Anal. Chem. **2005,** *77,* 7255−7264

Controlled Protein Precipitation in Combination with Chip-Based Nanospray Infusion Mass Spectrometry. An Approach for Metabolomics Profiling of Plasma

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Liquid chromatography−mass spectrometry (LC−MS) is a common method for profiling biological samples in metabolomics. However, LC−MS data of metabolomic studies are often affected by high noise levels, retention time shifts, and high variability in signal intensities. With a new chip-based nanoelectrospray source it becomes possible to directly infuse complex biological samples such as plasma without any chromatographic separation beforehand. In combination with highly diluted samples and long data acquisition times, the parallel analysis of hundreds of compounds is now possible. In a proof-of-concept study, 10 human plasma samples from females and males were analyzed with the intention to separate the two groups by their different metabolomes. The reproducibility was so high that statistical analysis of the data could be performed without prior normalization. Two groups of female and male samples were separated by a supervised machine learning algorithm, principal component analysis, and hierarchical clustering. Peaks contributing to the group separation were characterized by accurate mass measurement and MS−MS fragmentation and by spiking experiments. The feasibility of direct sample infusion using the new chip-based nanoelectrospray device opens a new dimension for the rapid parallel analysis of complex biological mixtures.

Metabolomics is the comprehensive analysis of the metabolome, which is defined as the sum of all non-protein small molecules ("metabolites") in a biological sample. As the latest addition to the "omics" family, metabolomics complements transcriptomics and proteomics. However, the metabolome can be considered as the final stage in the chain of events from genes to metabolism, and thus, the metabolic phenotype is the most direct reflection of the current state of a biological system. Consequently, metabolite analysis and metabolomics are likely to become the most relevant contributor to systems biology. In pharmaceutical drug development, metabolomics offers great potential for the identification of biomarkers of disease, drug efficacy, and toxicity.

Due to the complexity and chemical heterogeneity of biological samples, comprehensive metabolomics is a real challenge. Human plasma contains at least 2000 endogenous metabolites in a complex matrix.[1] Unlike transcriptomics and proteomics, which have been established for many years, there is to date no unique strategy toward metabolomics. Today, the targeted analysis of small molecules from biological samples by LC−MS is a routine method. A huge variety of different LC methods have been described in combination with different mass spectrometers, depending on the analytical question asked. All methods have in common the removal of proteins as an essential sample pretreatment step. Protein precipitation with acetonitrile prior to LC−MS analysis is one of the most common methods.[2]

In quantitative applications, it is very important that the protein precipitation does not pull out any analytes of interest.[3] It is known from many radioactive-labeled drug metabolism experiments and quantification based on the radioactivity counting in our laboratory that often only a slow protein precipitation can avoid unwanted sample loss. This becomes particularly important when performing metabolomic profiling of plasma, where hundreds of small molecules have to be analyzed out of samples with high albumin concentration. It is also known that, for quantification, LC conditions have to be carefully selected to avoid coelution of compounds. Unexpected ion suppression effects together with minor retention time shifts can dramatically change the measured ion intensities. Since at least relative quantification is a prerequisite for metabolomic studies, ion suppression effects in combination with retention time shifts become a very important issue. The use of long LC−MS runs to separate 400−900 different compounds without coeluting peaks may require up to 90 min/sample, which is tedious and time-consuming for medium- to high-throughput analysis. Subsequent statistical data analysis requires a sufficient number of samples, which easily leads to several hundred samples per experiment. The resulting time frame of several days or even weeks for the analysis of a single batch of samples directs the attention toward system stability, to minimize inevitable retention time shifts, which in turn cause variation in ion suppression. In addition, short run times (10−30 min) are preferred for metabo-

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10.1021/ac0508604 CCC: $30.25
Published on Web 10/14/2005

lomics, which places additional strain on the ion suppression effects.

For fast chromatography of samples as complex as plasma, coelution of compounds is the norm, resulting in a poor overall reproducibility. On the basis of these constraints, we also experienced high background noise in the LC−MS data, which hampered statistical analysis in several of our own metabolomics studies. In contrast, the corresponding GC−MS data of the same sample sets were highly reproducible due to the different ionization process that is less susceptible to ion suppression. GC/MS however, has its own restrictions since the analytes have to be volatile and most biomolecules require chemical derivatization. Automated nanoelectrospray infusion with a new chip-based electrospray system (The NanoMate, Advion BioSciences) is an alternative approach that has been successfully applied in different application areas.[5−15] Wickremsinhe et al. have shown that liquid−liquid extraction from plasma samples followed by infusion-MS/MS analyses provides greater sensitivity while using 80 times less plasma when compared to standard LC−MS/MS.[16] Extended linear dynamic range for their assay using the chip-based nanoelectrospray was demonstrated. Several groups have presented promising results here. Hao et al. have shown that it is feasible to infuse diluted serum for a proteomics approach.[20] They used a very fast method of only 1-min data acquisition time, and in so doing, they have limited their sensitivity. Scholz et al. have already shown with plant extracts that it is possible to do metabolomics by infusion mass spectrometry,[21] while Dethy et al. showed the feasibility of drug bioanalytics in plasma using chip infusion.[16,22] The increased buffer and salt tolerance of nanospray compared to regular higher flow electrospray was investigated by Karas et al. and others.[23−26] However, infusion also has its disadvantages as there may be vial background effects or the loss of structural isomer information due to the absence of chromatography. Despite these issues, the ease of use, sensitivity, and outstanding reproducibility make chip-based infusion mass spectrometry an attractive tool for expression profiling.

High-resolution mass spectrometry in combination with infusion offers several advantages that might be very useful in analysis of a given metabolome. With long data acquisition times, analytical sensitivity and dynamic range can be increased, making it possible to simultaneously quantify hundreds of compounds. Furthermore, under highly diluted conditions, the ion suppression effect seems to be reduced and the reproducibility is exceedingly improved too. These benefits become very important when expression profiling of plasma samples has to be done. In the following discussion, these aspects will be demonstrated. The final test of any new approach is of course a real-life metabolome analysis within a proof-of-concept study. Expression profiling of male and female plasma in combination with statistical analysis will be used to demonstrate the differences between LC−MS and the infusion-MS approach.

EXPERIMENTAL SECTION

Materials. Vials (Safe-Lock Tubes, 1.5 mL) were produced by Eppendorf (Hamburg, Germany), while the polypropylene V-bottomed 96-well plate was purchased as prewashed plates by Advion (Ithaca, NY). Progesterone and formic acid was produced by Fluka (Buchs, Switzerland), while the water (Lichrosolv) and acentonitrile (Lichrosolv hypergrade) came from Merk (Darmstadt, Germany). Horse myoglobin digest standard (23 peptides) was produced by Michrom BioResources, Inc. A centrifuge (model 5808) from Eppendorf was used for all experiments.

Human Plasma. Human plasma from 10 healthy female and male volunteers aged 32−55 years was used. Heparin was used as anticoagulant for all samples. After arrival in our laboratory the frozen plasma samples were thawed, split into 100 μL aliquots, and frozen at −80 °C. For analysis, the aliquot was thawed at 4 °C and later allowed to warm to room temperature.

Method a: The samples were centrifuged for 3 min at 2100*g*, and 4 μL were transferred into a new vial filled with 1 mL of water/acetonitrile (1:1) and 0.2% formic acid. The vial was sealed and kept at 4 °C for 2 h. Then the diluted plasma was centrifuged for 30 min at 20000*g*. From the supernatant, 100 μL was transferred into a 96-well plate. After all samples had been processed in this way, the well plate was heat sealed using aluminum foil. The 96-well plate was kept at 4 °C for additional 16 h. Before measurement of the samples, the well plate was centrifuged at 2100*g* for 10 min. During the mass spectrometric analysis, the well plate was kept always at 4 °C.

Method b: In another more standard way of slow protein precipitation, acetonitrile was slowly added in three steps to 100

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μL of plasma, resulting in a total acetonitrile concentration of 80% after 30 h, followed by centrifugation. For infusion, 10 μL of the supernatant was diluted with a mixture of water and acetonitrile (1:1) to a final dilution of 1:250. For LC−MS comparison in a proof-of-concept study, the other part of the supernatant was removed and evaporated down to ∼20 μL. Then the sample was redissolved in 20 μL of methanol and 80 μL of water.

Mass Spectrometer. For all analyses, an orthogonal hybrid quadrupole time-of-flight mass spectrometer (QTOF-Ultima, Micromass, Manchester U.K.) was used. All instrument settings were optimized for the mass range 100−3000 m/z to provide a mass resolution of ∼9000 fwhm. To keep the inlet system clean, cone gas was used with a flow rate of 200 L/h. The source block temperature was set to 60 °C. For chip-based nanoelectrospray, the Nanomate 100 (Advion) was attached to the QTOF. The automated chip-based spray system generated the nanoelectrospray at 1.6 kV and 0.35 psi head pressure. Typically 5 μL of sample solution was aspirated from the 96-well plate for each sample measurement. This amount corresponds to ∼20 nL of pure plasma.

While the mass spectrometer scan time was set to 10 s, mass spectra were always acquired over a period of 10 min. This was performed because of the highly diluted samples, and a standard 1-s scan did not provide enough information. The usage of a constant nanospray over a long time period permitted data accumulation and averaging that resulted in a dramatic increase in the signal-to-noise ratio. In a fully automated fashion, 30 samples were measured in ∼5 h. The mass spectrometer and the NanoMate were controlled by individual computers and their own respective software (Masslynx 4.0 and ChipSoft 6.1).

For potential identification of components of interest, tandem mass spectrometry experiments were performed. The QTOF precursor window for CID experiments was set to 1 Da, the smallest possible value. For the MS/MS measurements of these highly diluted plasma samples, the data acquisition time has to be extended to 30−60 min, depending of the precursor ion intensity. It should be noted that under these diluted conditions only very few ions were produced and even fewer ions were fragmented. Calculating backward from the total ion count, approximately every 90 s, a fragment ion will reach the detector, demonstrating the significant level of sample dilution. To create meaningful MS/MS spectra a collision energy of 19−26 eV were used.

In a comparison with liquid chromatography, the same set of samples were analyzed at the Waters application laboratory (Almere, NL) using their latest ultrahigh-performance liquid chromatography UPLC system (Aquity, Waters Corp., Milford, USA) coupled to an LCT Premier (Waters). This new single-stage mass spectrometer has a performance similar in sensitivity to the QTOF, but mass resolution is limited to 5000 fwhm in V-mode. An Acquity BEH column (C18, 2.1 × 100 mm, Waters) was used with a flow rate of 400 μL/min with a water/acetonitrile, 0.1% formic acid gradient. During the 40-min LC run, the gradient was controlled in the following way: 0.3 min 2% B; 7.0 min 20% B; 23.0 min 98% B; 28 min 98% B; 30 min 2% B; and 40 min 2% B. The column temperature was set to 40 °C. A 5-μL sample was injected. More details about this UPLC system and standard parameters were published recently.[17]

Statistical Spectra Analysis. For statistical spectra analysis, Expressionist Pro (Genedata, Basel, Switzerland) was used. The Spectools module was applied to preprocess the raw data by integrating the spectra over 10 min, correcting mass shifts within the low-ppm range, subtracting background, and preparing the data for multivariate analysis. Another important aspect was that the continuous spectra were not transformed into classical line spectra. Every data point was used for the statistical analysis. The Analyst module of Expressionist Pro was used to apply various statistical methods to our continuous spectra, such as hierarchical clustering, principal component analysis, and supervised machine learning. To handle up to 100 000 data points/spectrum, a p630 series IBM server with four RISC processors was used.

RESULTS AND DISCUSSION

Plasma Precipitation. Plasma is known as a very complex mixture. High albumin concentrations make the analysis even more of a challenge. Before starting any mass spectrometric investigation of the plasma metabolome, a sample pretreatment method for albumin and salt removal without affecting the metabolome itself is necessary. For our metabolomic investigations, the plasma was diluted and slow protein precipitation performed to partially separate the small molecules from the protein content. As mentioned before, when plasma is slowly and carefully diluted in 50% organic solvent, slow protein precipitation occurs without small-molecule loss. Fast precipitation (performed in 90% organic solution) will precipitate a lot of small molecules. Rapidly growing clouds of precipitating proteins disturb the metabolome more than gradual protein precipitation. This effect might be related to the fact that albumin and other proteins have an affinity to many low molecular weight compounds. Therefore, for good reproducibility, the precipitation step has to be done as slowly as possible. In Figure 1A a typical mass spectrum of infused fresh diluted plasma is shown. The multiple charged albumin peaks are very broad, and almost no individual peaks are visible in this mass range. Also, in the low-mass range between m/z 100 and 900, the mass spectrum does not exhibit good spectral quality. Even after averaging the spectra over 10 min, the resulting spectrum is disappointing. However, this might be due to the large amount of protein and salts within plasma. The total ion spectrum represents only 1090 counts. The embedded figures highlight the m/z 477 mass area. On the upper spectrum, the peak marked by x has 20 counts and the signal-to-noise ratio is poor. If one allows long incubation times for a slow partial protein precipitation of the same sample, the resulting mass spectrum will change. However, the solution will stay clear by eye. Figure 1B shows the same diluted plasma but now after 16-h incubation time at 4 °C. The total ion count has increased by more than a factor of 20. The multiple charged albumin peaks are resolved in a way that the deconvolutation leads to a clean albumin spectrum of m/z 66 429 (data not shown). Also in the mass range between m/z 100 and 900 there is improved spectrum quality. Many peaks are much more intense while only a few have disappeared. This typical signal increase is shown again in the embedded figure where the x marked peak has increased dramatically.

All experiments in this paper were obtained with heparin plasma. However, other anticoagulation reagents, like EDTA or citrate, gave similar mass spectra. For example, EDTA plasma shows a dominant EDTA peak, while citrate plasma shows a larger

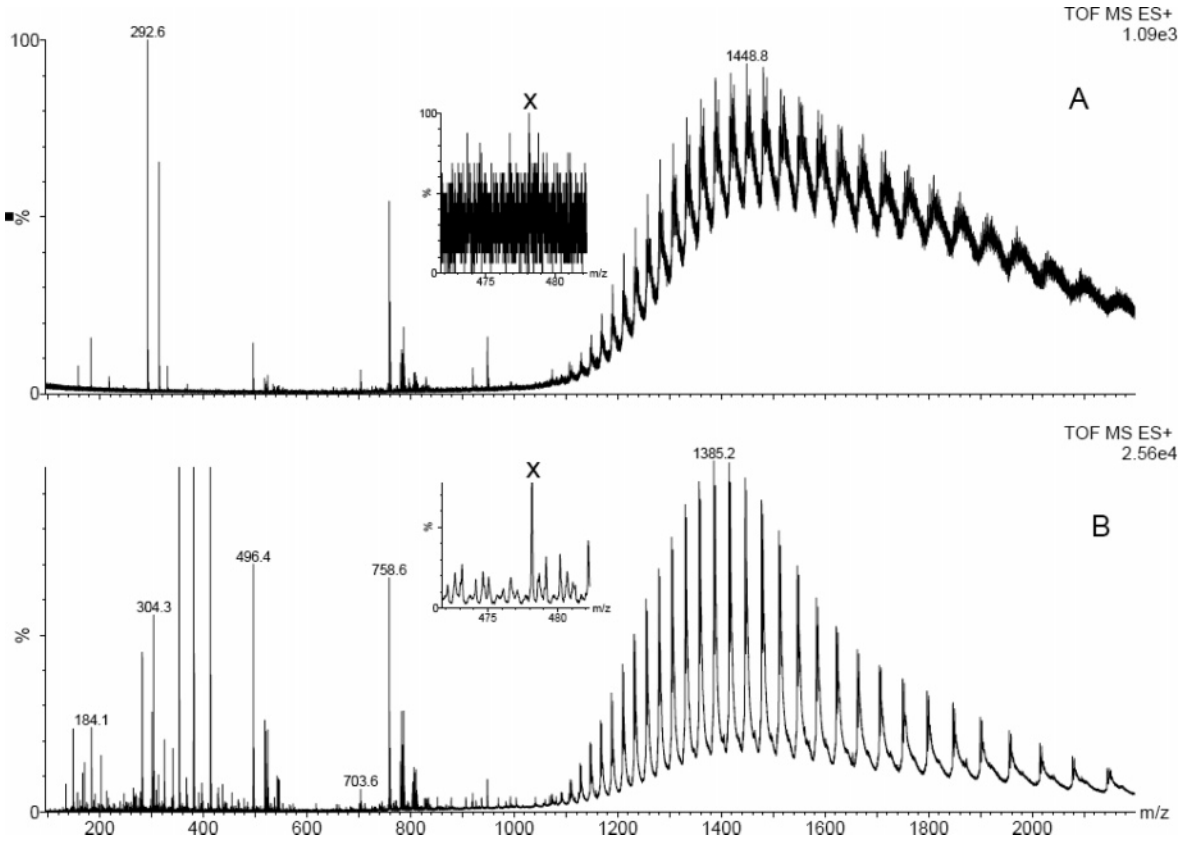

Figure 1. Typical mass spectrum after 10-min infusion of diluted human plasma (1:250) by 50% acetonitrile/water with 0.1% formic acid. (A) Fresh diluted plasma. (B) Typical mass spectrum under the same conditions but measured 16 h after dilution (method a). The embedded zoomed spectra show how the signal of low-abundant compounds increase with time. For the peak marked with x, there is an increase by a factor of 24 in intensity.

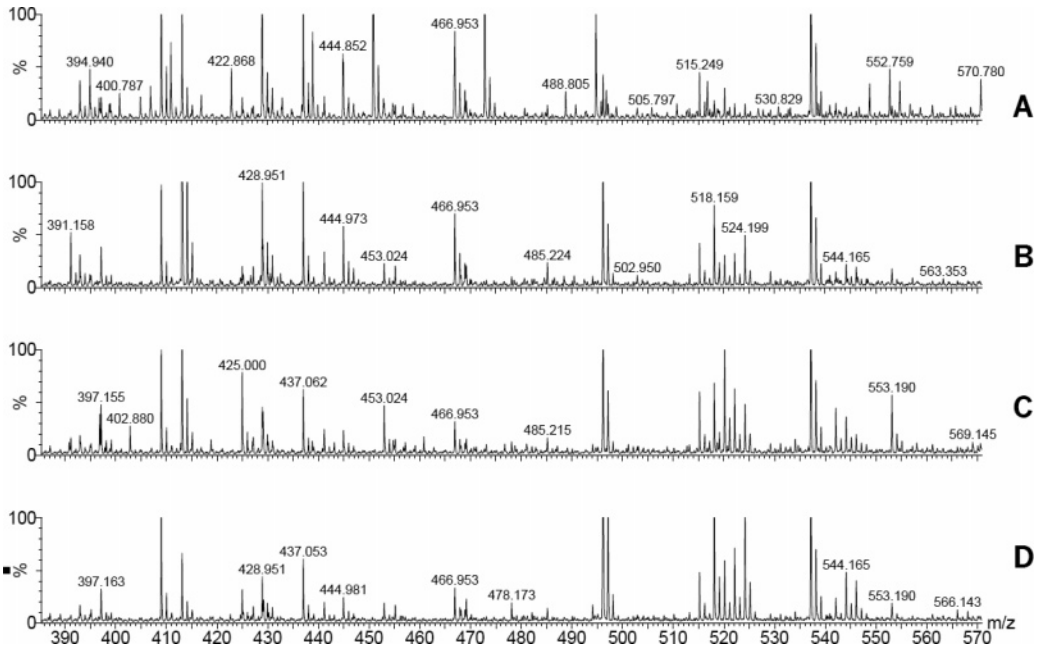

Figure 2. Comparison of infusion mass spectra of different plasma types and serum samples within the mass range of *m*/*z* 380−570 after dilution and 16-h incubation time. (A) Plasma with citrate as coagulant; (B) plasma with heparin; (C) plasma with EDTA; (D) serum.

number of more intensive peaks than the other reagents. Regardless, the reproducibility within one plasma type is very good as shown in Figure 2. Zoom view for the mass range from *m*/*z* 380 to 580 is presented. All spectra are displayed with the same intensity (counts). Using this highly diluted pretreatment method, fresh whole blood or serum can be also infused in this way for metabolomic studies as well as for small-molecule analysis.

After the slow precipitation of 16-h incubation time, the pattern of these spectra will not be dramatically changed during the following 8 h. Only a small decrease of the overall intensity can

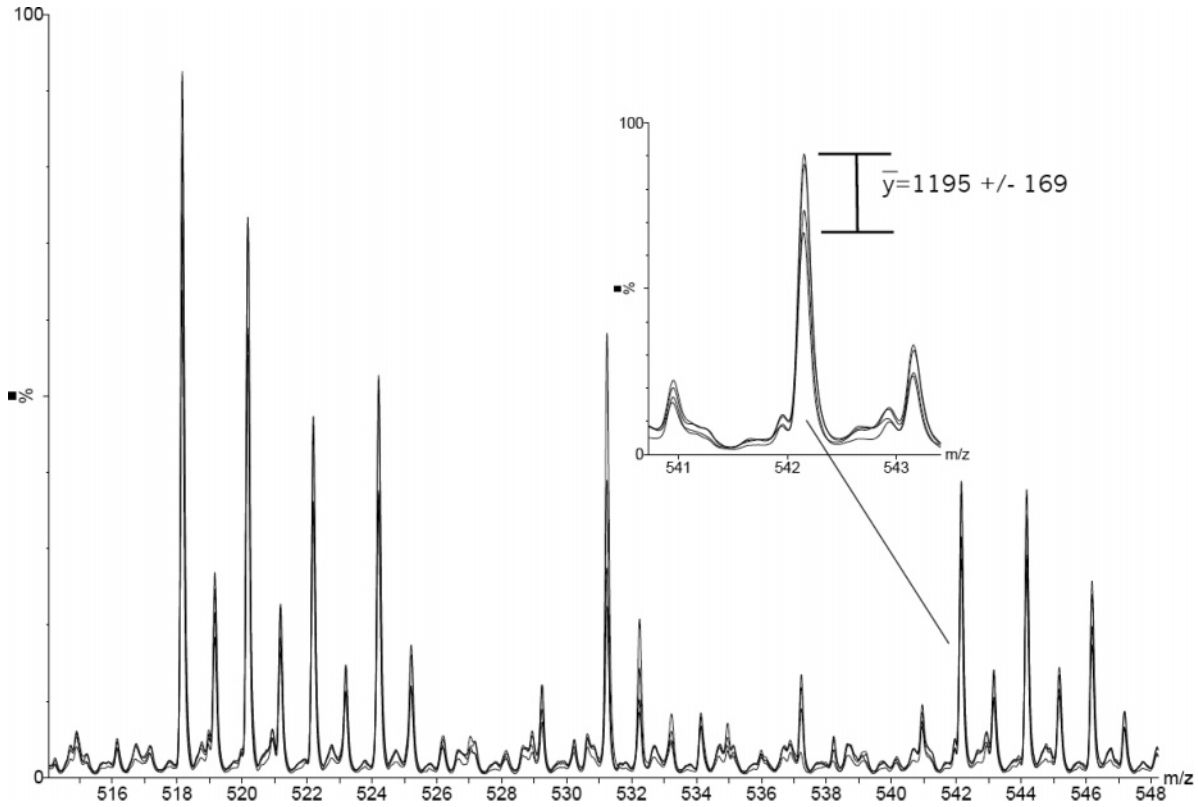

Figure 3. Four overlaid infused mass spectra of diluted plasma after 16-h incubation time of the same sample and processed in parallel. The embedded figure shows a typical peak for demonstrating the reproducibility of the method. No normalization was applied.

be observed. Measurement with different incubation times shows best results for 16 h at 4 °C. However, we still could use samples after 48 h total incubation time for profiling. The precipitation step and wall effects (some compounds may stick to surfaces) are the critical issues. As previously stated, it is very important that the complete set of plasma samples is always be treated in precisely the same way. Even after 48-h incubation time at 4 °C, the analysis and statistical separation of a metabolomic set of samples were possible, because all plasma samples had seen the identical treatment history. The slow precipitation step is a dynamic and ongoing process. In parallel, some compounds from the sample may stick to surfaces and their intensity will be reduced in the spectrum by time. It is also interesting that in the mass range between 50 and 700 m/z no multiple charged peptides were found. Between 700 and 900, there were only four peptides visible by their multiple charged isotope pattern. Another interesting effect of this dilution is that it seems that salts will be reduced under these conditions too.

A typical sign that protein precipitation occurs in the 1:250 dilutions is that the protein precipitations create little particles, which sometimes have clogged our chip nozzles. Additional centrifugation steps may reduce this but not completely. From this point of view, the chosen plasma diluting step is a good compromise. However, small changes within the sample pretreatment process may change some mass intensities relative to the each other, but such changes are reproducible. Figure 3 shows this reproducibility in an overlay of four mass spectra after infusion of the same plasma. All samples were diluted and incubated in parallel. Already without normalization, the different peak intensities are very similar. As this experiment demonstrates, it is more important for all samples of a given study to be treated in an identical way than the selection of the pretreatment method itself. Because of the physical pullout effect of precipitating protein clouds,[3] different precipitation methods may lead to different profiling results of the metabolome. Albumin and other serum proteins that act as molecular sponges are known to have affinity for a large number of small compounds. As such, they constitute a major compartment of the serum metabolome, and their adsorbed species must be reproducibly assessed for complete metabolomic studies. Thus, partial removal of albumin under equilibration conditions provides the most intact metabolome for further investigations. Reproducibility for albumin removal is shown in Figure 3, where four infusion spectra of the same plasma sample after dilution and incubation were applied in parallel and displayed as an overlay. After processing the same sample four times and measuring by infusion MS for a given peak (here m/z 542), a mean intensity of 1195 counts and a standard deviation of 169 counts could be determined. In comparison with our LC data (see proof of concept), the infusion data were so good that no normalization was necessary to allow males and females to be separated using statistics to find up- and downregulations between the two groups. A real-life example will be presented in the proof-of-concept section (Figure 4).

Comparison with LC. Mass spectra created by chip-based infusion show more than 1800 different mass peaks including isotopes over a mass range of up to 900 Da. This is similar to the results using a UPLC−MS system, which is one of the most powerful separation techniques of today.[17] Chromatographic peak widths of a few seconds in combination with a 40-min gradient resulted in excellent separation power. However, a fast gradient

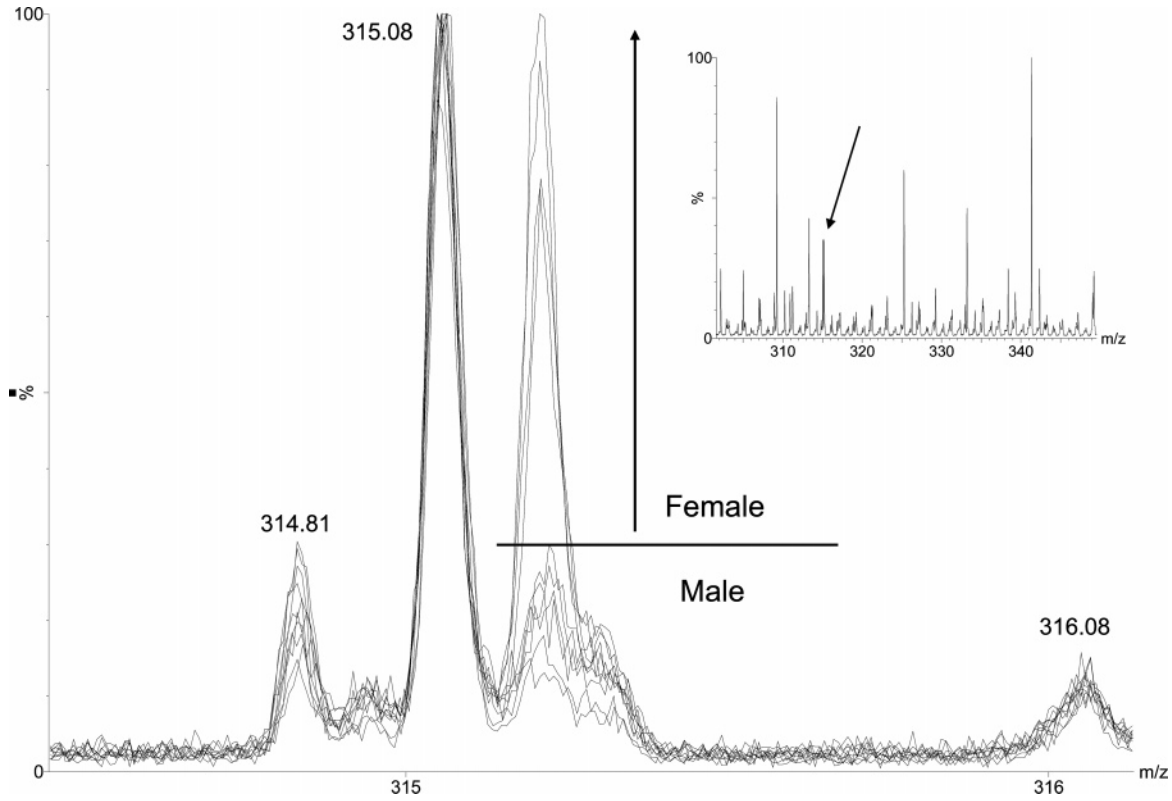

Figure 4. Comparison of infusion mass spectra for five female and five male plasma samples in the region of *m*/*z* 315. The overlay shows good reproducibility for all samples at *m*/*z* 315.08 and 316.08 without applying any normalization, while the mass *m*/*z* 315.21 shows different intensities for male (below 25%) and female samples. The embedded mass spectrum shows an overview of the selected mass range.

of 10 min had given much less peak information. HPLC or UPLC methods are standard approaches in the metabolomics analysis of various sample types. Therefore, a new approach has always to be compared with standard LC conditions, even when a different sample pretreatment method would be necessary, because at the end, any method has to deliver meaningful profiling results. A more real-life sample comparison will be presented in the proof-of-concept section, where female and male plasma samples will be compared by these two methods.

For better visualization and comparison, all single MS spectra of the complete LC run were accumulated into one single mass spectrum. Figure 5B shows the resulting spectrum together with the total ion chromatogram as an embedded figure, while Figure 5A shows an infusion mass spectrum. Already in this mass overview it becomes obvious that both methods present a similar number of mass peaks. Also in details (data not shown), many identical masses could be found within both spectra, while their relative intensities were always different. This is not surprising, because different sample preparation steps and different ESI sources were applied. The comparison of the same sample by LC and by infusion is also a question of sensitivity. For LC, a corresponding amount of 5 μL of plasma was injected after using a similar slow protein precipitation procedure (LC method b), while for an infusion mass spectrum, a corresponding amount of ~20 nL of plasma was spent (optimized infusion method a). Under these conditions, the LC does not show a higher sensitivity or more endogenous compounds compared to the simple infusion over 10 min. Similar pictures of LC and infusion were observed, when five male and five female plasma metabolomes were compared with each other under identical sample preparation (LC method b). The two groups of females and males could not be separated by statistical analysis (details are given in the proof-of-concept part) of the two-dimensional information (retention time and *m*/*z*) from LC−MS, nor did the infusion mass spectra provide enough information for group discrimination. This experiment shows that infusion and LC can provide the same results. However, applying the partial precipitation followed by infusion MS, could separate the females and males easily, as will be shown in the next section. It seems that clouds of precipitating proteins affect the metabolome. These experiments demonstrate that an optimized sample pretreatment is very important, especially for metabolomics.

One of the main advantages of LC−MS is that one can distinguish between background impurities and the target compounds. However, in the analysis of the complete metabolome, background peaks become a serious challenge because of the very high number of different compound peaks. A good separation between background compounds and plasma compounds is not always possible. By infusion mass spectrometry, the solvent background can be subtracted easily from the plasma background or just be compared. For example, the leaching of 3.3′-thiodipropionate (DDTDP) and its oxidation products from the polypropylene tubes could be found within the infusion spectra as minor peaks (*m*/*z* 531.41, 553.39, and 569.37). The corresponding MS/MS spectrum of the precursor ion *m*/*z* 531 was identical to that obtained by Franklin and Doss.[18] However, this background signal does not create any problems in our proof-of-concept infusion studies. Also within the METLIN metabolite database,[19] this peak could be found in the stored LC−MS chromatograms at 80% acetonitrile and *m*/*z* 531.4. Additionally, this compound was found

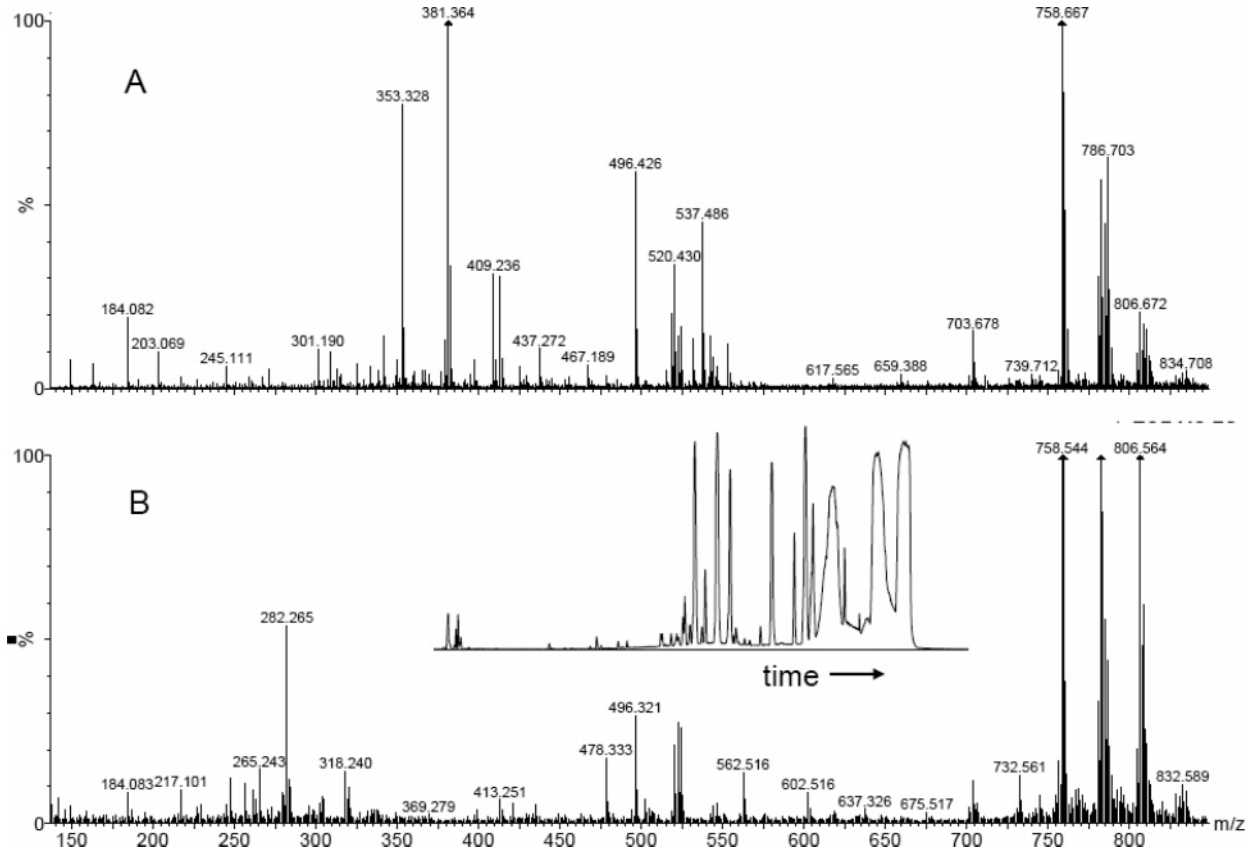

Figure 5. Comparison between infusion-MS and LC−MS using the same plasma sample and under best individual conditions for each method. Sample preparation was optimized for LC conditions (method b), where 5 μL samples corresponding to 5 μL of plasma were injected. (A) Infusion mass spectrum measured with the conditions as described under Figure 1. (B) Accumulated mass spectrum from a 40-min LC−MS run. The embedded figure shows the corresponding total ion chromatogram.

in the LC−MS run of Figure 5 as a very minor peak. This compound might actually be useful as an internal standard. We could also identify DDTDP within plasma samples, which did not have any contact with organic solvents prior to analysis. It seems that this compound can already leach into the samples during storage.

Proof of Concept. A new analytical approach has to be tested under realistic conditions. To prove this concept of diluted plasma infusion for metabolomics, we used plasma from five healthy females and five healthy males as mentioned. To increase the number of samples and to test the reproducibility of the method, every plasma sample was analyzed in triplicate. Three background samples of water/acetonitrile (without plasma) were also measured. However, all samples were randomized to avoid separation by instrument trends. This resulted in 33 mass spectra, similar to the spectrum in Figure 1B. A major differentiator in separating the two groups is the peak at mass m/z 315.17. Because of better visibility, only 10 of 30 spectra, randomly selected, are displayed in Figure 4 as a detailed overlay. In this figure, the good reproducibility between different samples becomes obvious. For the females, the medium intensity is 497 counts at the mass 315.17, while for the males, the medium intensity was found at 120 counts. The peak at m/z 315.08 in Figure 4, which seems to be constant over all samples, has a medium intensity of 565 counts over all 30 samples with a standard deviation of 122, which corresponds to 21.5%. If one takes into account that these are 30 data sets of 10 different plasma samples without any normalization, a deviation on peak heights (ion counts) of only 21.5% without normalization is quite respectable. Inside these spectra there are hundreds of different peaks that show similar behavior. Also, these samples have been measured over a time period of 5 h, which means that at least over this time period the conditions are quite stable. This experiment also highlights the high sensitivity of the infusion protocol. Figure 4 also shows that the mass intensities at m/z 315.08, 316.08, and 314.8 are very similar between all measurements, indicative of good sample-to-sample reproducibility.

Data Analysis. The main goal in metabolomics profiling is to find statistically significant up- or downregulations. In the framework of mass spectrometry, each compound is represented by one or several peaks, which have to be analyzed by sophisticated statistical means. A common approach is the transformation of the raw spectra into classical line spectra (centroids) to reduce the data set. However, the peak shapes of the individual masses are always lost and important peak shoulders might be interpreted incorrectly. In addition, the resulting line intensities often do not reflect the continuous peak intensities correctly. Without the visibility of a clear background line noise, peaks including chemical noise peaks will be difficult to recognize within a centroid spectrum. When no additional chromatographic information is available, such as this infusion approach, then the use of continuous mass spectra becomes important.

For these reasons, we decided to use the original raw data set (continuous spectra). Approximately 100 000 data points were used in the low-mass range of interest (50−1000 Da). On the raw data

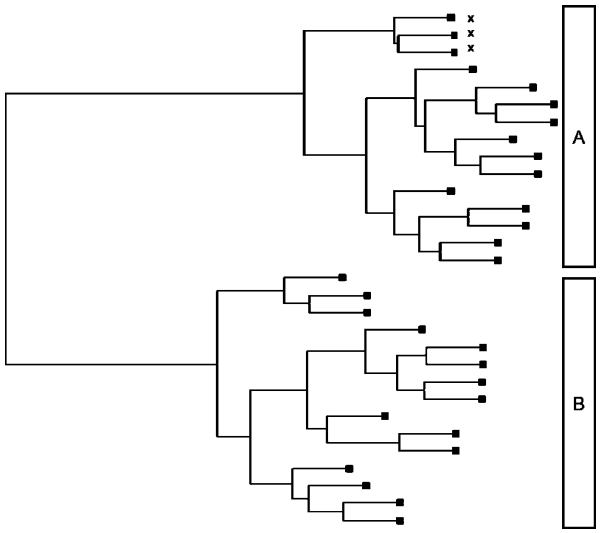

Figure 6. Hierarchical clustering after sparse linear discriminant analysis of 30 mass spectrometry spectra generated from five females and five males in triplicates. The upper large group represents the females (A), while the bottom represents the males (B). The three marked by x clusters are triplicates of the same female sample. (generated by Expressionist Pro).

level, every peak is determined by several data points, which can all be used for statistical analysis. It turned out that the most powerful method to analyze this complex data set was sparse linear discriminant analysis (SLDA), a supervised machine learning algorithm as a ranking method to reduce the number of data points. These methods use experimental grouping and are optimized for finding data points that can discriminate between groups. In the present study, SLDA identified 181 discriminating data points as statistical marker candidates. Subsequently, hierarchical clustering and principal component analysis (PCA) were applied, using only these markers. It should be noted that these data points in principle could not represent full mass peaks and can be spread over the complete spectrum. However when there is a real mass peak, a peak shoulder is involved and then the corresponding data points are consecutive. In these cases, a real mass can be used for further biomarker investigations, while the spread data points may be useful for pattern recognition.

Using the SLDA data set, hierarchical clustering was able to separate the two groups (male and female) completely, as shown in Figure 6. Even one specific sample (marked with a cross in Figure 6), for which the measurement had to be repeated 8 h later due to experimental problems (in the first run, the chip nozzle was clogged), clustered into the correct group. This again shows the excellent reproducibility of the method. Similar results have been observed in PCA. The two groups are again perfectly separated. The marker set lead us to a list of eight major m/z values, 259.21, 315.19, 365.15, 659.36, 695.53, 711.53, 727.49, and 987.70. The list contains only the base peaks, i.e., without ^{13}C isotopes. All peaks were more abundant in female plasma, and the differences are clearly visible in Figure 4 for one example. The results are consistent within all triplicates.

The comparison with the METLIN database showed that plasma samples from healthy individuals contain in general fewer peaks than samples taken from diseased patients.[19] Therefore, a comparison of samples within a healthy cohort will be more difficult than trying to discriminate healthy from diseased states. This can be one of the reasons why the present study resulted in a rather small number of discriminating peaks. Further studies are needed to confirm this finding.

Identification. The identification of the compounds in male and female plasma needs to be done with care to ensure the correct compound assignment. For example, the METLIN metabolomics database gave progesterone (m/z 315.217) as a close hit for a measured mass of m/z 315.19. For further confirmation, the diluted male plasma was spiked with 25 fg/μL progesterone, which represents 6.2 ng/mL in pure plasma and measured as described before. The resulting progesterone mass peak now had an intensity similar to the unknown peak with the same or similar molecular weight in the female plasma. Therefore, this progesterone concentration of 6.2 ng/mL gives an indication of the sensitivity of the method. From the intensity of progesterone, which does not have the best ionization efficiency in electrospray, it can be calculated that ~10 times less concentrated progesterone should be still visible. The spiking experiment gives evidence that it may be the same peak, but on closer inspection, there is a small difference of 80 mDa. Therefore, a MS/MS experiment with the native and spiked plasma was performed. For the mass spectrum in Figure 7A and B, 60 min was needed to acquire enough ion counts from these highly diluted samples. However, this example shows how effective the length of the infusion time can be. The fragmentation pattern of the pure progesterone (Figure 7C) was identical from the native and spiked plasma, as shown in Figure 7A and B. Almost every fragmented mass of the pure progesterone spectrum was found within the spiked (B) and the native plasma sample (A). However, the native spectrum shows a few additional masses that do not belong to progesterone. Here one has to take into account that a fixed precursor window of 1 Da is much too large for a single mass of 0.041-Da half-width within a high-resolution mass spectrum. In this case, other m/z values close to 315.2 would also be fragmented. A high-resolution tandem mass spectrometer with a narrow precursor window might produce much better results. Because of these MS system limitations, the MS/MS experiments do not provide absolute evidence for progesterone. It only confirms that in the region of m/z 315.2 $\pm$ 0.5 progesterone is present. As the example of DDTDP showed before, the identification of a known structure by MS/MS even within these diluted samples is often easily possible. MS/MS spectra from several other differentially expressed masses were acquired, but no structure could be elucidated from them until today.

Infusion. Chip-based infusion has the main advantage of almost unlimited data acquisition time whereas in chromatography only a few seconds are available for selecting data. Peak concentration and consequently peak intensities are much higher in chromatography than by infusion mode. However, there is a break-even point where long data acquisition times always provide better signal intensities of the accumulated data. With other electrospray systems, ion suppression reduces the gains that the long-term infusion provides. However, the electrospray chip may change this paradigm. Wickremsinhe et al. have already shown that the ion suppression effect of a given biological matrix in their infusion assay was minimal (less than $\pm$10%) by using the nanospray chip.[16] Gangi et al. could prove that ion suppression is reduced on

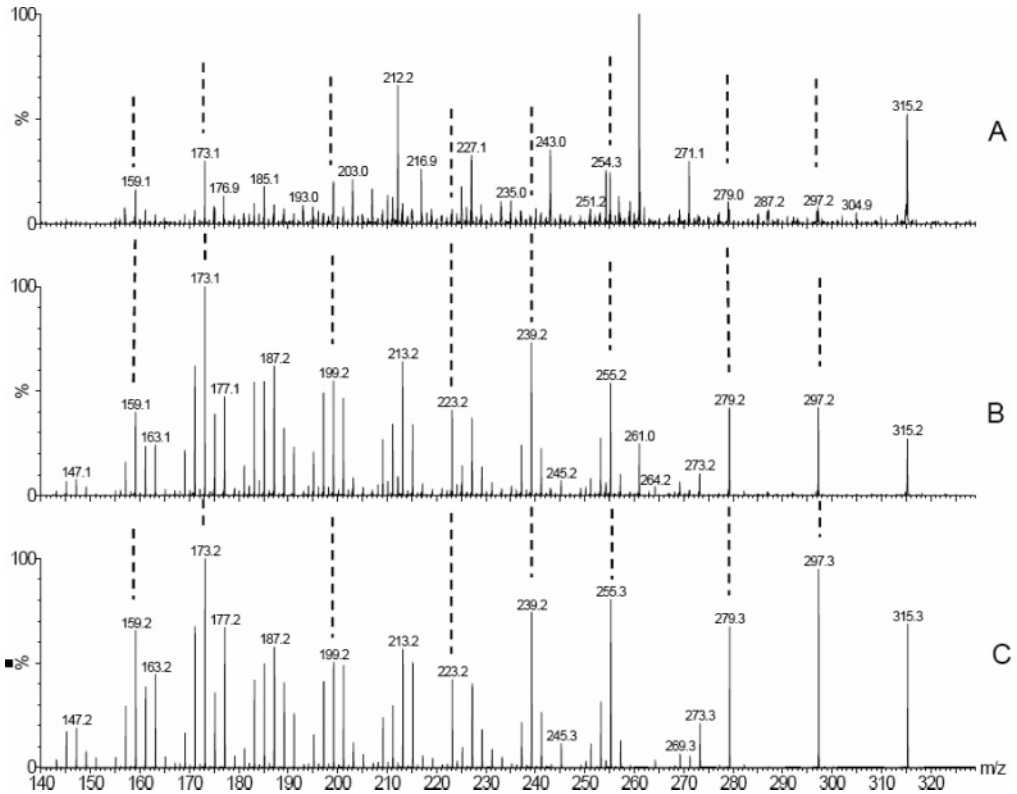

Figure 7. CID mass spectra for progesterone from native female plasma (A), spiked plasma (B), and pure progesterone (C). Spectra A and B: data accumulated during 60 min, while 10 ng/μL pure progesterone was measured for 2 min.

nanospray devices compared to standard ESI flow rates,[28] while on higher flow rates (0.4 mL/min), this effect can only be affected by solvents and buffers in a limited range.[29]

Also, in our laboratory, minimal impact of ion suppression has been previously observed during direct infusion of peptides in single and tandem MS studies of 2D-gel spot digests. However, here only 20−90 peptides were analyzed in parallel without notable ion suppression. In addition, chip-based infusion-MS of diluted plasma after a controlled slow precipitation step overcomes the difficulties of retention time shifts and changing intensities as no chromatographic step is performed.

In general, the large amount of different compounds within plasma helps stabilize the complete system and regulate suppression effects. Under these conditions, ion suppression becomes a constant factor. If one single compound out of this mixture is upregulated in a sample, the effect for ion suppression is too small to become visible. An explanation for this behavior may be the extremely high electrical field strength of the ESI chip, because the chip itself has its electrical ground at each individual nozzle and therefore the chip can spray without an MS in front of it. This high electrical field strength may therefore reduce the ion suppression effects. In our experiments, it seems that the ionization efficiency can be increased in extremely diluted samples. This would also explain the observed reduced ion suppression. If the compounds' concentration is low, then there are always enough protons available for ionization. With the current measurement of diluted samples and signal averaging into one single spectrum, the resultant intensity of the low-abundant peaks are in the range of 50−400 ion counts. This means that less than 1 ion count/s will be measured at the detector! This is far below standard mass spectrometry conditions, where several hundred ion counts per second will be measured. It should be noted that under these conditions detector saturation will never occur; hence, one would routinely enjoy full dynamic range detection. These numbers also show how diluted the samples really are. It is interesting that with this dilution factor several hundred compounds become visible in parallel. This can only be explained if the ionization efficiency is dramatically increased. However, because of the complex nature of human plasma, there is a wide distribution of ions over the complete mass range. Less diluted plasma samples do not give higher peak intensities, while higher diluted samples do not show significantly fewer ions. Samples where all proteins were precipitated showed increased signal intensities, because now there is no albumin in competition with the small molecules, resulting in higher ionization efficiency for the remaining molecules. A practical example for this may be the fact that for human plasma a dilution of 1:250 gives the best results. Dilutions of 1:100 do not increase the mass intensities of interest as expected, because in a higher concentrated solution the total number of ions is increased and therefore ion suppression will reduce the total ion intensity. However, less total ions also mean less ion suppression and concomitant relative increased signal intensities. These experiments also demonstrated that ion suppression is concentration dependent under such conditions. Dilutions of 1:500 will really dilute most of the MS signals and the beneficial effect of diminished ion suppression is already

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Table 1. Linearity of Diluted Horse Myoglobin Digests by Infusion

consumed within 10 min (fmol)	concn (fmol/μL)	ion counts on m/z 689.9; background subtracted (counts)	chemical background (counts)
0.6	0.3	673	159
2	1	1236	44
20	10	10398	102
200	100	99402	98

$r^2 = 0.999\ 997\ 75$

reduced. This effect can also be observed when only pure solvent as sample will be sprayed; here solvent impurities in the attomole range result in dramatically higher peak intensities. However, if a compound or a mixture of compounds will be added in sufficient concentration, then most of these solvent peaks will disappear from the resulting mass spectrum or their peak intensity will be decreased by more than 2 orders of magnitude. The TIC of $\sim 3 \times 10^5$ counts for the pure solvent mixture will only be increased to 3×10^6 counts when plasma is added. Two to three orders of magnitude more molecules or better ions lead to only a 10 times higher TIC. This can only be done if the low concentrated solvent impurities are suppressed by the more concentrated ones. Under this regime, ion suppression seems to be also a statistical effect.

As an example of sensitivity and linearity for this approach, horse myoglobin digest standard was measured by 10-min infusion-MS at different concentrations. Even in a real concentration of 300 amol/μL, several peptides of the digested myoglobin were visible, measuring a mass range from m/z 100 to 3000. The doubly charged peptide at m/z 689.9 was the most abundant one and used for calculations. In Table 1, below the measured ion counts are shown.

It should be noted that under these highly diluted conditions at a sample concentration of 300 amol/μL for a number of different peptides, the impurity of the solvent and plasticware becomes important as previously discussed. Therefore, the chemical noise was counted as background and subtracted. These data are in good agreement with Wickremsinhe et al. and their results of a bioanalytical assay using chip-based infusion on a triple quadrupole mass spectrometer.[16]

CONCLUSION

Metabolomics by infusion should not be compared with the targeted analysis of steroids or any other single compound, where optimized LC−MS methods with ultrahigh sensitivity are available.[27] The advantage of the infusion method is the potential to measure hundreds of compounds in parallel with excellent reproducibility and with little effort. Under these conditions, statistical analysis can focus on recognizing the biochemical differences and not on any variability of actual measurements. To maximize information and to ensure that all samples behave in an identical way, controlled slow protein precipitation is the most important step for these experiments. However, for a metabolomic study or any quantitative work, it is very important that all samples have precisely the same pretreatment history. Automated well plate-based sample pretreatment steps in combination with chip-based infusion seem to be a promising method for the future. It should be noted that the chip-based infusion-MS protocol presented here is not only useful for the metabolome within plasma samples but may also be used for other biofluids, such as urine and cerebral spinal fluid. Obviously different sample pretreatment procedures are required. Also, the measurement in the negative ionization mode will provide a more complete picture with complementary information.

The second major consideration is the nanospray chip nozzle itself. As ion suppression is reduced due to the nanospray flow rates from the chip, it becomes possible to see hundreds of compounds at the same time, with a good sensitivity. There will inevitably be ion suppression in such a complex sample, but the huge number of different compounds creates a constant ion suppression background that cannot be changed by a few single compounds of different concentrations. The main disadvantage of the infusion method is that all isomeric information is lost. This would be separated by time in chromatography. We propose that the described protocol using a controlled slow protein precipitation in combination with chip-based infusion offers the possibility to create high-quality data for important metabolomic profiling research. The combination of high-resolution ion mass spectrometers with the ESI chip might become a powerful tool for infusion mass spectrometry.

ACKNOWLEDGMENT

The authors thank Stephanie Richer from Waters for providing us with the corresponding LC−MS spectra of our samples. Mark Flesch from Genedata is thanked for his contributions to the statistical analysis. We also thank Gary Schultz and Alistair Sterling from Advion for fruitful discussions. Thanks to Scot Weinberger and Mark Allen for critical reading of the manuscript. For excellent technical assistance, we thank Guido Wahl.

Received for review May 18, 2005. Accepted August 26, 2005.

AC0508604