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Precision and sensitivity of the measurement of ^{15}N enrichment in D-alanine from bacterial cell walls using positive/negative ion mass spectrometry

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Summary

Sensitive detection of cellular components from specific groups of microbes can be utilized as 'signatures' in the examination of microbial consortia from soils, sediments or biofilms. Utilizing capillary gas chromatography/mass spectrometry and stereospecific derivatizing agents, D-alanine, a component localized in the prokaryotic (bacterial) cell wall, can be detected reproducibly. Enrichments of D-[^{15}N]alanine determined in *E. coli* grown with [^{15}N]ammonia can be determined with precision at 1.0 atom%. Chemical ionization with methane gas and the detection of negative ions $(M - HF)^{-\cdot}$ and $(M - F \text{ or } M + H - HF)^{-\cdot}$ formed from the heptafluorobutyryl D-2 butanol ester of D-alanine allowed as little as 8 pg (90 fmol) to be detected reproducibly. This method can be utilized to define the metabolic activity in terms of ^{15}N incorporation at the level of $10^3 - 10^4$ cells, as a function of the $^{15}N - ^{14}N$ ratio.

Key words: *Bacterial cell wall – Capillary gas chromatography/mass spectrometry – D-Alanine – Positive/negative ion chemical ionization – Selected ion monitoring – Stable isotope labeling*

Introduction

Certain microbial cellular components ('signatures') are restricted to subsets of the microbial community and their determination, in particular by capillary gas chromatography (GC) and GC combined with mass spectrometry (GC/MS) [1], have made possible studies of shifts in microbial biomass and community structure [2]. Such shifts may result from predation, bioturbation, biocides, surface chemistry and microtopology as well as mechanical disturbance [3–11]. Furthermore, incorporation of isotopes in the

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communities enables studies of formation and turnover of the signatures resulting in insight into the dynamics of groups of organisms.

Stable isotope-labeled precursors have advantages over radioactive isotopes in being efficiently detectable by MS and in having a high specific activity. The latter enables use of concentrations of signature precursors at just above the natural levels and avoids distortion induced by the additions of high concentrations of substrate required for many radioactive precursors. Furthermore, mass spectrometric analysis of stable isotopes permits studies of particular atoms in labeled specific signatures in contrast to the overall measurements usually accomplished in work with radioactive material.

Because it is most often confined to a single locus in the signature molecule, ^{15}N is the most suitable stable isotope for studying marked molecules. If alternatives like ^{2}H or ^{13}C are used, molecules with multiply labeled sites will often result during the course of biosynthesis and the MS will be considerably more complex to interpret.

The bacteria-specific cell wall amino acids D-alanine, diaminopimelic acid and *N*-acetylmuramic acid are important N-containing signatures. Of these, the former have proved particularly convenient to determine by combined capillary gas chromatography/mass spectrometry (GC/MS) [12, 13]. The enantiomers of alanine can be separated either on a capillary column with a chiral stationary phase or on a conventional, optically inactive column by forming diastereoisomeric derivatives using an optically active alcohol [12, 13]. Extremely high sensitivity (below 1 pg) and selectivity can be achieved if soft ionization methods (chemical ionization, CI) and selected ion monitoring (SIM) techniques are employed [12].

Traditionally MS determinations of the ^{15}N abundance of amino acids have involved isolation of individual amino acids by chromatographic or electrophoretic methods, subsequent conversion of the amino acid to N_2 followed by MS measurements of the N_2 isotopes (e.g., Ref. 14). The method is time consuming and requires careful corrections for the background levels of atmospheric nitrogen. To achieve satisfactory accuracy of the measurements microgram levels of amino acids are necessary.

The sensitivity of the isotopic enrichment measurements can be increased by GC/MS analyses of a suitable volatile derivative of the intact amino acid. Using electron impact/mass spectrometry (EI/MS) Nissim et al. [15] determined, for example, [*guanidino*-^{15}N]-arginine in plasma whereas Rodes et al. [16] studied the assimilation of $^{15}NH_4^+$ in *Lemna minor* by measuring on the heptafluorobutyryl (HFB) esters of amino acids. CI-MS has been used to estimate ^{15}N levels in derivatized amino acid standards [17] and the ^{15}N enrichment in plasma amino acids [18]. It can be concluded from these investigations that current MS technology allows determination of ^{15}N increments ranging from 0.2 to 1 mol% excess, the figures largely depending on the absolute amounts studied.

Enrichment experiments involving CISIM studies of polyhalogenated amino acids monitoring negative ions have not been reported in the literature. The present paper demonstrates that ^{15}N enrichment in bacterial cell wall-specific D-alanine using capillary GC/MS and negative ion monitoring of ions formed using CI with methane as reagent gas can be performed with high precision and sensitivity.

Materials and Methods

Reagents

[^{15}N]L-alanine (99 atom%) and $^{15}NH_4Cl$ (95 atom%) were obtained from Merck Sharp & Dohme, Canada Ltd. (Montreal). D-Alanine, D-2-butanol (optical purity 98%), heptafluorobutyric anhydride (HFBA), Dowex 50 W X8 100/200 mesh were from Fluka AG, Switzerland. Solvents and reagents were of analytical grade and used without further purification.

Cultivation of labeled and unlabeled Escherichia coli *cells*

The bacteria were cultured at 37°C in a defined glucose medium [19]. The nitrogen source in the medium for labeled cells was substituted for $^{15}NH_4Cl$.

Standard curves

To demonstrate the precision and accuracy of the method for ^{15}N determination, a standard dilution curve of L-[^{15}N]alanine with final concentrations of 0.00, 0.11, 1.09, 2.68, 5.26 and 9.94 mol% ($^{15}N/^{15}N+^{14}N$) alanine was prepared by mixing labeled and unlabeled L-alanine. A second standard curve was prepared by mixing maximally labeled and unlabeled *E. coli* cells to achieve the weight proportions ($^{15}N/^{15}N+^{14}N$) of 0.00, 1.5, 8.9, 25.2, 48.9, 70.7, 90.2, 98.7 and 100.0%.

Sample preparation and derivatization

The samples of *E. coli* cells were hydrolysed in 6 M hydrochloric acid and purified on a Dowex cation exchange column [12]. Diastereoisomeric derivatives of D- and L-alanine were formed by esterification with D-2-butanol and by acylation with HFBA. Details of the procedures are given in [12].

Gas chromatography/mass spectrometry (GC/MS)

The N-HFB D-2-butyl ester derivatives of D- and L-alanine were separated on a 25 m (i.d. 0.2 mm) fused silica capillary column statically coated with SE-54 [20] connected directly to the ion source of the MS.

The instrument was a Ribermag R10-10C quadrupole GC/MS/data acquisition system equipped with a Carlo Erba model 4160 GC. Helium was used as carrier gas at a flow rate of 40 cm s^{-1}. The injector temperature was 230°C. The initial oven temperature was 80°C and after 5 min linearly increased by 5° min^{-1} to 220°C. Splitless injections were performed and the split vent opened 30 s after injection.

In EI ionization the electron energy was 70 eV and the ion source temperature was 140°C. The CI reagent gases were ionized with electrons of 94 eV. Positive ion CI (PICI) was performed by using ammonia (>99.96%) as reagent gas at an ion source temperature of 110°C and a pressure of 0.1 torr. Methane at 0.06 torr and 180°C (>99.995%) served as reagent gas in the negative ion CI (NICI) studies. The monitored ions were in EI m/z 241.1/242.1, in PICI m/z 359.1/360.1 and in NICI m/z 321.1/322.1.

Isotopic enrichment of ^{15}N was calculated from the signals given by the respective labeled and unlabeled fragments according to the formulas of Biemann [21].

Results and Discussion

The initial enrichment studies were performed with L-alanine since no labeled D-alanine was available. The mass spectra of the N-HFB D-2-butyl ester of alanine using EI ionization and different CI methods have been published elsewhere [12, 22]. Data of importance for the present study are summarized in Table 1, where the base peaks (m), all containing N, form the basis for our considerations. Notably, the different ($m+1$) ions in all ionization methods consisted of a mixture of the most abundant ions plus a hydrogen atom and the next higher isotopic ions.

The ratio of the abundances of the ($m+1$) and m ions were considerably higher in EI than in the two CI methods. Since it was assumed that small increments in the ($m+1$) peaks due to enrichment of ^{15}N could be measured with higher precision on a small ratio than on a large, the ($m+1$) and ($m+2$) ions in EI were investigated. It was thereby found that the peak at ($m+2$) (m/z = 242, 4.7%) corresponded only to the natural isotopic distribution. Therefore, despite a slight decrease in absolute sensitivity, the ions of m/z 241 and 242 were chosen for the precision studies in EI.

The precision in the determination of the signal ratio of the selected ions, ($m+1$) and m in CI, ($m+2$) and ($m+1$) in EI, obtained from unlabeled alanine derivative was compared (Table 2). Even though the ($m+1$) fragments in CI, in contrast to the ($m+2$)

TABLE 1

SUMMARY OF MASS SPECTROMETRIC CHARACTERISTICS OF THE N-HFB D-2-BUTYL ESTER DERIVATIVE OF [^{14}N]ALANINE $CH_3CH[HNCOCF_2CF_2CF_3]$ $COOCH(CH_3)$ CH_2CH_3, M = 341

Ionization mode	m (Most abundant ions)			($m+1$)		
	m/z	Assignment	%	m/z	Assignment	%
EI	240	$(M-OCOC_4H_9)^+$	100	241	$(M-OCOC_4H_8)^+$	60 (7.0)[a]
PICI[b]	359	$(M+NH_4^+)^+$	100	360	$(M+H+NH_4)^+$	19 (13.1)[a]
NICI[c]	321	(M-HF)	100	322	(M-F) or (M+H-HF)	20 (12.6)[a]

[a] Contribution from the natural abundance of heavy isotopes [23].

[b] Ammonia as reagent gas.

[c] Methane as reagent gas.

TABLE 2

PRECISION IN THE DETERMINATION OF THE SIGNAL RATIO OF SELECTED IONS FROM NON-LABELED ALANINE USING DIFFERENT IONIZATION METHODS

800 pg of the N-HFB D-2-butyl ester derivative of L-alanine was injected. n = 5, SD = standard deviation, CV = coefficient of variation.

Ionization mode	Monitored ions (m/z)	Mean	SD	CV (%)
EI	242.1/241.1	0.0790	0.0065	8.23
PICI (ammonia)	360.1/359.1	0.1860	0.0097	5.22
NICI (methane)	322.1/321.1	0.1958	0.0102	5.21

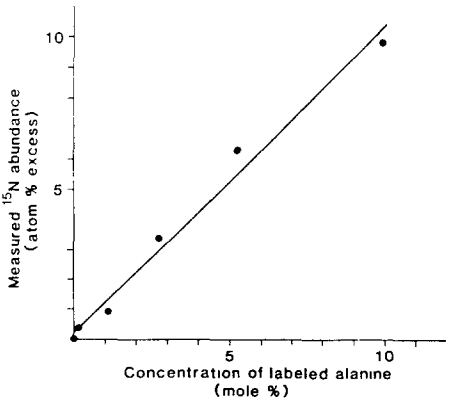


Fig. 1. Standard curve of ^{15}N enriched L-alanine. The signal ratio of the fragments of m/z 322.1/321.1 was used for the calculation of ^{15}N abundance. Injected amount corresponded to 500 pg of alanine.

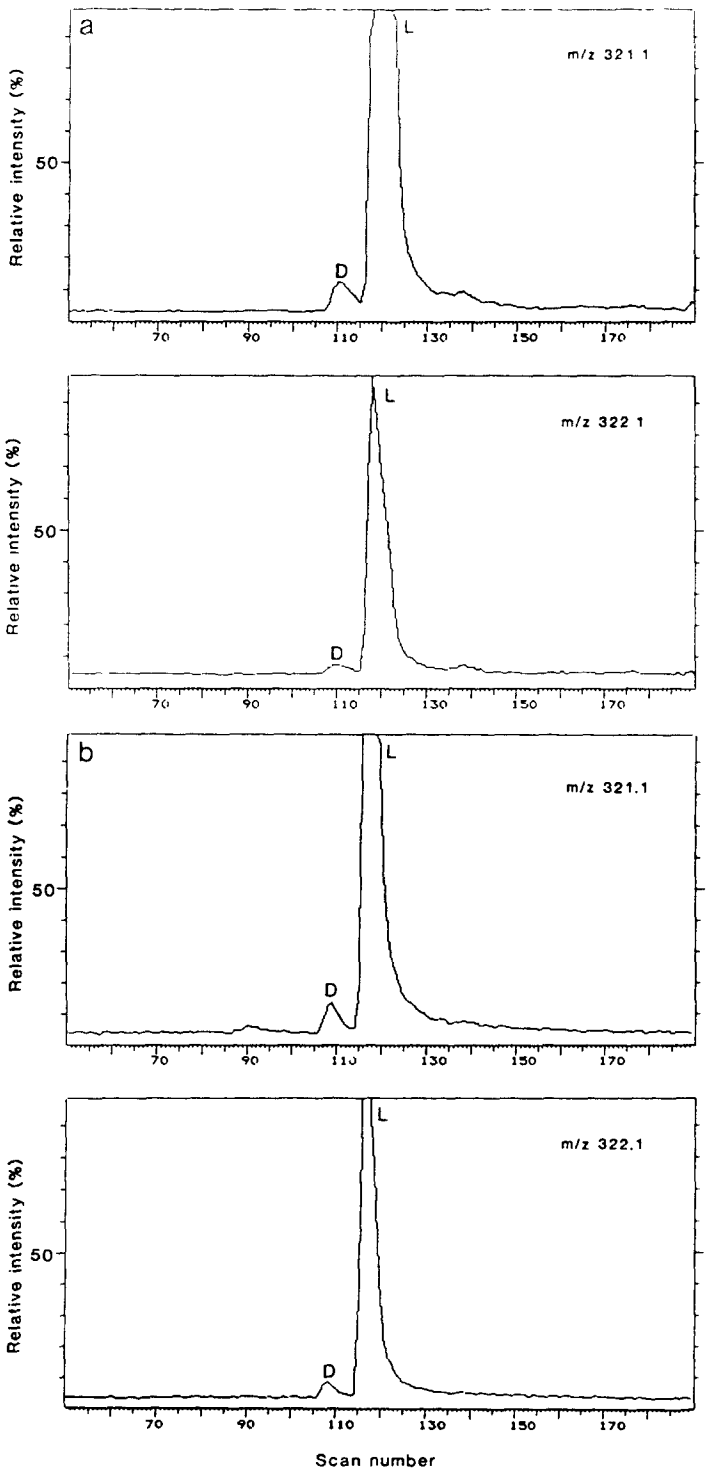
fragments in EI, consisted mainly of rearrangement ions, the formation of which could be sensitive to variations of the ion source parameters [24], the coefficient of variation (CV) was lower in CI than in EI. The CV for the two CI methods were similar. Since it was previously shown that NICI offered the highest absolute sensitivity [12], it was chosen for the following experiments.

In the next series of measurements a standard dilution curve, containing mixtures of ^{15}N-labeled and unlabeled L-alanine in the picogram range, was constructed (Fig. 1). It showed satisfactory agreement between measured ^{15}N abundance (y) and concentration of labeled alanine (x). The regression line was $y = 1.00X + 0.29$, correlation coefficient $r = 0.993$.

^{15}N enrichment in D-alanine from bacteria was also determined. Mass fragmentograms of a sample of unlabeled and a sample of a mixture of labeled and unlabeled *E. coli* cells are reproduced in Fig. 2a and b. The plot, based on the analysis of samples with known concentrations of labeled bacterial cells, showed good linearity (Fig. 3). The regression equation was $y = 0.94X - 0.01$; $r = 0.999$.

To test the precision of these measurements, five replicate analyses were performed with the *E. coli* sample with the concentration of 8.9% (w/w) labeled cells. The signal ratios of the fragments of m/z 322.1/321.1 was 0.257 ± 0.0025 (mean ± standard error (SE)). The sensitivity in measuring small changes in ^{15}N levels may be evaluated from these data [25]. The SE value of ±0.0025 predicts with 95% probability that all measurements made in the same way will fall within the range of ±0.005 of the mean value found. This implies that at the same confidence level, mean values differing by 1.0 atom% ^{15}N will be significantly resolved.

Another important point to be considered is the amount of sample needed to obtain good signal responses for the ratio measurements. The above precision study was performed with samples containing about 800 pg of D-alanine. An enrichment of 1.0 atom% ^{15}N reflects an actual increment of about 8 pg of alanine, an amount one order of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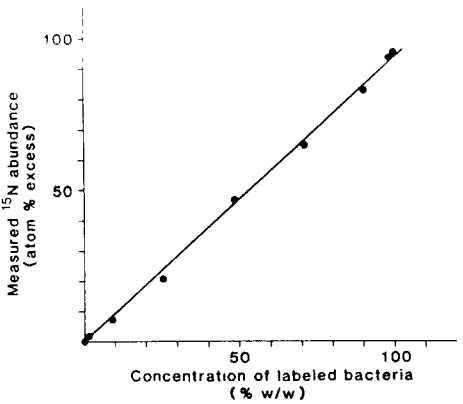

Fig. 3. Standard curve of ^{15}N enrichment in D-alanine from samples of mixed ^{15}N-labeled and unlabeled *E. coli* cells. Injected amount corresponded to about 800 pg of D-alanine.

magnitude above the found detection limit of alanine using NICI MS [12]. This implies possibilities to measure enrichments of ^{15}N in considerably smaller samples or samples with lower amounts of D-alanine. However, the precision in the measurements will decrease as the detection limit is approached.

The present method, using CI and monitoring of negative ions has a precision for the measurement of ^{15}N enrichment in the cell wall-specific D-alanine comparable to that obtained in GC/MS methods reported for the common protein amino acids using EI [15, 16] or CI with positive ion detection [17, 18]. Notably, this study was performed with at least one order of magnitude less amount of material. Using 15 μmol of D-alanine/g dry wt based on the muramic acid content [1], the detection of 8 pg (90 fmol) of D-alanine provides the sensitivity to detect 6 ng or 6×10^3 bacteria the size of *E. coli*.

Conclusion

Quantitative mass spectrometry using CI and negative ion detection provides a high sensitive and selective means of determining ^{15}N enrichment in D-alanine from bacteria. The same technique should be possible to apply to measurements of the incorporation of ^{15}N in other specific (e.g., muramic acid and diaminopimelic acid) or non-specific amino acids of microbial origin.

In ecological microbiology the method can be used to measure the rate of formation or turnover of bacterial biomass in environments with minute amounts of microorganisms. Such environments might include microbes attached to surfaces in biofilms and to plant roots in the rhizosphere.

Fig. 2. Mass fragmentograms of derivatized D- and L-alanine in a hydrolysate of *E. coli* cells. (a) Sample of unlabeled cells. (b) Sample of a mixture of ^{15}N-labeled and unlabeled cells; concentration of labeled cells 25.2% (w/w). The injected amount corresponded to about 800 pg of D-alanine.

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