Simultaneous Quantitative Analysis of Metabolites Using Ion-Pair Liquid Chromatography–Electrospray Ionization Mass Spectrometry

ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2006

Impact Factor: 5.64 · DOI: 10.1021/ac0607616 · Source: PubMed

6 AUTHORS, INCLUDING:

Richard Bas
TNO
11 PUBLICATIONS **247** CITATIONS
SEE PROFILE

Sonja Jespersen
Ferring Pharmaceuticals
23 PUBLICATIONS **781** CITATIONS
SEE PROFILE

Elwin Verheij
TNO
66 PUBLICATIONS **2,440** CITATIONS
SEE PROFILE

Thomas Hankemeier
Leiden University
181 PUBLICATIONS **4,146** CITATIONS
SEE PROFILE

Anal. Chem. 2006, *78*, 6573−6582

Simultaneous Quantitative Analysis of Metabolites Using Ion-Pair Liquid Chromatography−Electrospray Ionization Mass Spectrometry

Leon Coulier,* Richard Bas, Sonja Jespersen, Elwin Verheij, Mariët J. van der Werf, and Thomas Hankemeier†

Analytical Research Department, TNO Quality of Life, Utrechtseweg 48, 3700 AJ, Zeist, The Netherlands

We have developed an analytical method, consisting of ion-pair liquid chromatography coupled to electrospray ionization mass spectrometry (IP-LC-ESI-MS), for the simultaneous quantitative analysis of several key classes of polar metabolites, like nucleotides, coenzyme A esters, sugar nucleotides, and sugar bisphosphates. The use of the ion-pair agent hexylamine and optimization of the pH of the mobile phases were critical parameters in obtaining good retention and peak shapes of many of the above-mentioned polar and acidic metabolites that are impossible to analyze using standard reversed-phase LC/MS. Optimum conditions were found when using a gradient from 5 mM hexylamine in water (pH 6.3) to 90% methanol/10% 10 mM ammonium acetate (pH 8.5). The IP-LC-ESI-MS method was extensively validated by determining the linearity (R^2 > 0.995), sensitivity (limit of detection 0.1−1 ng), repeatability, and reproducibility (relative standard deviation <10%). The IP-LC-ESI-MS method was shown to be a useful tool for microbial metabolomics, i.e., the comprehensive quantitative analysis of metabolites in extracts of microorganisms, and for the determination of the energy charge, i.e., the cellular energy status, as an overall quality measure for the sample workup and analytical protocols.

Metabolomics is the most recent addition to the applied genomics toolbox and involves the nontargeted, comprehensive determination of changes in the complete set of metabolites in a biological system, i.e., the metabolome.[1−3] The metabolomics approach is only recently technically feasible due to the enormous improvements made in two critical areas of research, i.e., analytical chemistry and bioinformatics. As the biochemical level of the metabolome is closest to that of the function of a cell, i.e., the phenotype, it is expected that the study of the metabolome will be very relevant in order to understand biological functioning.[1,2] Various strategies have been proposed and applied to analyze metabolites, as recently reviewed by Dunn and Ellis.[3]

The development of a metabolomics platform with the aim to analyze quantitatively as many metabolites as possible with as few analytical methods as possible is considered to be a promising strategy in the field of metabolite analysis compared to e.g. target analysis or metabolite profiling.[1] However it is also the most challenging strategy with respect to analytical chemistry and bioinformatics.[1,3]

It is questionable whether it is possible to detect all metabolites ranging widely in polarity and molecular weight with one analytical method only.[1] Therefore, a comprehensive analytical platform was set up in which both GC/MS and LC/MS methods were developed to quantitatively analyze as many metabolites in microorganisms as possible. Recently, a validated GC/MS method was described with which several classes of small polar metabolites, i.e., sugars, amino acids, and sugar phosphates, could be analyzed quantitatively in extracts of microorganisms.[4] In addition to this GC/MS method, a LC/MS method was developed for the simultaneous quantitative analysis of different classes of important metabolites that could not be analyzed by GC/MS, such as nucleotides, coenzyme A esters, and sugar nucleotides.[4] The advantages of LC/MS are its compatibility with commonly used solvents such as water and methanol and the fact that large (nonvolatile) polar compounds can be analyzed without derivatization. Polar metabolites, such as ATP, a nucleotide, require special attention due to the strong polarity of these metabolites resulting in irreversible adsorption or peak tailing. These polar metabolites are impossible to be analyzed by standard reversed-phase LC/MS. Previous studies showed that the use of an ion-pair agent is essential for the analysis of nucleotides.[5−8] However, care must be taken when using ion-pair agents in combination with LC/MS due to the possibility of, for example, ion suppression, contamination of the MS source, and adduct formation, which will have significant consequences for the robustness of the method.[9−11]

* Corresponding author. E-mail: leon.coulier@tno.nl. Phone: +31 30 694 4888.
† Current address: LACDR Analytical Biosciences, Leiden University, Einsteinweg 55, 2333 CC, Leiden, The Netherlands.

(1) Van der Werf, M. J.; Jellema, R. H.; Hankemeier, Th. J. *Ind. Microbiol. Biotechnol.* 2005, *32*, 234−252.
(2) Van der Werf, M. J. *Trends Biotechnol.* 2005, *23* (1), 11−16.
(3) Dunn, W. B.; Ellis, D. I. *Trends Anal. Chem.* 2005, *24* (4), 285−294.
(4) Koek, M. M.; Muilwijk, B.; van der Werf, M. J.; Hankemeier, Th. *Anal. Chem.* 2006, *78*, 1272−1281.
(5) Allinquant, B.; Musenger, C.; Schuller, E. *J. Chromatogr.* 1985, *326*, 281−291.
(6) Federn, H.; Ristow, H. *Chromatographia* 1986, *22* (7−12), 287−291.
(7) Uesugi, T.; Sano, K.; Uesawa, Y.; Ikegami, Y.; Mohri, K. *J. Chromatogr., B* 1997, *703*, 63−74.
(8) Lopez, S. L. B.; Moal, J.; Serrano, F. S. J. *J. Chromatogr., A* 2000, *891*, 99−107.
(9) Bonfiglio, R.; King, R. C.; Olah, T. V.; Merkle, K. *Rapid Commun. Mass Spectrom.* 1999, *13*, 1175−1185.

10.1021/ac0607616 CCC: $33.50
Published on Web 07/22/2006

Therefore, the type of ion-pair agent and the subsequent concentration of the ion-pair reagent should be optimized. The ion-pair agent acts as a counterion for the acidic nucleotides, thereby facilitating the elution of nucleotides from the column with good peak shape. Furthermore, the ion-pair method should preferably be compatible with the simultaneous analysis of other (polar) metabolites such as coenzyme A esters, sugar bisphosphates, purines/pyrimidines, organic acids, and sugar nucleotides.

One of the difficulties in the analysis of microorganisms is the presence of high-abundant substances, such as proteins, (residual) substrate, and product compounds in the fermentation medium and buffer components. Although some of these components can be removed during sample workup, others will be present in the final extracts and might interfere or influence the performance of the analytical methods applied. Therefore, it is necessary to develop robust and reproducible analytical methods to analyze large numbers of extracts of different microorganisms quantitatively.

In this paper, an ion-pair LC-ESI-MS method is described, that is complementary to our previously described GC/MS method[4] and that allows the simultaneous analysis of important classes of metabolites in microorganisms, such as nucleotides, coenzyme A esters, sugar nucleotides, and sugar bisphosphates, with good analytical performance with respect to sensitivity, linearity, repeatability, and reproducibility. The application of the ion-pair liquid chromatography coupled to electrospray ionization mass spectrometry (IP-LC-ESI-MS) method is demonstrated for different microorganisms such as *Bacillus subtilis* and *Escherichia coli* and the determination of the energy charge for the quality control of the overall procedure, from sampling and quenching of microorganisms up to the analysis.

EXPERIMENTAL SECTION

Chemicals and Reagents. Reference standards of the various metabolites were obtained from Sigma-Aldrich Chemie (Zwijndrecht, Netherlands). Isotopically labeled quality control standards of AMP (^{13}C,^{15}N), GMP (^{13}C,^{15}N), GTP (^{13}C, 5 ^{15}N), and deuterated quality control standards of cholic acid-d_4 and phenylalanine-d_5 were obtained from Cambridge Isotope Laboratories (Andover, MA). Stock solutions of the various metabolites were prepared in an appropriate solvent, preferably methanol/water (1:3 v/v). Calibration solutions were prepared by diluting the stock solutions in methanol/water (1:3 v/v).

Bacterial Strains and Sample Workup. *B. subtilis* strain 168 (ATCC 23857) and *E. coli* NST 74 (ATCC 31884) were obtained from the ATCC (Manassas, VA). *B. subtilis* and *E. coli* were grown under controlled conditions in a batch fermentor (Bioflow II, New Brunswick Scientific) at 30 °C. The fermentors contained 2 L of mineral salts medium D[12] containing 50 mg/L L-tryptophan and 10 g/L glucose or MMT12 medium[13] at pH 6.8 and 6.5, respectively. The oxygen tension was maintained at 30% by automatic increase of the stirring speed in the fermentor. Samples were taken from the *B. subtilis* and *E. coli* bioreactors at the midlogarithmic phase.

Lactobacillus plantarum WCFS1 is maintained at TNO Quality of Life. *L. plantarum* was grown in a continuous culture at a dilution rate of 0.3 h^{-1} on MRS medium as described by Pieterse et al.[14] Samples were taken from the *L. plantarum* culture once the steady state was reached.

Samples (~0.5 g DWT) were taken as quickly as possible from the bioreactor and immediately quenched, to halt cellular metabolism, at −45 °C in methanol as described previously.[14] Prior to extraction, quality control standards were added and a sample was taken for biomass determination. The intracellular metabolites were extracted from the cell suspensions by chloroform extraction at −45 °C as described by Ruijter and Visser.[15] These samples were deproteinated by filtration using an Amicon Centriprep YM-10 filter centrifuged at 3000*g* and −20 °C for 16 h. Subsequently, quality control standards were added, and all samples were lyophilized and dissolved in methanol/water (1:3 v/v). Prior to analysis by IP-LC-ESI-MS, a quality control standard was added for correction of eventual variations of the MS response.

Instrumentation. All experiments were carried out on a Thermo Finnigan LTQ linear ion-trap system consisting of a Surveyor AS autosampler, Surveyor MS pump, and LTQ LT-10000 mass detector with an Opton ESI probe (Thermo Electron Corp., San Jose, CA). All system control, data acquisition, and mass spectral data evaluation were performed using XCalibur software version 1.4 (Thermo Electron Corp.).

LC-ESI-MS Conditions. For the IP-LC-ESI-MS method, a Chrompack Inertsil 5-μm ODS-3 cartridge column (100 × 3 mm, Middelburg, The Netherlands) was used with a Chrompack Inertsil 5-μm ODS-3 guard column. A mobile-phase gradient was used with a flow rate of 0.4 mL/min in which mobile phase A consisted of 5 mM hexylamine (Aldrich) in Nanopure water adjusted to pH 6.3 with acetic acid (Merck) and mobile phase B consisted of 90% methanol (Biosolve)/10% 10 mM ammonium-acetate (Aldrich) adjusted to pH 8.5 with ammonia (Merck). A mobile-phase gradient was used starting at 100% A for 2 min, followed by a linear gradient from 100 to 80% A in 4 min, followed by a linear gradient from 80 to 69% A in 11 min, followed by a linear gradient from 69 to 40% A in 19 min, followed by a linear gradient from 40 to 0% A in 5 min, and finally 10 min at 0% A. The flow was reduced to 100 μL/min prior to MS detection using a T-split and a restriction column. The column temperature was maintained at 30 °C, and the injection volume was 10 μL. Mass detection was carried out using electrospray ionization in the negative mode using the following conditions: ESI spray voltage 3−4 kV, heated capillary 250 °C, sheath gas 20, auxiliary gas 0, full-scan range m/z 150−1200, number of microscans 3, maximum injection time 300 ms. The system was tuned using a stock solution of ATP (c ~10 μg/mL) in methanol/water (1:3 v/v).

RESULTS AND DISCUSSION

Method Development. An IP-LC-ESI-MS method was developed using a reversed-phase LC C18 column (Inertsil ODS-3) and a mobile-phase gradient from water containing 5 mM hexylamine (mobile phase A) to methanol/10 mM NH_4Ac (9:1 v/v) (mobile

(10) Muller, C.; Schafer, P.; Strotzel, M.; Vogt, S.; Weinmann, W. *J. Chromatogr., B* **2002**, *773*, 47−52.
(11) Hsieh, Y.; Chintala, M.; Mei, H.; Agans, J.; Brisson, J.-M.; Ng, K. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2481−2487.
(12) Hartmans, S.; Smits, J. P.; van der Werf, M. J.; Volkering, F.; de Bont, J. A. M. *Appl. Environ. Microbiol.* **1989**, *55*, 2850−2855.
(13) Tribe, D. E. U.S. Patent 4,681,852, 1983.
(14) Pieterse, B.; Jellema, R. H.; van der Werf, M. J. *J. Microbiol. Methods* **2006**, *64*, 207−216.
(15) Ruijter, G. J. G.; Visser, J. *J. Microbiol. Methods* **1996**, *25*, 295−302.

phase B). Alkylamines were shown to be promising ion-pair agents for acidic analytes,[16,17] especially compared to established ion-pairing agents such as tetraalkylammonium salts which are incompatible with LC/MS.[18] In principle, several alkylamines can be used as the ion-pair agent, but larger, less volatile alkylamines are preferred as they retain more strongly acidic, polar compounds.[16,17,19] Therefore, hexylamine was chosen in this method. It was observed that the amount of hexylamine could be minimized to 5 mM while still obtaining good retention of acidic, polar compounds and minimal contamination in the MS source. It was essential that the pH of mobile phase A was optimized in order to obtain good retention and peak shapes.[7] A pH of 6.3 was found to be the optimum for the various classes of metabolites, especially for ion-pair formation of hexylamine with nucleotides. Furthermore, mobile phase B with a high pH (pH 8.5) provided good results for metabolites eluting at higher retention times. The same was true for mobile phase B with hexylamine (pH 8.5), thereby indicating that the pH of mobile phase B is important rather than the presence of an ion-pair reagent.

It was concluded that a gradient from 100% mobile phase A (5 mM hexylamine in water adjusted to pH 6.3 using acetic acid) to 100% mobile phase B (90% methanol/10% 10 mM NH_4Ac adjusted to pH 8.5 using ammonia) resulted in the best separation and peak shapes for nucleotides and coenzyme A esters (see Figures 1–3). Note that the solvent gradient comprises both an ion-pair gradient, i.e., from 5 to 0 mM, and a pH gradient, i.e., from pH 6.3 to pH 8.5. This combination of two gradients leads to separation of very polar and acidic metabolites such as nucleotides via an ion-pair mechanism and more nonpolar compounds such as CoA esters via a reversed-phase mechanism (see Figures 1–3). This is difficult if not impossible to achieve using standard reversed-phase, normal-phase, cation/anion exchange or mixed mode, i.e., combination of reversed-phase and cation/anion exchange columns; see, for example, Figure 4. No other additives besides the ion-pair agent are necessary to improve the peak shape or sensitivity.[7,16,20] The amount of ion-pair agent was significantly lower compared to previous studies, especially taking into account the column dimension used in this study.[17]

The optimal MS response was obtained with ESI in negative mode providing mainly $[M - H]^-$ ions. As a result, no interference by hexylamine was observed as hexylamine is only visible in positive ionization mode. Furthermore, no significant adduct formation was observed in negative ionization mode with the LC method described above, thereby facilitating both quantification and identification.

Ion Suppression. Ion suppression in LC/MS may occur when coeluting compounds suppress the ionization of the metabolites to be determined in the mass spectrometer. Ion suppression has a negative effect on the robustness and repeatability of the LC/MS method and makes quantification of metabolites troublesome. Changes in the mobile-phase gradient or selective sample cleanup are the preferred methods to prevent ion suppression by separating the analyte(s) of interest and the compound(s) causing ion suppression. However, both options are not viable in our case, because sample cleanup will result in loss of some metabolites, which is in contradiction with the comprehensive requirement for this analytical method, and moreover, the mobile-phase gradient was very critical (cf. above). Therefore, we studied whether ion suppression played a significant role during the IP-LC-ESI-MS analysis anyway.

To this end, a metabolite, e.g., ATP, was added at a constant rate to the mobile phase eluting from the HPLC system prior to MS detection via a T-piece.[9–11] This resulted in a constant "background" signal of, for example, ATP in the MS. Subsequently, a cell extract of *B. subtilis* was injected, and if any component in the cell extract would suppress the ionization of ATP, this would result in a drop in the constant ATP background signal (data not shown). This procedure was repeated for other metabolites that showed significant retention with the IP-LC-ESI-MS method, i.e., AMP, ADP, UDP glucose, CoA, and acetyl CoA. No significant ion suppression, i.e., less than 10% reduction of the metabolite signal, could be observed for various metabolites in the part of the chromatogram where these metabolites eluted (t_r > 5 min).

It should be stressed that several components are generally present at higher concentrations in extracts of microorganisms depending on the type of medium, buffer, salt, etc. However, these types of components, suck as phosphate, sulfate, and the buffer used for quenching the cells, such as HEPES, are not significantly retained in the IP-LC-ESI-MS method and, therefore, elute at the start of the gradient. As a result, ion suppression may occur in this part of the chromatogram, but this is not expected to interfere with the ionization of metabolites that are retained with the IP-LC-ESI-MS method.

Hence, it is concluded that ion suppression does not play a significant role in the analysis of those classes of metabolites that are specifically analyzed with the IP-LC-ESI-MS method.

Application Range. The applicability of the IP-LC-ESI-MS method is demonstrated in Figures 1–3. Figure 1 shows the selected ion chromatograms of four different nucleosides and their mono-, di-, and triphosphates. It can be clearly seen that all 16 compounds can be detected with good peak shapes and can be separated from each other based on retention time and m/z value. Figure 2 emphasizes the applicability for guanosine and its nucleotides, showing that nucleoside tetraphosphates and deoxy- and cyclic nucleotides can also be analyzed and separated from each other. Furthermore, a large number of coenzyme A esters, from CoA to decanoyl CoA, could also be separated from each other using the IP-LC-ESI-MS method (see Figure 3). The IP-LC-ESI-MS method was found to be suitable for at least 68 nucleosides and nucleotides and 24 coenzyme A esters that were commercially available, including biologically important nucleotides, such as FAD, FMN, NAD^+, NADH, $NADP^+$, and NADPH. Furthermore, other classes of metabolites, such as sugar nucleotides, e.g., UDP-glucose, sugar bisphosphates, e.g., fructose 1,6-bisphosphate, aromatic amino acids, organic acids, alkyl phosphates, and some purines/pyrimidines, e.g., folic acid, can also be analyzed adequately with the IP-LC-ESI-MS method. The current database of compounds that can be adequately analyzed by the IP-LC-ESI-

(16) Tuytten, R.; Lemiere, F.; van Dongen, W.; Esmans E. L.; Slegers, H. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1205–1215.
(17) Qian, T.; Cai, Z.; Yang, M. S. *Anal. Biochem.* **2004**, *325*, 77–84.
(18) Witters, E.; van Dongen, W.; Esmans, E.l.; van Onckelen, H. A. *J. Chromatogr., B* **1997**, *694*, 55–63.
(19) Verheij, E. R.; Tjaden, U. R.; Niessen, W. M. A.; van der Greef, J. *J. Chromatogr.* **1991**, *554*, 339–349.
(20) Appfel, A.; Chakel, J. A.; Fischer, S.; Lichtenwalter K.; Hancock, W. S. *J. Chromatogr., A* **1997**, *777*, 3–21.

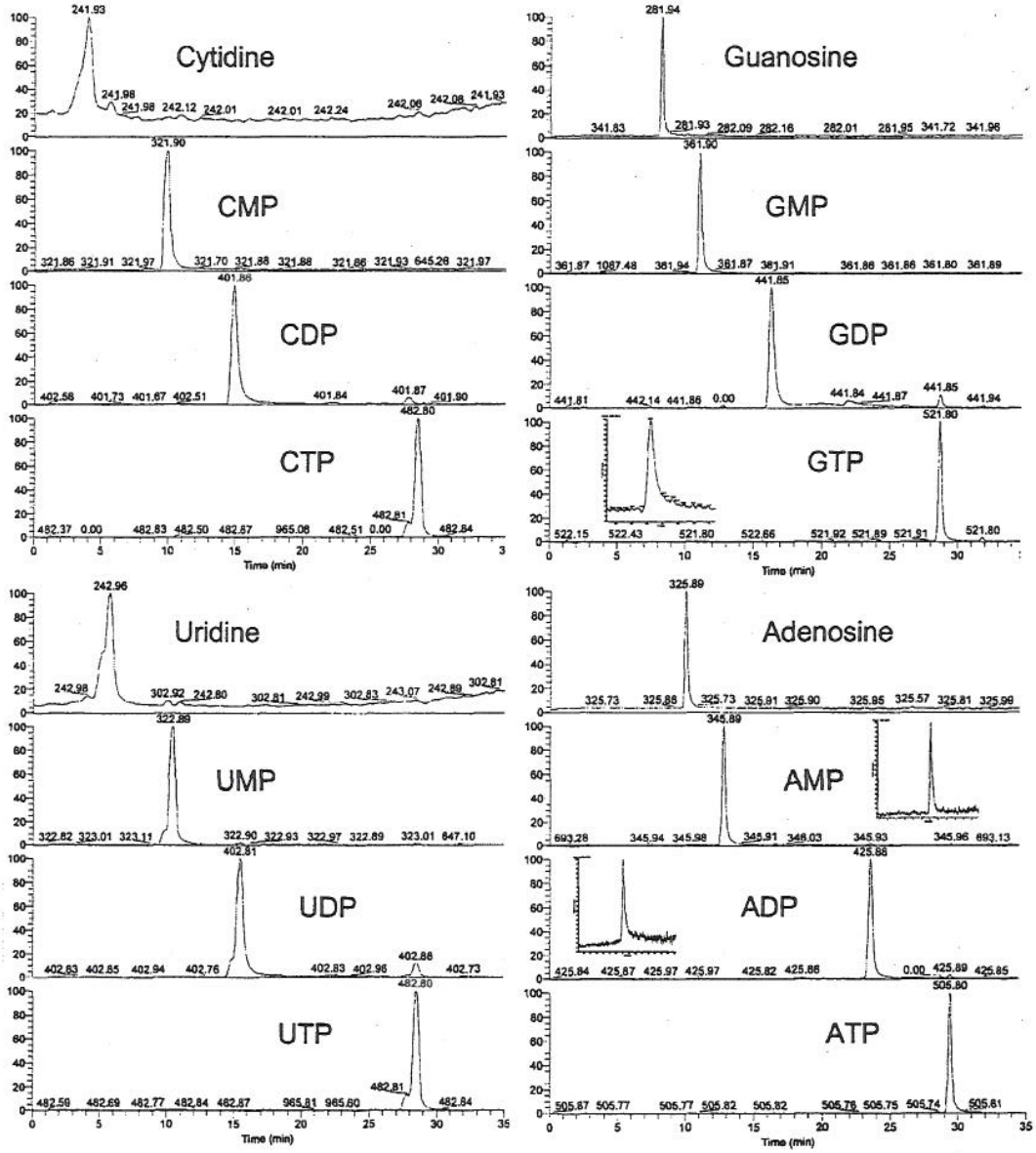

Figure 1. Selected reconstructed ion LC/MS chromatograms of standard solutions of several nucleotides ($c \sim 10$ μg/mL) and insets of low-concentration standard solutions ($c \sim 0.05$ μg/mL) for some nucleotides using the IP-LC-ESI-MS method.

MS method consists of more than 150 commercially available metabolites. It should be noted that small polar metabolites, such as amino acids, sugars, and small organic acids, do not have significant retention with the IP-LC-ESI-MS method. However, these metabolites can be analyzed by GC/MS[4] thereby showing the added value of the IP-LC-ESI-MS and the GC/MS method.

In summary, the IP-LC-ESI-MS method can be applied to detect simultaneously a large number of key classes of metabolites in microorganisms, such as nucleotides, coenzyme A esters, sugar bisphosphates, and sugar nucleotides, with excellent peak shapes and separation efficiency.

Analytical Performance. To apply the developed IP-LC-ESI-MS method for metabolomics studies of large numbers of extracts of biological samples from different biological origins, the analytical performance of the method should be satisfactory with respect to sensitivity, linearity, repeatability, and reproducibility.

Sensitivity and Linearity. The limit of detection (LOD) and linearity of the IP-LC-ESI-MS method were determined for several metabolites by analyzing calibration solutions with concentrations ranging from approximately 0.05 to 10 μg/mL, i.e., corresponding to an absolute amount of 0.5−100 ng at an injection volume of 10 μL in full-scan MS mode. From the calibration curves, the linearity (R^2) and LOD were determined (see Table 1). The linearity was generally higher than 0.995, while the LOD, i.e., corresponding to a concentration with S/N ~3, varied between 0.01 and 0.2 μg/mL. In general, a somewhat higher LOD was observed for coenzyme A esters compared to nucleotides and sugar nucleotides. In Figures 1 and 3, reconstructed ion LC/MS chromato-

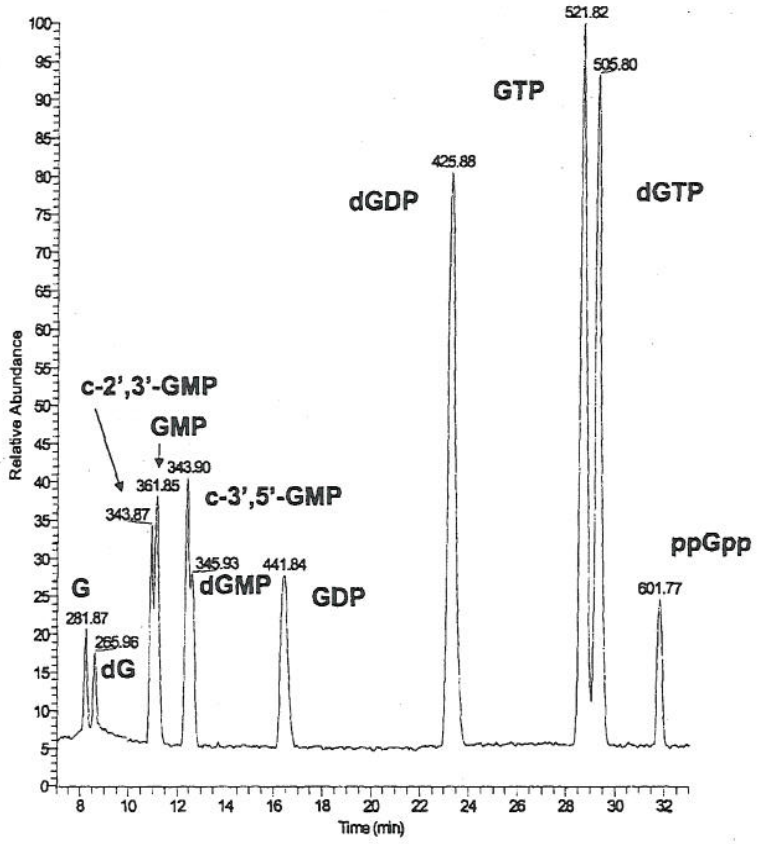

Figure 2. Selected reconstructed ion LC/MS chromatograms of standard solutions of several guanosine nucleotides ($c \sim 10\ \mu g/mL$) using the IP-LC-ESI-MS method.

grams are inset of calibration solutions ($c \sim 0.05\ \mu g/mL$) of AMP, ADP, GTP, butyryl CoA, and octanoyl CoA to show representative peaks obtained for low concentrations.

Similar experiments were carried out with quality control standards, consisting of, for example, isotopically labeled metabolites, spiked to complex cell extracts containing cell medium and buffer (see Table 2). These labeled metabolites have the advantage that they are not naturally occurring in any microorganism and can be spiked at various concentrations to cell extracts to determine the analytical performance of the method using representative samples. The linearity and limits of detection of these metabolites in cell extracts are in close agreement with the values obtained for standard solutions of metabolites shown in Table 1. This indicates that the complex cell extract has no significant effect on the linearity and limits of detection of metabolites.

Repeatability and Reproducibility. The repeatability and reproducibility of the IP-LC-ESI-MS method is studied by determining the variation in retention time and (relative) MS response when analyzing larger numbers of samples of extracts from microorganisms.

The standard deviation (SD) in the retention time is ≤ 0.07 min and the relative standard deviation (RSD) in the peak area is $\leq 10\%$ for several intracellular metabolites and quality control standards present in an extract of *B. subtilis* analyzed repeatedly (total analysis time ~12 h). This demonstrates the good repeatability of the IP-LC-ESI-MS method (Table 3).

It should be noted that when analyzing large numbers of samples with IP-LC-ESI-MS, both the retention time (e.g., $\Delta t_r \sim 0.5$ min after 3–4 days) due to, for example, small changes in the state of the column, and the MS response due to contamination of the MS source might slightly change with time. The same is observed for standard reversed-phase LC/MS methods of complex samples. To minimize contamination of the MS source, the first 5 min of the analytical run, i.e., the part where all nonretained compounds elute, is sent to waste when large series of biological extracts are analyzed.

However, if one is able to correct for these changes in retention times and MS response using data preprocessing software packages,[21] it is still possible to apply the IP-LC-ESI-MS to large numbers of samples without losing important information. In our case, deoxyfluridine is used as a MS response marker, and all quality control standards in Table 2 are used for correction of shifts in the retention time using homemade software.[22,23]

(21) Davidov, E.; Clish, C. B.; Oresic, M.; Meys, M.; Stochaj, W.; Snell, P.; Lavine, G.; Londo, T. R.; Adourian, A.; Zhang, X.; Johnston, M.; Morell, N.; Marple, E. W.; Plasterer, T. N.; Neumann, E.; Verheij, E.; Vogels, J. T. W. E.; Havekes, L. M.; van der Greef, J.; Naylor, S. *Omics* **2004**, *8*, 267–288.
(22) van der Greef, J.; Vogels, J. T. W. E.; Wulfert, F.; Tas, A. C. U.S. Patent 2004267459, 2004.

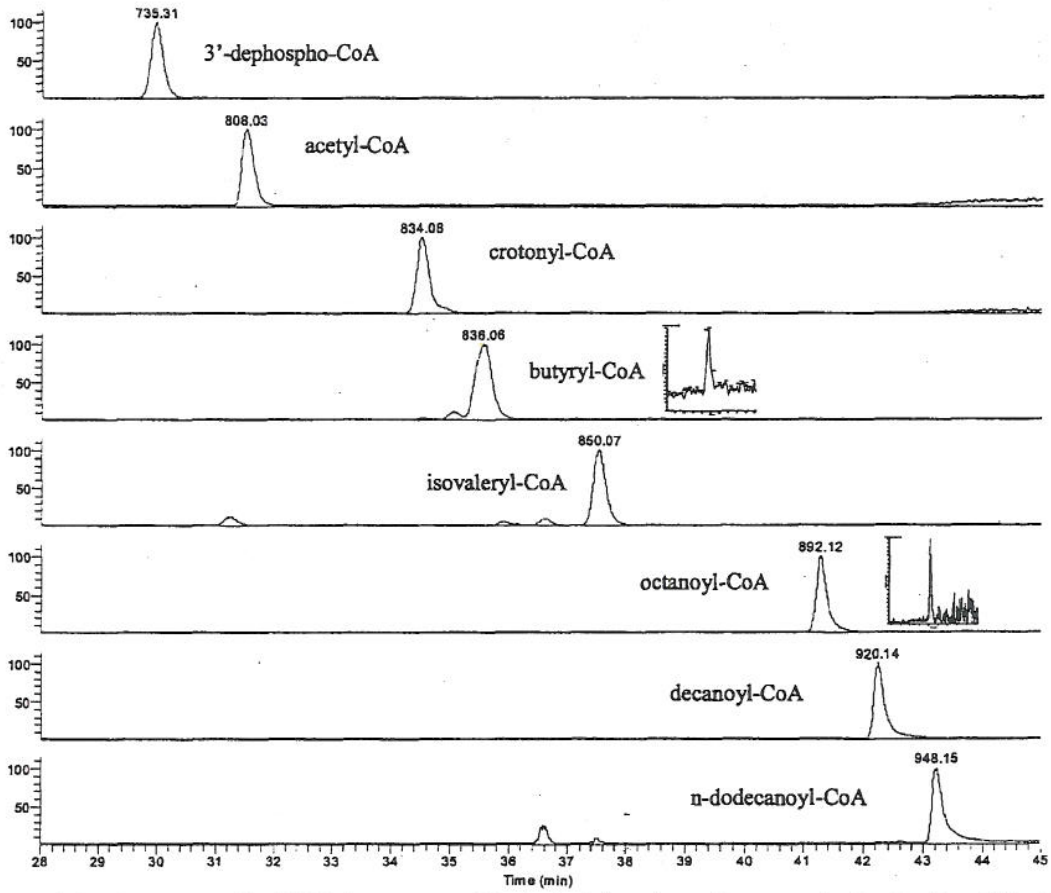

Figure 3. Selected reconstructed ion LC/MS chromatograms of standard solutions of several coenzyme A esters ($c \sim 10$ μg/mL) and insets of low-concentration standard solutions ($c \sim 0.05$ μg/mL) for some coenzyme A esters using the IP-LC-ESI-MS method.

A demonstration of the robustness of the IP-LC-ESI-MS method is shown in Table 4 where the RSD of the relative peak area, i.e., the peak area corrected for the change of the MS response via the peak area of deoxyfluridine, is calculated for some selected intracellular metabolites, i.e., nucleotides, coenzyme A esters, and sugar bisphosphates, present in both standard solutions and cell extracts of *E. coli* and analyzed repeatedly between a series of in total 80 measurements of cell extracts (total analysis time ~85 h). For both standard solutions and cell extracts, the RSD of the metabolites shown in Table 4 was better than 10%. From these results, the robustness of the IP-LC-ESI-MS method is clear as well as its applicability to different microorganisms, i.e., *B. subtilis* (Table 3) and *E. coli* (Table 4).

In metabolomics studies, it is likely that one would want to compare the concentration of metabolites in samples acquired in one analytical sequence with concentrations found in separate analytical sequences. Therefore, the reproducibility, i.e., the precision of quantification in sequences of samples acquired at various moments in time, the so-called interbatch precision, was determined next to the repeatability within one sequence, the so-called intrabatch precision (see Table 5). Quantification was carried out using response factors in a database as determined from standard solutions at the start and end of the experiments. The interbatch precision was determined from 3 different batches of 10 experiments carried out within a period of 6 months. It can be seen that both the intrabatch precision (≤7%) and the interbatch precision (≤12%) were good, thereby demonstrating that quantification of metabolites in cell extracts with good precision using response factors stored in a database is possible. This enables the quantitative comparison of the metabolite profile measured within larger studies conducted at various points in time.

It can be concluded that the method was highly reproducible and robust, as the method was applied for the analysis of many sample series for various microorganisms with good performance, which was assessed by spiking labeled quality standards, such as AMP ($^{13}C/^{15}N$), GMP ($^{13}C/^{15}N$), and GTP ($^{13}C/^{15}N$), to the samples for control of the overall analytical performance.

Application. The final aspect of the IP-LC-ESI-MS method is its use in analyzing as many metabolites as possible, especially compared to standard reversed-phase LC/MS methods. Figures 4 shows full-scan LC/MS chromatograms of an extract of *E. coli* analyzed by a standard reversed-phase LC/MS method (Figure 4C) and the IP-LC-MS method (Figure 4B). The added value for using an ion-pair LC/MS method is clearly visible.

(23) Vogels, J. T. W. E.; Tas, A. C.; Venekamp, J.; van der Greef, J. *J. Chemom.* 1996, *10*, 425–438.

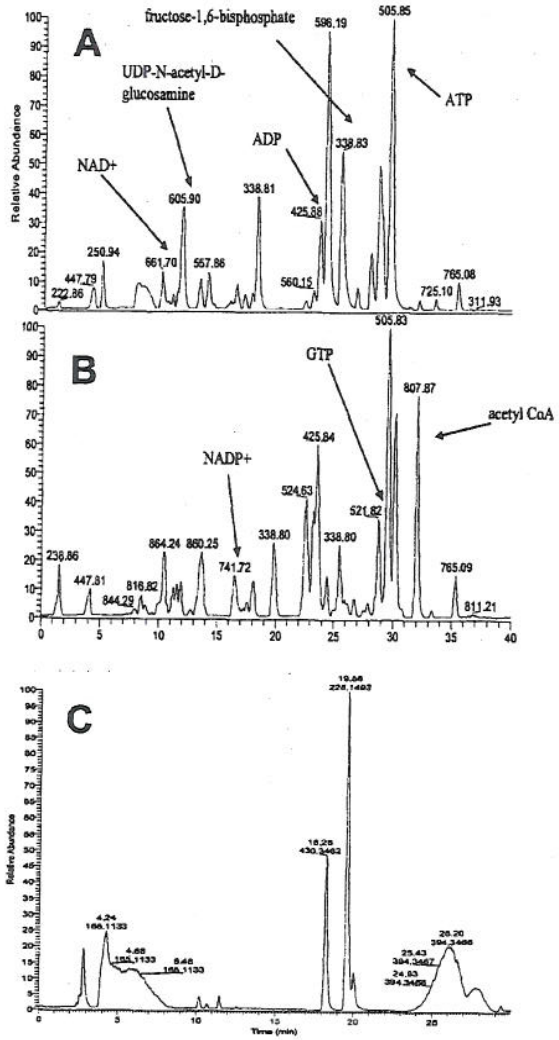

Figure 4. (A) Full-scan LC/MS chromatogram of an *L. plantarum* cell extracted and analyzed by IP-LC-ESI-MS, (B) full-scan LC/MS chromatogram of an *E. coli* cell extract analyzed by IP-LC-ESI-MS, and (C) full-scan LC/MS chromatogram of an *E. coli* cell extract analyzed by standard reversed-phase LC-ESI-MS.

Furthermore, it is important that the developed IP-LC-ESI-MS method can be applied to various microorganisms. Panels A and B in Figure 4 show full-scan LC/MS chromatograms of extracts from *L. plantarum* and *E. coli*, respectively. For both microorganisms, many peaks can be observed with retention times being spread over the whole LC/MS chromatogram. Some metabolites that could be identified in the extracts are indicated. The pattern of peaks between the two microorganisms showed large differences. The dominating peaks in the extract of *E. coli* were identified as $NADP^+$, ATP, and acetyl CoA, while for the extract of *L. plantarum* the main peaks were identified as NAD^+, ADP, fructose 1,6-bisphosphate, and ATP. From the peak patterns and peak shapes, it can be concluded that the IP-LC-ESI-MS method can be applied to highly different microorganisms.

The full-scan IP-LC-ESI-MS chromatogram of *E. coli* is dominated by some high-abundant peaks, some of which are indicated

Table 1. Retention Time (t_r), Quantification Mass (m/z), Concentration Range (μg/mL), Limit of Detection (LOD), and Linearity (R^2) Obtained from Calibration Curves of Standard Solutions of Various Metabolites

	t_r (min)	quantifn mass (m/z)	concn range (μg/mL)[a]	LOD (μg/mL)[b]	R^2 ($n = 6$)
adenosine	10.0	266	0.05−11	0.01	0.9982
AMP	12.8	346	0.05−10	0.02	0.9998
ADP	23.6	426	0.05−10	0.02	0.9998
ATP	29.4	506	0.05−9	0.02	0.9994
guanosine	8.2	282	0.06−12	0.01	0.9962
GMP	11.0	362	0.06−11	0.06	0.9998
GDP	16.3	442	0.05−10	0.02	0.9980
GTP	28.7	522	0.05−11	0.03	0.9954
dG	8.6	266	0.05−10	0.01	0.9998
dGMP	12.4	346	0.04−9	0.03	0.9994
dGDP	23.3	426	0.06−12	0.02	0.9998
dGTP	29.3	506	0.05−9	0.03	0.9952
c-2′,3′-GMP	10.9	344	0.05−10	0.02	0.9992
NADH	10.1	664	0.06−12	0.06	0.9998
NADPH	16.5	742	0.05−10	0.05	0.9998
UDP-glucose	11.9	565	0.05−11	0.01	0.9998
UDP-*N*-acetyl-D-glucosamine	11.8	606	0.06−11	0.01	0.9998
ADP-glucose	13.8	588	0.05−11	0.01	0.9998
UDP-glucuronate	16.6	579	0.06−11	0.01	0.9998
fructose 1,6-bisphosphate	25.4	339	0.05−10	0.01	0.9998
CoA	30.4	766	0.05−10	0.10	0.9994
dephosphoCoA	30.0	686	0.06−12	0.12	0.9954
butyryl CoA	35.6	836	0.05−11	0.01	0.9998
malonyl CoA	30.3	852	0.07−13	0.07	0.9998
octanoyl CoA	41.3	892	0.06−12	0.03	0.9964
decanoyl CoA	42.2	920	0.05−10	0.21	0.9948

[a] Concentration range of standards used for establishment of calibration curve. [b] Extrapolated from calibration curves acquired in full-scan MS mode; LOD corresponded to concentrations with S/N ~3.

Table 2. Retention Time (t_r), Quantification Mass (m/z), Concentration Range (μg/mL), Limit of Detection (LOD), and Linearity (R^2) for Various Metabolites, Used for Quality Control, Spiked to a Cell Extract of *E. coli* Containing Growth Medium and Quench Buffer at Various Concentrations

	t_r (min)	quantifn mass (m/z)	concn range (μg/mL)[a]	LOD (μg/mL)[b]	R^2 ($n = 6$)
deoxyfluridine	10.6	245	0.1−11	0.05	0.9998
AMP ($^{13}C/^{15}N$)	13.0	361	0.1−14	0.03	0.9974
GMP ($^{13}C/^{15}N$)	11.3	377	0.1−14	0.03	0.9998
toluenesulfonic acid	20.1	171	0.1−10	0.01	0.9998
GTP ($^{13}C/^{15}N$)	28.7	537	0.1−12	0.03	0.9960
cholic acid-d_4	42.2	411	0.1−10	0.1	0.9986

[a] Concentration range at which metabolites were spiked to cell extract. [b] Extrapolated from calibration curves acquired in full-scan MS mode; LOD corresponded to concentrations with S/N ~3.

in Figure 4B. However, further investigation of the chromatogram revealed the presence of at least 100 different peaks, ranging widely in peak intensity. This is further demonstrated in Figure 5, where several selected reconstructed ion chromatograms are shown for different peaks eluting around 15−20 min. This demonstrates that the full-scan IP-LC-ESI-MS chromatogram is dominated by the high-abundant metabolites, but many more metabolites, with low abundance, are present in the chromato-

Table 3. Relative Standard Deviation (RSD) of the Peak Area and Standard Deviation (SD) of the Retention Time (t_r) of Metabolites in an Aliquot of a *B. subtilis* Extract and Quality Control Standards Spiked (c = 10 μg/mL) to a Cell Extract of *B. subtilis* Determined from Repeated Analysis

	RSD peak area (n = 12) (%)	SD t_r (n = 12) (min)
Quality Control Standards (c = 10 μg/mL)		
deoxyfluridine	6	0.02
AMP ($^{13}C/^{15}N$)	7	0.03
GMP ($^{13}C/^{15}N$)	6	0.03
toluenesulfonic acid	7	0.04
GTP ($^{13}C/^{15}N$)	6	0.03
cholic acid-d_4	7	0.02
Metabolites		
AMP	3	0.06
ADP	6	0.07
ATP	6	0.03
GDP	5	0.07
GTP	7	0.05
NAD^+	10	0.03
$NADP^+$	5	0.03
UDP-glucose	7	0.05
CoA	9	0.07
acetyl CoA	8	0.07
isovaleryl CoA	5	0.05

Table 4. Relative Standard Deviation (RSD) of the Peak Area of Several Metabolites Analyzed Repeatedly in Standard Solutions and in an Aliquot of the Same *E. coli* Extract between a Series of 80 Measurements of Extracts of Microorganisms[a]

component	RSD peak area (%) standard solutions (n = 8)	RSD peak area (%) biological extracts (n = 6)
fructose 1,6-bisphosphate	9	8
UDP-glucose	6	9
NAD^+	9	6
$NADP^+$	8	7
AMP	6	4
ADP	5	7
ATP	5	7
acetyl CoA	8	9

[a] Total analysis time ~85 h.

Table 5. Repeatability[a] and Reproducibility[a] of the Quantification of Five Metabolites in *B. subtilis* Extracts Analyzed by Ion-Pair LC/MS

	metabolite				
	GMP ($^{13}C/^{15}N$)	AMP ($^{13}C/^{15}N$)	GTP ($^{13}C/^{15}N$)	cholic acid-d_4	toluene-sulfonic acid
repeatability[b] RSD (%)	5	7	6	6	6
reproducibility[c] RSD (%)	9	12	6	10	10

[a] Repeatability (intrabatch precision) and reproducibility (interbatch precision) were calculated according to analysis of variance calculation (one-way ANOVA).[24] [b] One batch consisted of 10 measurements. [c] Three batches were analyzed within a period of 6 months.

Table 6. Energy Charge (EC) Obtained by IP-LC-MS of *B. subtilis* Extracts Obtained from Six Independent Fermentations and Harvested at Two Different Growth Stages

	optical density at harvesting	AMP (μM)	ADP (μM)	ATP (μM)	EC[a]	average ±SD
1	mid logarithmic (8.5)	86	86	322	0.74	0.79 ± 0.04
2	mid logarithmic (7.8)	78	117	518	0.81	
3	mid logarithmic (7.8)	37	43	241	0.82	
4	early stationair (15)	201	91	496	0.69	0.68 ± 0.01
5	early stationair (13.5)	77	28	179	0.68	
6	early stationair (15)	56	21	124	0.67	

[a] Energy charge was calculated as ([ATP] + 0.5[ADP])/([AMP] + [ADP] + [ATP]).

Table 7. MS^n Conditions for Separation of AMP, ADP, and ATP from DGMP, DGDP, and DGTP

	parent (m/z)	characteristic MS^n transition (m/z)
AMP	346	346 → **211** ($\Delta m/z$ = 135), loss of adenine
dGMP	346	346 → **195** ($\Delta m/z$ = 151), loss of guanine
ADP	426	426 → **291** ($\Delta m/z$ = 135), loss of adenine
dGDP	426	426 → **275** ($\Delta m/z$ = 151), loss of guanine
ATP	506	506 → 408 ($\Delta m/z$ = 98), loss of H_3PO_4
		408 → **273** ($\Delta m/z$ = 135), loss of adenine
dGTP	506	506 → 408 ($\Delta m/z$ = 98), loss of H_3PO_4
		408 → **257** ($\Delta m/z$ = 151), loss of guanine

grams obtained by IP-LC-ESI-MS, even in regions where upon visual inspection no peaks are observed in the full-scan trace. This is an important prerequisite in metabolomics studies where the aim is to analyze many metabolites in a wide range of concentrations. Together with GC/MS[4], the IP-LC-ESI-MS method is capable of analyzing quantitatively a large amount of different classes of metabolites ranging widely in concentrations and can therefore be ideally used for metabolomics studies.[24]

An additional parameter that can be derived from the data obtained by the IP-LC-ESI-MS method is the so-called energy charge (EC).[25] The energy charge (EC = ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]), all in moles per liter) involves the determination of several metabolites with the highest turnover in a cell; e.g., ATP has a half-life of less than 0.1 s.[25] The determination of the energy charge of different samples of cells obtained from fermentation at different growth stages can be used to validate the sample workup and analytical protocols. Theory states that exponentially growing cells have an EC of ~0.8 while an EC of <0.5 is indicative of dead cells.[26]

From the IP-LC-ESI-MS measurement, the concentrations of AMP, ADP, and ATP can be determined and thus the energy charge can be calculated. This has been carried out for fermentations of *B. subtilis* extracts harvested at two different growth stages (see Table 6). For each growth stage, biological triplicates were analyzed. An energy charge value of 0.68 ± 0.01 was obtained for

(24) Smilde, A. K.; van der Werf, M. J.; Bijlsma, S.; van der Werff-van der Vat, B. J. C.; Jellema, R. H. *Anal. Chem.* **2005**, *77*, 6729–6736.
(25) Walsch, K.; Koshland, D. E. *J. Biol. Chem.* **1984**, *259*, 9646–9654.
(26) Chapman, A. G.; Fall, L.; Atkinson, D. E. *J. Bacteriol.* **1971**, *108*, 1072–1086.

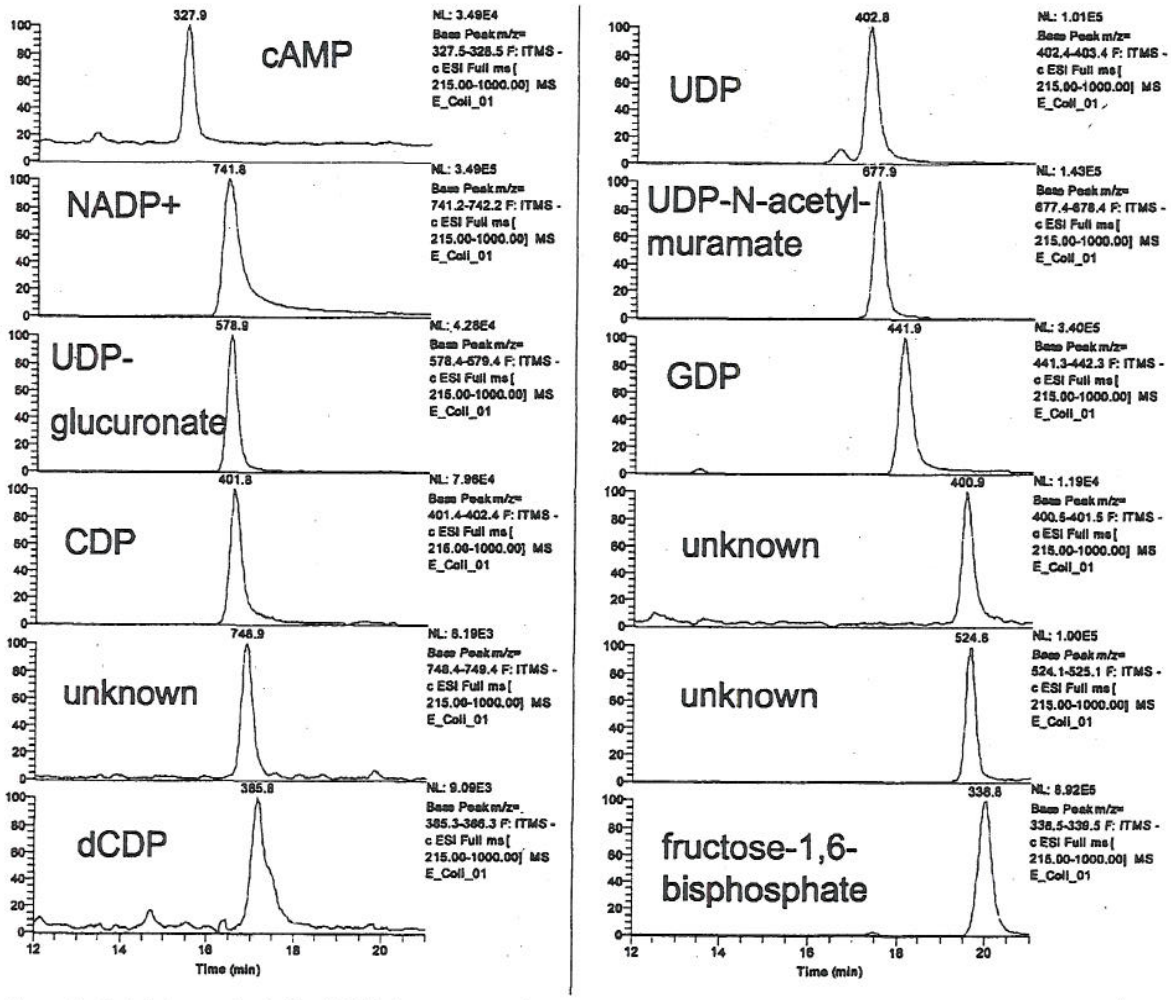

Figure 5. Selected reconstructed ion LC/MS chromatograms from the full-scan chromatogram shown in Figure 4B in the region t_r ~15–20 min.

early stationary phase and an energy charge of 0.79 ± 0.04 for logarithmically growing cells.

A complicating factor in the determination of the energy charge is the fact that deoxy-GMP, deoxy-GDP, and deoxy-GTP have the same elemental composition and thus molecular mass as AMP, ADP, and ATP (see Table 1). Furthermore, these metabolites have very similar retention times as can be seen in Table 1 and are, therefore, difficult to distinguish. To determine the exact value for the energy charge, the concentrations of AMP, ADP, and ATP should be corrected for the contributions of dGMP, dGDP, and dGTP. Therefore, a LC/MS^n method was developed using the IP-LC-ESI-MS method to distinguish AMP, ADP, and ATP from dGMP, dGDP, and dGTP by identifying specific fragments that are characteristic for the individual metabolites (see Table 7). For AMP, dGMP, ADP, and dGDP MS^2, detection is sufficient while for ATP and dGTP MS^3 detection is necessary to obtain specific fragments. It can be seen that the two types of nucleotides can be separated after loss of the purine unit, i.e., adenine ($\Delta m/z$ 135) and guanine ($\Delta m/z$ 151). MS^2 and MS^3 detection was carried out next to full-scan acquisition in the same run.

It can be concluded that the IP-LC-ESI-MS method can be used to determine the energy charge as an overall quality control parameter with respect to sample workup and analytical protocols.

CONCLUSION

A robust and inert IP-LC-ESI-MS method was developed that allows for the first time the simultaneous quantitative analysis of several key classes of metabolites, such as nucleotides, coenzyme A esters, sugar nucleotides, and sugar bisphosphates, that are impossible to be analyzed by standard reversed-phase LC/MS methods. The pH of the mobile phases and the use of hexylamine as ion-pair agent are critical parameters for obtaining good separation and peak shapes, especially for acidic metabolites such as nucleotides. Finally, a gradient in pH and ion-pair concentration appeared to be most suitable for the analysis of a wide range of metabolites. With these two gradients next to the solvent gradient, reproducible results were obtained: the IP-LC-ESI-MS method was successfully validated by determining the linearity, sensitivity, repeatability, and reproducibility and eventual ion suppression. The method proved to be reliable for the analysis of highly diverse

microorganisms such as *B. subtilis* and *E. coli*. Furthermore, the IP-LC-ESI-MS method was successfully applied to determine the energy charge, i.e., the energy status of cells. Using LC/MS/MS and LC/MS^3, nucleotides with identical masses but slightly different structures could be distinguished and quantified, e.g., ATP versus deoxy-GTP. The reliable determination of the energy charge emphasizes the potential of the method for the analysis of metabolites with a high turnover.

The developed IP-LC-ESI-MS method is a valuable addition to the analytical platform applied to microbial metabolomics studies with the aim of identifying and quantifying as many metabolites as possible in microbial extracts.

ACKNOWLEDGMENT

The authors thank Roelie Bijl, Machtelt Braaksma, and Karin Overkamp for cultivating the microorganisms and extracting the samples, Roche Vitamins (currently DSM Nutritional Products), DSM Food Specialties, and Friesland Foods for financial support, and Markus Wyss and Werner Bretzel (currently DSM Nutritional Products) for fruitful discussions.

Received for review April 22, 2006. Accepted June 19, 2006.

AC0607616