaf material was stabilized in liquid nitrogen, freeze–dried, weighed for dry matter determination, and ground.

Polyamines and betaines were extracted from either fresh or freeze–dried plant materials. The equivalent of 50 mg dry material was homogenized in 500 μl 1 *M* HCl on ice for 1 h. The homogenate was centrifuged for 20 min at 9000 rpm at 4°C and the supernatant collected. The pellet was resuspended in 500 μl 1 *M* HCl and incubated 1 h at ice temperature. A second resuspension of the pellet was done under the same conditions. The combined supernatants constituted the crude extract for characterization and determination of free polyamines, water soluble conjugated amines and betaines.

For ^{1}H-nuclear magnetic resonance (NMR) studies of betaines the crude extracts were freeze–dried and resuspended with 1 ml $^{2}H_2O$ 99.8% containing *tert.*-butanol as an internal standard.

Free amides and amino acids were extracted by incubating 50 mg dry leaf powder in ethanol at 95°C until the complete evaporation of ethanol. The insoluble residue was treated with 1 ml of ultrapure cold water to obtain the free amino acids.

2.3. Chromatographic analysis

2.3.1. Aliphatic and aromatic amines

The polyamines were derivatized by dansylation according to the method of Flores and Galston [12], modified by Smith and Davies [13]. The dansylated polyamines were further extracted with ethyl acetate. The organic phase containing polyamines was dried under nitrogen and the residue solubilized in 1 ml of methanol and stored at −20°C until analysis.

The high-performance liquid chromatography (HPLC) design consisted of a LDC Milton Roy SM 4000 ternary gradient pump, a TSP Autosampler (Spectraseries AS 100) with a 20-μl injection loop, a Beckman C_{18} Ultrasphere column (250×4.6 mm, 5 μm), and a LDC fluoromonitor III with an excitation wavelength of 336 nm and an emission wavelength of 486 nm. Signals were computed and analysed though the APEX data acquisition program (SRA Instruments, France). Samples were eluted from the column with a programmed water–methanol solvent gradient, which changed from (40:60, v/v) to (5:95, v/v) in 23 min and to 100% methanol in 2 min at a flow-rate of 1 ml min^{-1} and a temperature of 27°C. Elution was completed by 25 min. The column was washed with 100% methanol for 5 min and re-equilibrated at 60% methanol for 10 min before the next sample was injected. Polyamines were quantified with external calibration curves of corresponding standards.

2.3.2. Amides and amino acids

Amides and amino acids were characterized and quantified with HPLC after pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (Waters Accq-Tag amino acid analysis system) and reversed-phase liquid chromatographic separation as described by Cohen and Michaud [14].

Ten to 20-μl aliquots of the crude aqueous extracts were reacted with AQC using the procedure optimized by Cohen and Michaud [14].

The HPLC system consisted of a TSP ternary pump (Spectraseries P200), a 20-μl injection loop, a 4 μm Nova-Pak C_{18} column (Waters Accq-Tag column: 150×3.9 mm) heated to 37°C and a TSP spectrophotometer (Spectraseries UV 100) adjusted to 254 nm. The flow-rate was 1 ml min^{-1}. The working eluent A was prepared as described by Cohen and Michaud [14]. Eluent B was acetonitrile–water (60:40, v/v). The gradient elution program was as follows: initial, 100% A; 0.5 min 98% A; 15 min, 98% A; 25 min, 90% A; 35 min, 70% A; 45 min, 100% B (all linear). The column was washed with 100% eluent B for 5 min then re-equilibrated with 100% A for 10 min. Amides and amino acids were characterized by co-chromatography of individual standards and quantified by comparison of individual external calibration curves.

2.3.3. β-Alanine betaine

The procedure of HPLC measurements of acrylic acid and dimethylsulfoniopropionate (DMSP) developed by Osinga et al. [15] was adapted for the quantification of β-alanine betaine, which yields trimethylamine and acrylate under alkaline conditions [16]. Aliquots of 200 μl from HCl extracts were incubated with 300 μl 10 *M* NaOH for 30 min

at 50°C for quantitative chemical conversion of β-alanine betaine to acrylate and trimethylamine. The complete mixture was added with 300 μl 10 *M* HCl prior filtration through a 0.2-μm cellulose acetate filter and HPLC analysis of released acrylate.

The HPLC design consisted of an isocratic LDC Analytical pump (Consta-Metric 3200), a 20-μl injection loop, a Spherisorb ODS-2 column (250×4.6 mm) and a LDC/Milton Roy SM 4000 spectrophotometer adjusted to 205 nm. The eluent consisted of 12.5% (v/v) methanol in water adjusted to pH 2 with phosphoric acid and delivered at a flow-rate of 1 ml min^{-1}. The temperature was kept at 28°C.

2.4. ^{1}H-NMR analysis of betaines

Betaines, especially β-alanine betaine, glycine betaine, choline-*O*-sulfate and choline, were characterized and quantified through ^{1}H-NMR analysis of $^{2}H_2O$ extracts with *tert*.-butanol as an internal standard. A procedure was developed allowing the connection of an autosampler (B-ACS 60) to the spectrometer. ^{1}H-NMR spectra were measured on a Brucker AC 300 P Fourier transform spectrometer operating at a frequency of 300 MHz at an ambient probe (QNP type) temperature. A FID of 32 K using a spectral width of 6024 Hz induced a resolution of 0.368 Hz per point. Brucker ''presat.au'' and Brucker ''win-nmr'' software were used for solvent pre-saturation and data treatments, respectively. In cases where the residual water peak was large, water peak suppression by homonuclear gated decoupling was used. Peak identities were confirmed by addition of commercial or synthetic standards and quantification was obtained by comparing integrated peak intensities against standard curves with synthetic standards or using *tert*.-butanol as an internal standard.

3. Results and discussion

3.1. Optimization of chromatographic procedures for the analysis of low-molecular-mass nitrogenous compounds in Limonium sp.

3.1.1. Water-soluble polyamine analysis

The dansylated amines from different *Limonium* species were analysed using a reversed-phase liquid chromatographic procedure with methanol as the eluent as described by Flores and Galston [12] and Smith and Davies [13]. Seven species of *Limonium*, most of them used for horticultural purposes, were under study. Dependent on the organ, the developmental stage or the environmental conditions of culture, major changes could be observed on the accumulation of individual soluble free amines. Our first goal was to characterize the amine profiles of each species to identify the most abundant compounds. Typical chromatograms of dansylated polyamines extracted from leaves of *L. vulgare* and *L. perezii* are shown in Fig. 1. Side products of the dansyl reaction were eluted before 14 min and were clearly separated from dansylated polyamines which were completely eluted by 30 min. The *L. vulgare* polyamine profile was by far the most complex and for which at least 30 different dansylated compounds could be clearly isolated. Ten of them were definitively identified. These were agmatine (peak 6), tryptamine (7), 1,3-diaminopropane (8), putrescine (9), cadaverine (10), octopamine (12), methoxytyramine (16), tyramine (17), spermidine (18) and spermine (24). This identification was achieved by co-chromatography with typical standards and by thin-layer chromatographic control (results not shown). Moreover, an acidic hydrolysis of aqueous extracts prior to the dansylation reaction clearly demonstrated the lability of tyramine (peak 17) and peaks 20, 21, 22 and 23 which could correspond to acylated compounds. The comparative analysis of the different polyamine profiles obtained from the different *Limonium* species revealed that the major occurring compounds were those corresponding to peak 1 (unidentified), peak 2 (unidentified), peak 6 (agmatine), peak 7 (tryptamine), peak 8 (1,3-diaminopropane), peak 9 (putrescine), peak 17 (tyramine), peak 18 (spermidine) and peak 24 (spermine). Of particular interest was that 1,3-diaminopropane and tyramine and/or spermidine was relatively high in all species. Putrescine and spermine, which are usually considered as very common aliphatic polyamines, seemed to be poorly represented in these species except for *L. perezii* in which spermine was abundant (Fig. 1). The remarkably high level of tyramine in leaves of the halophytic salt marshes species, *L. vulgare*, was also noteworthy. The general occurrence of 1,3-diaminopropane

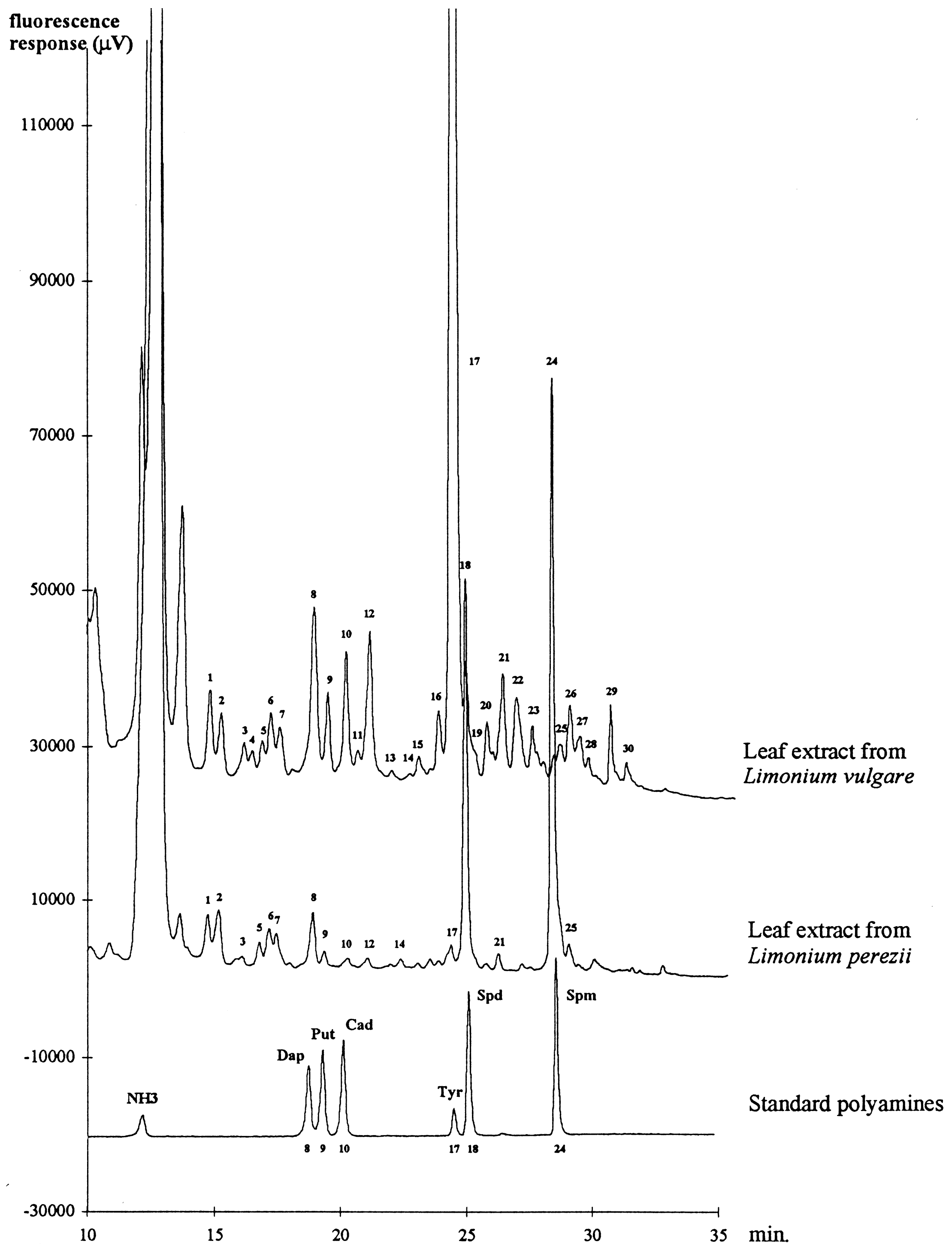

Fig. 1. HPLC chromatograms of dansylated water soluble amines from a standard mixture and from leaf extracts of *Limonium perezii* and *Limonium vulgare* (for details, see Experimental). Identified peaks: agmatine, 6; tryptamine, 7; 1,3-diaminopropane (Dap), 8; putrescine (Put), 9; cadaverine (Cad), 10; octopamine, 12; methoxytyramine, 16; tyramine (Tyr), 17; spermidine (Spd), 18; spermine (Spm), 24.

pointed out the catabolic processes which might operate at the spermidine and the spermine levels.

To obtain more information about acylated compounds, especially those found in *L. vulgare* leaves, a methanol extraction of amines was performed on freeze–dried material. Conjugated polyamines are poorly soluble in water [17]. Neutral conjugated amines were partially discarded from this methanolic extract through phase partition with ethyl acetate and water. The aqueous fraction was fractionated through cation-exchange chromatography on an Amberlite Serva (CG 50, H^+ form) conditioned column. The elution was performed through the successive applications of water, 40% ethanol and 40% acetic acid. The unidentified compounds shown to be labile after acidic hydrolysis and numbered 20, 21, 22 and 23 (Fig. 1) were localized in the acidic fraction, indicating that they could be cationic. This could be the case for monoconjugated aliphatic polyamines covalently linked to phenolic compounds, which are commonly found in plants [17, and references therein]. The main products obtained after the acidic hydrolysis of this cationic chromatographic fraction were 1,3-diaminopropane, putrescine and spermidine. The nature of the acyl moiety could not be determined but further investigations were initiated to determine the structure of the corresponding amine for each unidentified conjugate. This was achieved through thin-layer chromatography (TLC) of the cationic ion-exchange chromatographic fraction, which enabled the isolation of four main ninhydrin responsive spots at R_F 0.18, 0.34, 0.46 and 0.70 in a butanol–ethanol–water (4:1:2, v/v) eluent system. Elution of the isolated spots and dansylation of the isolated compounds, prior to and after an acidic hydrolysis, allowed the chromatographic determination of the amine constituent. Thus it appeared that spot at R_F 0.18 probably corresponded to HPLC peak 20 and could be a 1,3-diaminopropane conjugate. A similar conclusion can be drawn for peak 22 which gave the TLC spot at R_F 0.46. Compounds with R_F 0.34 and 0.70 that corresponded to peaks 21 and 23 respectively gave putrescine after acidic hydrolysis. We now need to complete the characterization of the acidic moiety involved in the biosynthesis of these conjugates. We assume that conjugation could be an efficient storage mechanism for these compounds. In addition these forms may either be translocated or they may constitute potential substrates for oxidation, since it has been observed in animals that acetylated amines are oxidized by amine oxidases [18].

3.1.2. Amides and amino acids

For a comprehensive approach to the metabolic pathways leading to production and accumulation of nitrogenous solutes induced by environmental stress in plants, an accurate analysis of free amides and amino acids was needed. Liquid chromatography is now the most widely used technique for such determinations. Recently a novel pre-column derivatization reagent, AQC was developed by Cohen and Michaud [14] and used for the analysis of amino acids in protein hydrolysates, peptides and other biological samples while using fluorescence or absorbance reaction [19–23]. This method was shown to be highly sensitive, accurate and reproducible for the determination of most amino acids in protein hydrolysates [22]. Our main objective was to transpose this procedure to the analysis of free amino acids from crude extracts of plant materials. In addition to the rapid characterization of most proteinic amino acids we tried to develop a technique for the determination of amino acids related to amine and polyamine metabolism (i.e., glutamate, ornithine, lysine, arginine, methionine, tyrosine, phenylalanine, tryptophane, γ-aminobutyric acid and β-alanine). The aminograms shown in Fig. 2 were established through the chromatographic analysis of AQC derivatized amino acids of a calibration mixture with standard amino acids and those of a crude aqueous extract from *L. vulgare* leaves. They reveal the efficiency of the procedure and show that most of proteinic amino acids could be recognized and quantified. However, glutamine was not well resolved from histidine and or were arginine from threonine. The precursor amino acids used for the synthesis of the main aliphatic and aromatic amines were characterized (glutamate: peak 6; arginine: peak 10; tyrosine: peak 19; methionine: peak 22; ornithine: peak 23; lysine: peak 24; phenylalanine: peak 27 and tryptophane: peak 28). This technical approach allowed us to characterize and quantify γ-aminobutyric acid (peak 15), a well known plant stress metabolite with an undetermined function and β-alanine (peak 11), the precursor for β-alanine

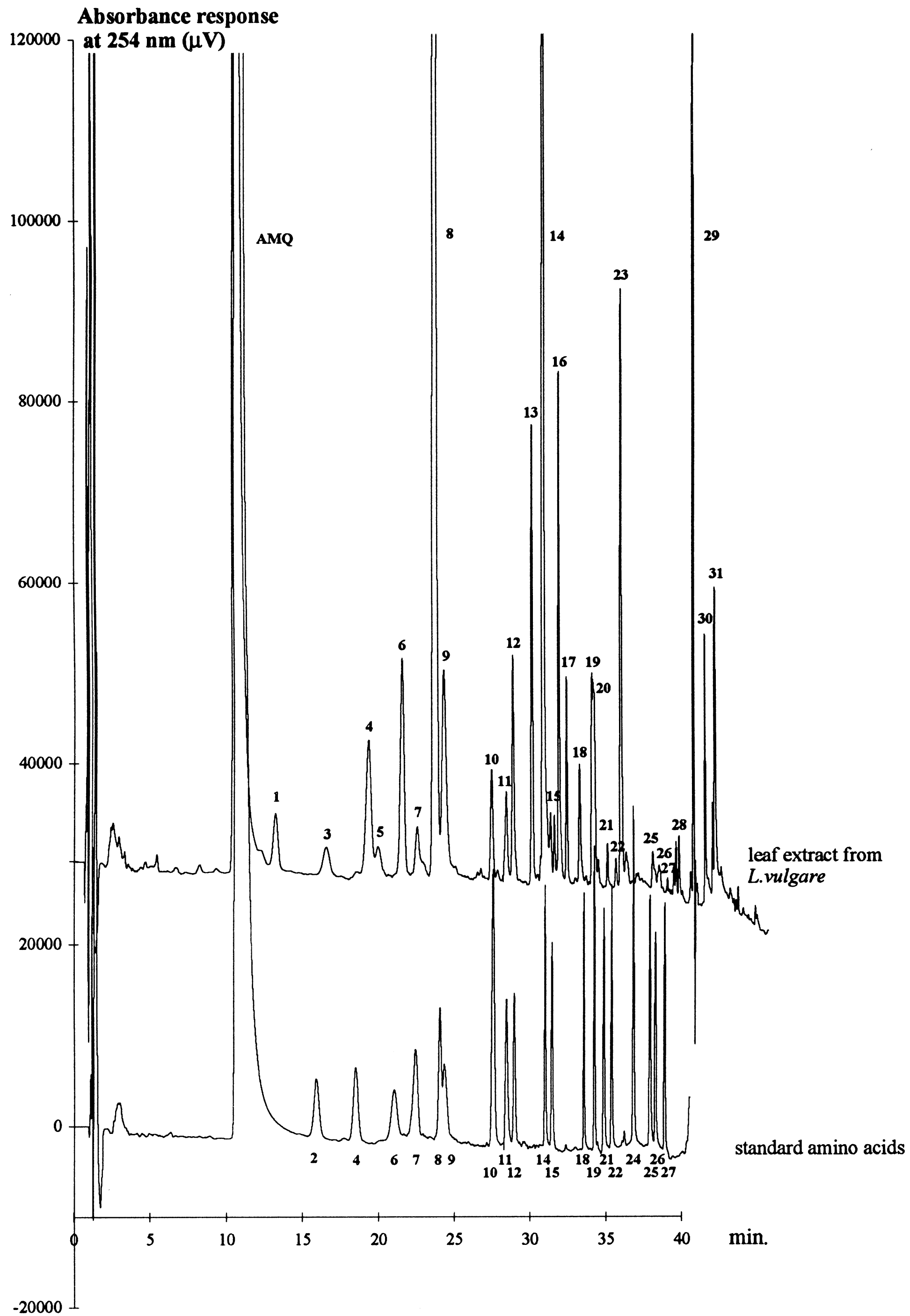

Fig. 2. HPLC chromatograms of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate-derivatized compounds from a standard mixture of amino acids and from an aqueous leaf extract of *Limonium vulgare* (for details, see Experimental) (conversion factor for absorbance scale: 50 000 μV=0.040 $AU_{254\ nm}$). Identified peaks: 6-aminoquinolyl, AMQ; Asp, 2; Asn, 3; Ser, 4; Glu, 6; Gly, 7; His+Gln, 8; NH_3, 9; Arg+Thr, 10; β-Ala, 11; Ala, 12; Pro, 14; Gaba, 15; Cys, 18; Tyr, 19; Val, 21; Met, 22; Orn, 23; Lys, 24; Ile, 25; Leu, 26; Phe, 27.

betaine accumulated in *L. vulgare*. The main advantages of this procedure lies in its simplicity and quickness and in the reproducibility of the derivatization reaction. It is shown here for the first time to be effective with plant crude extracts. The detection threshold of derivatized amino acids through AQC reaction at the pmol level is also a criterion of effectiveness. Furthermore the detection sensitivity can be enhanced by fluorimetry [14]. Some of the compounds responsive to the AQC derivatization and resolved by our chromatographic procedure in the different *Limonium* species extracts could not be identified. The unknown compounds best represented were numbered as peaks 5, 13, 16 and 17 (Fig. 2), for which we do not have clear evidence about their amino acid nature.We only know that the compound corresponding to peak 13 was destroyed when the crude extract was incubated for 12 h in 6 *M* HCl (110°C) whereas that corresponding to peak 17 was shown to be stable. Nevertheless, efforts will be devoted towards their identification as some of these compounds are highly concentrated in some species (i.e., peak 13 in *L. latifolium*, peaks 13 and 17 in *L. gmelini* and peaks 13, 16 and 17 in *L. dumosum* and *L. vulgare*).

Concurrently to the analysis of amino acids, it has recently been demonstrated that high resolution reversed-phase HPLC is achievable *via* the present procedure for the analysis of polyamines and their monoacetyl conjugates using fluorescence detection after derivatization with *N*-hydroxysuccinimidyl 6-quinolinyl carbamate [24]. This was used in the determination of the polyamine content of cultured pancreatic cancer cells and of tissue from colorectal adenocarcinoma. Preliminary investigations would indicate that, under our analytical conditions, derivatized polyamines can be resolved without interfering with the analyses of amino acids which have lower retention times (i.e., up to 40 min, peaks 30 and 31) (Fig. 2). We are currently optimising this procedure to allow the simultaneous identification and quantification of amino acids and polyamines.

3.1.3. β-Alanine betaine

Hanson et al. [9] recently reported the occurrence of several quaternary ammonium compounds in *Plumbaginaceae*: glycine betaine, β-alanine betaine, proline betaine, hydroxyproline betaine and choline-*O*-sulfate. Unlike glycine betaine which is quite common in higher plants [25], proline betaine and hydroxyproline betaine were shown to occur only in a small number of plant families. In particular, choline-*O*-sulfate and β-alanine betaine were found to accumulate in the *Plumbaginaceae*. The latter was discovered in higher plants in *L. vulgare* [16]. The role of some of these compounds as so-called compatible solutes (which facilitate osmotic adjustment to dry or saline environments) has been proposed and the pathways leading to their biosynthesis established. The biosynthetic routes for β-alanine betaine, which is often found in species particularly well adapted to harsh environments, has been shown to be very complex. In *L. vulgare*, β-alanine betaine is synthesized by the methylation of β-alanine, with the methyl groups coming from methionine [8]. Nevertheless the origin of the β-alanine moiety is not known. As indicated previously, the β-alanine skeleton needed for β-alanine betaine synthesis could be derived from polyamine catabolism. Thus our current efforts are focused on the optimization of a simple HPLC procedure to quantify β-alanine betaine. Currently the only efficient method for this compound requires the use of fast atom bombardment mass spectrometry (FAB-MS) [26,27]. We have extended the technique described by Osinga et al. [15] for measuring dimethylsulfoniopropionate. In this procedure, dimethylsulfoniopropionate is converted to acrylate and dimethylsulfide, with the former being measured by HPLC. In the case of alkaline treated β-alanine betaine, the β-elimination of Hoffman also takes place giving rise to trimethylamine and acrylate in an equimolar ratio [16]. This process was used for indirect estimation of β-alanine betaine concentrations through acrylate level measurements. Moreover, the suitability of the chromatographic analysis and determination of acrylate was demonstrated by concurrent ^{1}H-NMR quantification of β-alanine betaine (as described by Jones et al. [28]) using a *tert*.-butanol internal reference with the sample peak position measured relative to that obtained with the synthetic compound.

We first assayed the conversion yield of synthetic β-alanine betaine into acrylate under alkaline conditions. It appears that within 30 min at 50°C in presence of 6 *M* NaOH (final concentration) chemical splitting was complete within the range of β-

alanine betaine level found in the plant extracts. We have also shown that HPLC with UV detection at 205 nm leads to reliable calibration curves using standards of acrylate. This compound was previously demonstrated to be stable following alkaline treatment. Similar results were obtained with standards of β-alanine betaine and the three sets of calibration curves were accurately superimposed. Therefore β-alanine betaine could be measured in *Limonium* plant extracts after its conversion to acrylate, providing that this compound could be efficiently resolved under our chromatographic conditions. Chromatograms of standard acrylate and extracts of *L. vulgare* leaves, before and after alkaline hydrolysis, are shown in Fig. 3. In crude plant extracts there was a compound whose chromatographic retention time was identical to that of acrylate (Fig. 3). The relative abundance of this compound (in terms of signal) differed among the *Limonium* species, ranging from 4% of total acrylate quantified after hydrolysis in *L. gmelini* to 35% in *L. tataricum* and *L. vulgare* (data not shown). As a consequence the accurate estimation of β-alanine betaine content through acrylate quantification was performed by subtracting (at the surface level) the amount of the interfering compound from the total level registered after hydrolysis. However, in order to validate this indirect measurement of β-alanine betaine, an ^{1}H-NMR quantifica-

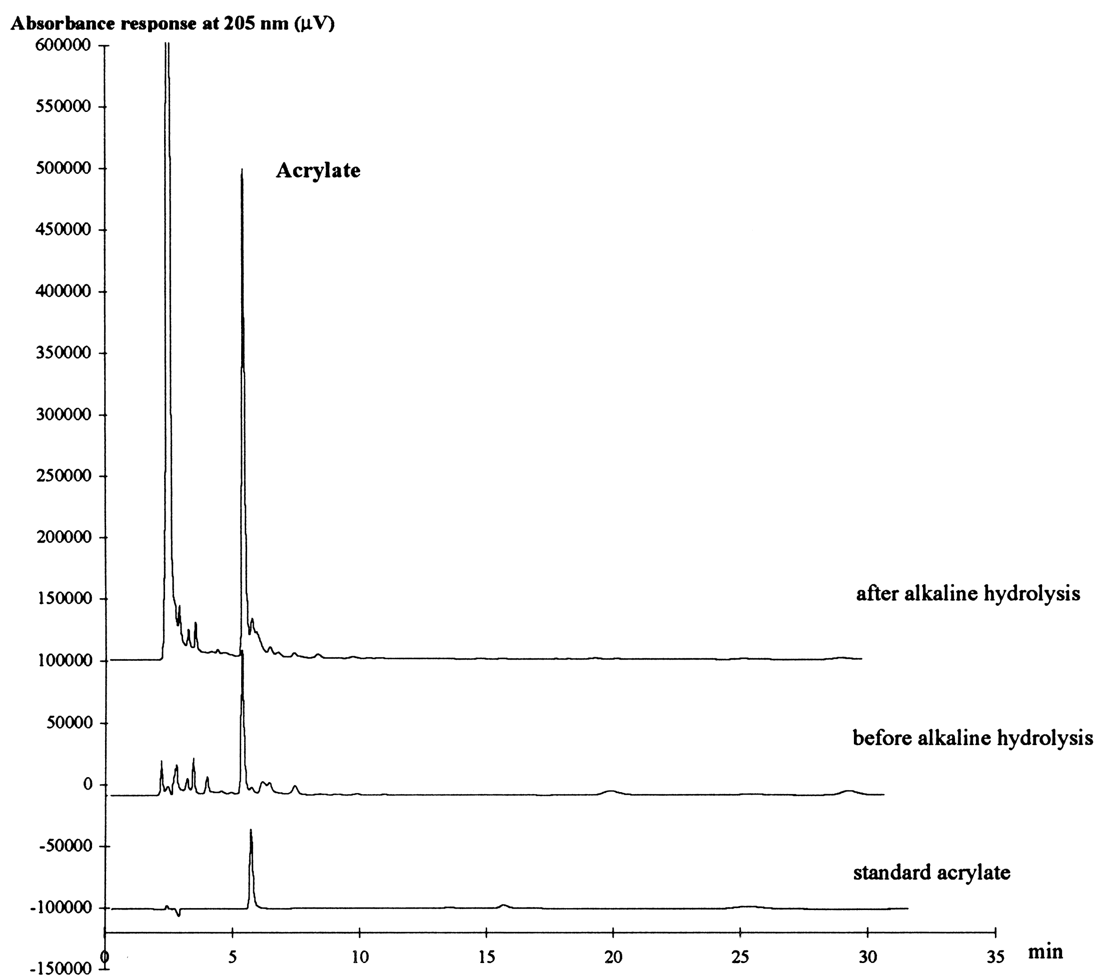

Fig. 3. HPLC chromatograms of standard acrylate and aqueous leaf extracts from *Limonium vulgare* before and after alkaline hydrolysis (for details, see Experimental) (conversion factor for absorbance scale: 50 000 μV=0.243 $AU_{205\ nm}$).

tion of β-alanine betaine was developed. The integrated area of the singlet signal at 3.14 ppm caused by the nine protons of the trimethylated ammonium group of β-alanine betaine was used to estimate the levels of these compound as compared with the integrated area of the peak corresponding to the 9 protons of the internal standard, *tert.*-butanol (Fig. 4). The suitability of the calibration curve based on the relative areas of both *tert.*-butanol and the *N*-methyl protons was checked. Peak position was measured relative to the synthetic standard product (3.14 ppm). This peak was absent or weakly represented in *L. perezii* and *L. sinuatum* suggesting that β-alanine betaine did not accumulate in these species. Nevertheless in these two species and to a lesser extent in *L. vulgare,* a peak at 3.25 ppm was characterized that could be attributable to that of the *N*-trimethyl protons of glycine betaine. Additional peaks with chemical shifts of 3.18 and 3.21 ppm occurred in ^{1}H-NMR spectra from all *Limonium* species extracts (Fig. 4). They could be associated with the occurrence in these species of choline and choline-*O*-sulfate, respectively. The presence of these QACs was confirmed by paper high-voltage electrophoresis and Dragendorff revelation (data not shown). At the quantitative level, we have focused our attention on β-alanine betaine determination but the same procedure is suitable for other quaternary ammonium compounds (i.e., glycine betaine, choline, choline-*O*-sulfate) occurring in the various species of *Limonium* under study. Thus, as an example, the β-alanine betaine levels of three different *Limonium* species, which differ in their capacity to accumulate this compound, were determined by ^{1}H-NMR of the intact betaine concurrently with HPLC of the derived acrylate (Table 1). Both methods led to similar results indicating that chromatographic analysis of acrylate constitutes a simple and convenient method for a fast, reliable and cheap quantification of β-alanine betaine.

3.2. Application of the optimized chromatographic techniques to the quantification of related nitrogenous compounds in Limonium species

Table 2 summarizes the quantitative investigations

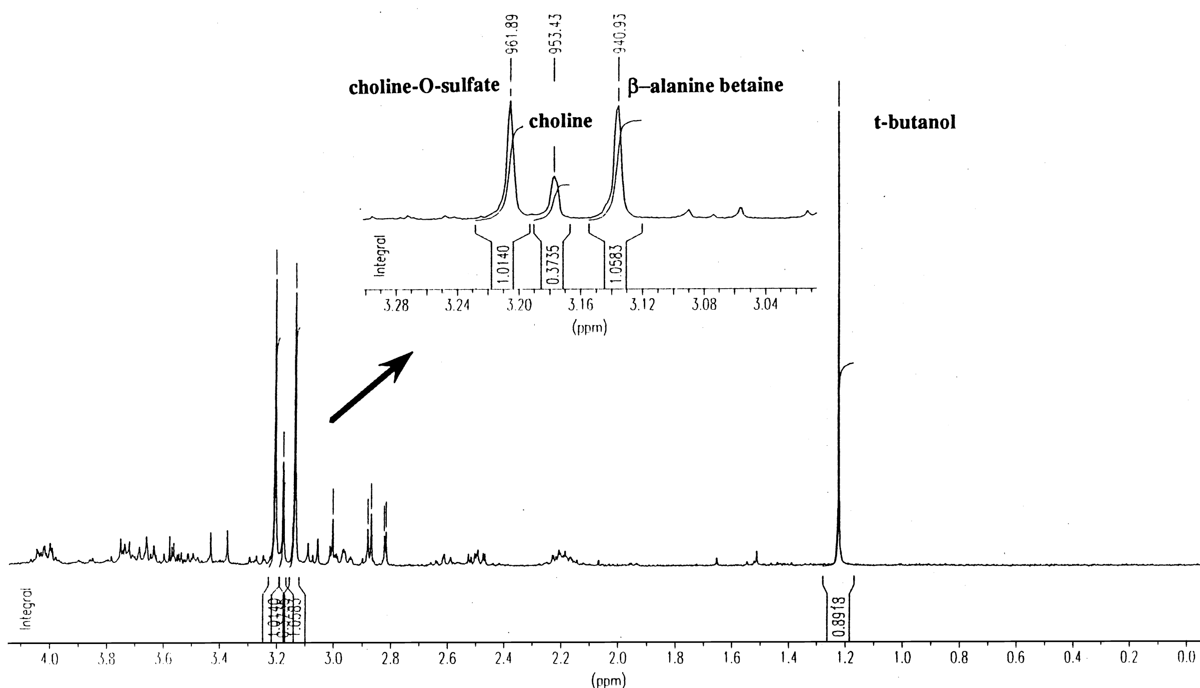

Fig. 4. ^{1}H-NMR spectrum of an aqueous extract from leaves of *Limonium vulgare* and *tert.*-butanol as an internal standard (for details, see Experimental).

Table 1
Comparative quantification of β-alanine betaine through ^{1}H-NMR of crude aqueous extracts and HPLC of alkaline hydrolysates from leaves of different *Limonium* species (see Experimental)

Limonium species	^{1}H-NMR determinations (μmol g^{-1} dry mass)	HPLC determinations (μmol g^{-1} dry mass)
L. vulgare	154.4±5.4	153.0±6.8
L. tataricum	31.5±3.2	27.7±1.5
L. dumosum	13.5±0.7	10.8±0.8

performed in the vegetative parts from a few *Limonium* species at the amino acid, polyamine and betaine levels. Species were chosen according to their horticultural or ecological interest as they were shown to display rather high degrees of tolerance to drought or salt stress [29]. These species have been shown to display a high flexibility of their nitrogen metabolism in relation to environmental salinity. This flexibility is characterized through an accumulation of heterocyclic amino acids (proline, pipecolic acid) and storage of betaines (glycine betaine, β-alanine betaine) [8,29]. Polyamine metabolism is far less well known in these species. Considering their crucial roles during plant growth and development and metabolic links with amino acid metabolism and β-alanine betaine production, special emphasis was directed towards their analysis (Table 2). We did not try to establish close comparisons between species as they were not grown under the same conditions or for the same duration. As already described, 1,3-diaminopropane and tyramine are the main stored aliphatic and aromatic amines, respectively (Table 2). Because the other aliphatic amines are poorly accumulated it suggests there is tight regulatory processes at both the biosynthetic and the catabolic levels. Cadaverine, which results from lysine decarboxylation, was poorly detected in these species. Among amino acid precursors of these polyamines (i.e., arginine, ornithine, glutamate, tyrosine, lysine, methionine) no major accumulation was observed except for methionine, for which levels were rather high in these species. Arginine content could not be estimated due to its poor resolution from threonine under our analytical conditions.

Table 2
Free polyamine, related amino acid and betaine contents (μmol g^{-1} dry mass) of leaf extracts from different *Limonium* species (see Experimental)

Nitrogenous compounds	*L. vulgare*	*L. latifolium*	*L. tataricum*	*L. dumosum*	*L. gmelini*	*L. perezii*	*L. sinuatum*
1,3-Diaminopropane	0.7	2.3	1.3	2.0	0.5	0.3	0.8
Putrescine	0.3	tr	0.1	0.2	0.1	tr	0.1
Cadaverine	0.5	tr	tr	tr	tr	tr	tr
Tryamine	33.6	1.2	1.7	8.3	3.1	0.4	2.1
Spermidine	0.3	0.1	0.2	0.3	0.2	1.0	0.1
Spermine	0.1	tr	tr	0.2	0.1	1.5	0.1
Glutamate	3.2	2.0	2.3	1.1	3.2	25.4	4.5
β-Alanine	0.7	1.1	tr	tr	tr	tr	0.1
Proline	12.1	19.1	4.8	7.4	tr	0.3	0.3
γ-Aminobutyrate	0.3	0.5	0.1	0.2	tr	tr	0.1
Tyrosine	0.8	tr	0.8	0.1	tr	2.4	0.4
Methionine	0.3	5.0	2.1	2.0	6.9	3.0	3.4
Ornithine	2.8	0.5	0.2	0.2	0.2	2.0	1.4
Lysine	tr[a]	tr	3.5	tr	tr	3.8	tr
β-Alanine betaine	153.0	102.5	27.7	10.8	121.7	nd	nd
Glycine betaine	tr	tr	nd[b]	nd	tr	175.0	80.5
Choline-*O*-sulfate	61.1	47.3	36.1	13.0	85.5	222.5	56.7

[a] tr=Traces.
[b] nd=Non-detectable.

No salt or drought treatments were applied to the plants. Nevertheless the β-alanine betaine levels were quite substantial ranging from 11 μmol g^{-1} dry mass in *L. tatarium* to 153 in *L. vulgare*. As already described [29], *L. perezii* and *L. sinuatum* did not accumulate β-alanine betaine, but were noted to contain glycine betaine which was not or very poorly detected in the other species. With respect to the accumulation of β-alanine betaine in the species studied, three groups can be distinguished: a first group characterized by high β-alanine betaine levels (*L. vulgare*, *L. latifolium* and *L. gmelini*), a second with low concentrations (*L. tataricum* and *L. dumosum*) and a third one where β-alanine betaine could not be detected (*L. sinuatum* and *L. perezii*). In the latter group, β-alanine betaine is replaced by glycine betaine (this work and [29]). Accumulation of choline-*O*-sulfate is a general fate. Considering taxonomical and ecological characters, high β-alanine betaine accumulators are in the sub-genus *Eulimonium* and found in sandy or dry inlands or littoral habitats. In contrast low β-alanine betaine accumulators are classified in the sub-genus *Goniolimon* and found in cold habitats [30]. Hanson et al. [9] observed variability of QAC osmoprotectant systems by noting QAC accumulation in relation with habitats of various members of the *Plumbaginaceae*. Thus in contrast with glycine betaine accumulation, β-alanine betaine synthesis was regarded as an advantageous metabolic pathway for choline economy or under hypoxic conditions. Recent data were obtained about salt resistance-related traits in *Plumbaginaceae* by comparing different populations of *Armeria maritima* from salt marshes and from inland sites [31]. It was concluded, that among these traits, the capacity to accumulate betaines, which is constitutive in this species, is of special interest for salt tolerance and might have facilitated the colonization of salt marshes [31]. These authors also observed that, by contrast to betaine storage, no proline accumulation was found under long-term-salt stress and they deduced that proline was not involved in adjustment to long-term-salt stress. Here, at the interspecific level, in absence of apparent salt or drought stress, β-alanine betaine accumulating species were shown to display a constitutive accumulation of this compound which is not correlated with the amount of proline accumulated (Table 2). This raises the concern of the metabolic velocity of β-alanine betaine accumulation during the drought or the salt stress response from precursors which remain to be elucidated. Low levels of putrescine, spermidine and spermine and especially high levels of 1,3-diaminopropane (the first catabolic product of spermidine and spermine) suggested the occurrence of active diamine and polyamine oxidases in these species. The point that arises is whether or not 1,3-diaminopropane does constitute an end product. If it does not its fate is unknown because the degradative products of diamine and polyamine catabolism such as γ-aminobutyrate and β-alanine were not found to accumulate. In attempting to learn the route of synthesis of β-alanine, insofar as several metabolic pathways for this amino acid production are conceivable through decarboxylation of aspartic acid, oxidative metabolism of propionic acid or pyrimidine catabolism [8,25], we postulate that, in *Limonium* species, it may be through polyamine catabolism a way-out of γ-aminobutyrate to the Krebs cycle, with β-alanine partly or totally directed to β-alanine betaine biosynthesis.

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