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Neutral loss analysis of amino acids by desorption electrospray ionization using an unmodified tandem quadrupole mass spectrometer

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A new method to analyze free amino acids using desorption electrospray ionization (DESI) has been implemented. The method is based on the neutral loss mode determination of underivatized amino acids using a tandem quadrupole mass spectrometer equipped with an unmodified atmospheric interface. Qualitative and quantitative optimization of DESI parameters, including ESI voltage, solvent flow rate, angle of collection and incidence, gas flow and temperatures, was performed for amino acids detection. The parameters for DESI analysis were evaluated using a mixture of valine, leucine, methionine, phenylalanine and tyrosine standards. A few microliters of this mixture were deposited on a slide, dried and analyzed at a flow rate of 2 μL/min. The optimal ionization response was obtained using laboratory glass slides and an equivalent solution of water/methanol doped with 2% of formic acid. The method specificity was evaluated by comparing product ion spectra and neutral loss analysis of amino acids obtained either by DESI or by electrospray ionization flow injection analysis (ESI-FIA). To evaluate the quantitative response on amino acids analyzed by DESI, calibration curves were performed on amino acid standard solutions spiked with a fixed amount of labelled amino acids. The method was also employed to analyze free amino acids from blood spots, after a rapid solvent extraction without other sample pretreatment, from positive and negative subjects. The method enables one to analyze biological samples and to discriminate healthy subjects from patients affected by inherited metabolic diseases. The intrinsic high-throughput analysis of DESI represents an opportunity, because of its potential application in clinical chemistry, for the expanded screening of some inborn errors of metabolism. Copyright © 2007 John Wiley & Sons, Ltd.

Ambient mass spectrometry techniques are used to analyze many compounds on intact samples, either directly from complex matrices or after a simple solvent dilution, as in matrix-assisted laser desorption/ionization (MALDI).[1,2] Desorption electrospray ionization (DESI) has been recently introduced by Cooks and his group where electrospray-charged droplets and ions of solvent are directed onto a solid sample placed on a surface. The impact of the charged particles on the surface produces gaseous ions from material deposited on the surface.[3–5] The resulting mass spectra are similar to those obtained by liquid electrospray[6] where the analytes produce mainly singly or multiply protonated molecules. DESI can be considered an atmospheric pressure version of secondary ion mass spectrometry (SIMS), similar to other ambient mass spectrometry techniques recently introduced such as direct analysis in real time (DART),[7] desorption atmospheric pressure chemical ionization (DAPCI),[8] electrospray-assisted laser desorption/ionization (ELDI)[9] and the atmospheric solids analysis probe (ASAP).[10] Compounds ranging from non-polar small molecules, such as alkaloids and small drugs, to polar compounds, such as peptides and proteins, can be selectively analyzed using DESI on solids, on biological matrices and *in vivo*.[4] Less than a few seconds are necessary for the acquisition of a spectrum by DESI making this mass spectrometric technique of potential interest for *in situ* analyses in a wide variety of fields such as anti-terrorism, environmental, agriculture, pharmaceutical and biomedical. Analysis of explosives, toxic compounds, drugs and biomarkers from complex matrices such as plants,[3,11] tissue,[12] blood,[3] and urine,[13,14] have been already implemented by DESI. Most analytical methods in these fields are at present performed by ESI-MS/MS, which is considered the technique of choice for the quantitative analysis of metabolites with high specificity and efficiency and lower in-source fragmentation of molecular species.[15,16]

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The ambient ionization method of DESI shows the same analytical performance as ESI and is also able to analyze samples directly from a complex matrix. In particular, the application of DESI is interesting to study the inherited errors of metabolism (IEM) that often require a fast turnaround time analysis for their diagnosis or their exclusion in suspected patients. The standardization of quantitative DESI methods is still lacking in this field and there is also a lack of standard methods in other fields of application. A recent paper reports the analysis of the urine of patients with IEM[14] and its application to the study of the impairments of metabolism *in vivo* or *in vitro*. Improved DESI methods could represent a new application of mass spectrometry for the analysis of metabolic profiles, directly *in vivo* on skin surface or as a bed-side test to analyze freshly collected complex biological matrices (e.g. blood or urine), by procedures and instrumentation previously standardized to study the metabolic diseases. The IEM are metabolic diseases, genetically determined, due to the structural change of a single enzyme or protein. Usually, they lead to the abnormal catabolism of certain carbohydrates, amino or fatty acids, or organic acid derivatives. Technological advances have revolutionized the way in which we can diagnose and screen whole populations for IEM. Improvements in technology, and hence in assay sensitivity, have allowed more tests to be performed on a single dried blood spot sample, expanding the ability to screen for more than one disorder in the neonatal period.[17,18] Enormous advances have been made in neonatal blood spot screening since the initial introduction of biological methods in the early 1960s to detect a disorder of the biochemistry of the amino acid phenylalanine. This prototype of disorders, known as phenylketonuria, produces profound mental retardation if it is not detected and treated early in life as many others.[19,20] Early screening programs that relied on population screening techniques have been replaced by more accurate analytical methods such as tandem mass spectrometry (MS/MS). Since the 1990s the evolution of MS/MS has improved our diagnostic capabilities and we can now readily detect multiple diseases on a single sample, using a single analytical procedure.[21,22]

The use of MS/MS enables a multianalyte approach to the detection of biochemical disorders so that a metabolic profile is obtained rather than a single analyte measurement. The provision of a metabolic profile has led to clear improvements in the detection of diseases such as phenylketonuria and several disorders arising from errors in fatty acids oxidation and organic acids metabolism. It has also been demonstrated that the analysis of multiple metabolites better characterizes a particular metabolic disease or other iatrogenic influences such as therapeutic nutrition than a one-method, one-metabolite, one-disease approach.[18] The use of two analyzers in tandem (MS/MS) enables control of the formation of precursor and product ions. This permits measurement of a particular chemical class or subset of molecules, without detecting the hundreds of molecules that are not of interest, maintaining selectivity and speed of analysis in a 2-min assay.[19,20] Most DESI methods have been developed using ion trap MS analyzers and are mainly focused on the qualitative analysis of target compounds as above mentioned.[4,11,12,14,23,24] However, it is still lacking a DESI-MS/MS method that can yield results to compare with those from reference methods (e.g. isotope-dilution MS/MS) already employed for the analysis of metabolic profiles.

In the present study we implement a DESI method, using an unmodified tandem mass spectrometer, to standardize the analysis of some amino acid (AA) profiles using a bench-top triple stage quadrupole mass spectrometer. The qualitative and quantitative optimization of many parameters for DESI analysis has been evaluated on AA standards. Chemical specificity has been achieved by the acquisition of product ion spectra and the neutral loss analysis of AA by DESI; these results were also compared with those obtained by electrospray ionization flow injection analysis (ESI-FIA). Experiments with blood samples have been performed to evaluate the performance of DESI for the AA analysis of positive and negative samples rapidly extracted from paper spots without other sample pretreatment. The method is suitable for biological samples analysis in healthy subjects and in patients affected by inherited metabolic diseases.

EXPERIMENTAL

Materials

Phenylalanine (Phe), leucine (Leu), valine (Val), tyrosine (Tyr) and methionine (Met) were purchased from Sigma Aldrich (Steinheim, Germany), prepared as a stock solution of 20 mM in 0.01 N HCl and frozen at −20°C. A labelled standards mixture, containing ^{15}N;2-$^{13}C_2$-glycine, $^{2}H_4$-alanine, $^{2}H_8$-valine, $^{2}H_3$-leucine, $^{2}H_2$-ornithine, $^{2}H_3$-aspartate, $^{2}H_3$-glutamate, $^{2}H_3$-methionine, $^{13}C_6$-phenylalanine, $^{2}H_2$-citrulline, $^{2}H_4$;5-$^{13}C_5$-arginine and $^{13}C_6$-tyrosine, was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA), and a stock standard solution was prepared at a concentration of 500 μM. The analytical solvents, of HPLC grade, acetonitrile, formic acid and methanol, were from J.T. Baker (Deventer, The Netherlands). Purified water was generated by a Milli-Q Reagent water system (Millipore Corporation, Bedford, MA, USA). A working solution for tuning the mass spectrometer containing Val, Leu, Met, Phe, and Tyr and internal labelled standards was prepared at equimolar concentrations of 50 μM in water. Calibrators were prepared in water from the stock standard solution to obtain concentrations of 100, 50, 25 and 12.5 μM of Val, Leu, Met, Phe and Tyr, respectively, and of 50 μM for relative internal labelled standards. Dried blood spots were prepared by spotting whole blood from healthy and maple syrup urine disease (MSUD) subjects followed in our hospital laboratory for the analysis of AA.

Instrumentation

A triple quadrupole mass spectrometer (Micromass QuattroMicro, Waters, Milford. MA, USA) was equipped with a DESI device that does not involve any modification of the configuration of the original mass spectrometer (Fig. 1). The device was built in order to support a sample holder without modifying any part of the source enclosure. The mass spectrometer was operated in the positive ion mode and a water/organic solvent solution was sprayed through the ESI

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Parameter	Optimal value
Sprayer – surface distance	9 mm
Inlet – sample distance	4 mm
Inlet – surface distance	2 mm
Collection angle	30°
Incidence angle	57°

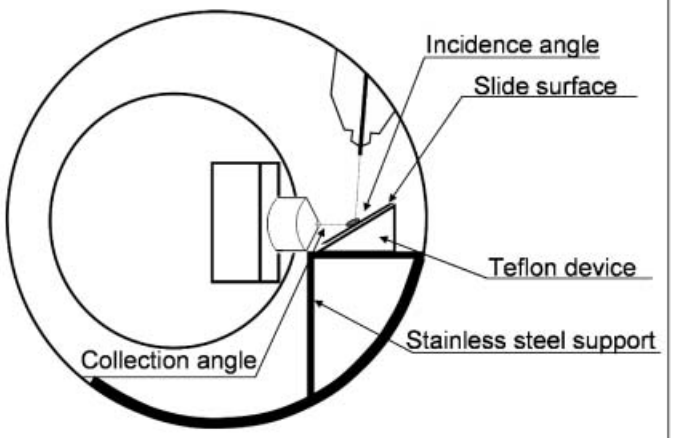

Figure 1. Parameters optimized for the analysis of amino acids and DESI device representation.

probe by a syringe pump directly onto the sample or onto the standard solution spotted on the sample holder. The investigated mass range was from *m/z* 115–195 and the scan time and interscan delay were 1 and 0.1 s, respectively. A neutral loss scan of H_2O and CO (46 Da) was used setting the cone voltage at 25 V, collision energy at 12 eV and the gas cell Pirani gauge at 2×10^{-3} mbar using argon as the collision gas; these values represent the best compromise of settings investigated for each AA that ranged from 20 to 35 V, 8 to 20 eV and 1.5 to 3.5×10^{-3} mbar for cone voltage, collision energy and gas cell Pirani, respectively. The DESI-MS parameters were optimized by varying the collection angle between the inlet of mass spectrometer and the sample from 5 to 50° while the incidence angle between sprayer and sample was varied from 90 to 40°. The sample-inlet distance, inlet-surface distance and sprayer-surface distance were set at about 4, 2 and 9 mm, respectively (table inset in Fig. 1). The source temperature, desolvation gas flow rate, desolvation gas temperature, cone gas flow rate and gas nebulizer pressure were set at 150°C, 400 L/h, 400°C, 100 L/h and 80 psi, respectively. A slide of glass, steel, PTFE or a piece of paper, as the sample holder (20 mm × 25 mm) was located on the Teflon device (Fig. 1). An equivalent mixture of water/acetonitrile or water/methanol containing a variable percentage of formic acid (from 0.2% to 2%) was used as the spray solvent for all experiments. Evaluation of different solvent mixtures was performed spotting onto a glass slide 4 μL of the AA solution at concentrations of 300, 160, 35, 70 and 100 μM for Val, Leu, Met, Phe and Tyr, respectively. These concentrations were selected because they represent the mean or the upper borderline limit of AA concentration in the human plasma of reference subjects. The DESI-MS settings were evaluated by spotting 4 μL of the working solution onto a glass slide. The electrospray ionization parameters for flow injection analysis (ESI-FIA) were optimized at a capillary voltage of 3.30 kV and source temperature, desolvation gas flow rate, desolvation gas temperature, cone gas flow rate and gas nebulizer pressure of 100°C, 300 L/h, 250°C, 40 L/h and 80 psi, respectively. ESI-FIA spectra were acquired for 2 min using water/acetonitrile/formic acid (50/50/2%) at a flow rate of 2 μL/min, analyzing an equimolar working solution (50 μM).

Sample preparation

Working solutions and calibrators were spotted directly onto a glass slide (4 μL) without sample preparation. Extraction of amino acids from a dried blood spot (3 mm Ø, 3.1 μL of whole blood) was performed by adding 20 μL of methanol containing internal standards to a blood spot and incubating for 10 min in an ultrasonic bath. Then, 4 μL of the supernatant were spotted onto a glass slide.

RESULTS AND DISCUSSION

DESI device and sample holder surfaces optimization

A device to support the sample holder was built in-house using stainless steel and a Teflon block as depicted in Fig. 1. This device was placed into the source enclosure of a triple quadrupole mass spectrometer. The geometric form of the device permits us to change angles and distances from the cone source and ESI tip simply by rotating its base. Different angles and distances were tested and then optimized with a mixture of AA standards. The DESI-MS analysis was optimized by rotating the base, clockwise or counter-clockwise, to change both the incidence angle of the ESI spray and the ion collection angle, to obtain the best signal intensities (box inset in Fig. 1). In addition, fine regulation of the incidence angle was obtained by moving the external micrometer of the ESI probe. The angle of incidence was set at 57° and the angle of collection at 30°; the distances of the sprayer from surface, of the inlet from the sample and of the inlet from the surface were set at 9, 4 and 2 mm, respectively. These parameters are in agreement with those reported by others,[3–5] although their results were obtained with different devices and mass spectrometers. We found that the ionization and desorption efficiency of the DESI system also depends strongly on instrumental parameters such as distances and angles among the ESI probe, ion selector

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and sample (surface) and it also depends on surface materials, solvent mixtures and flow rate, in agreement with that has been reported in other studies.[3–5,24] PTFE, microscope glass, stainless steel, common paper and Whatman paper were tested as surfaces. We obtained best results using glass slides. The ion signal intensity was a maximum for glass (100%) compared with PTFE (30%), stainless steel (13%) and paper (10%). According to Kauppila *et al.*,[25] less porous surface provides a better support because it is very difficult to desorb the analytes from a more porous surface like paper. Optimized parameters for DESI and the signal intensity of each amino acid were obtained in positive ion mode using the neutral loss scan function (4 μL of AA mix spotted onto a glass slide). Table 1 summarizes the results obtained from different solvent mixtures on mass spectrometer ion signal intensities; these show that by increasing the amount of formic acid from 0.2 to 2% it is possible to improve the ion signal intensities, especially when using a (50:50) water/methanol solution. The intensities of the ions produced are influenced both by the capillary voltage (Fig. 2, left panel) and by the solvent flow rate (Fig. 2, right panel). As also reported by others, in our experiments the ion signal intensities are directly correlated to capillary voltage and inversely correlated to flow rate when using glass surfaces. The maximum capillary voltage achieved by our instrument is 5 kV, so it was not possible to investigate the signal intensity response with voltages greater than 5 kV. The poorer signal intensities obtained at high flow rates could be due to the splashing effect described by Cooks and co-workers, who showed that the solvent sprayed at high flow rate can cause the removal of sample from less porous surfaces.[3–5,25] The spot position and spot size on the slide (especially when liquid samples are investigated) are very important and can affect the ion signal intensity. Wrong positioning of deposited sample can change distances and angles, while a spot that is too large in size will not be completely covered by the spray. Both the wrong position and a too large spot size can cause a reduction in desorption efficiency and consequently a loss of sensitivity and reproducibility. When liquid samples are investigated, a drop of solution is spotted onto the slide surface; if the solvent has a high relative surface tension (such as water) it will produce small droplets and a correct spot position and spot size compared with a solvent with lower surface tension (such as methanol). The average spot diameter for our experiments was less than 4 mm.

MS specificity evaluation and optimization

To evaluate the specificity of our method a tandem mass spectrometry collision-induced dissociation study was performed by spotting the mix of amino acids onto a glass slide, selecting the protonated molecule of each amino acid and fragmenting this precursor ion to give its product ion spectrum. The spectra obtained by DESI were compared with those obtained by ESI-FIA performed with the same solution (2 μL/min) using the optimized mass spectrometry settings applied routinely in our laboratory for these amino acids. The product ion spectra for Phe and Met are depicted in Fig. 3; in both compounds the spectra obtained by DESI (Figs. 3(a) and 3(b)) and by ESI-FIA (Figs. 3(c) and 3(d)) are similar. Moreover, it is known that a protonated AA produces a neutral loss of 46 Da. In our DESI and ESI-FIA experiments, the spectra of all AA display an abundant $[M+H-46]^+$ ion; e.g. the Phe and Met spectra, reported in Fig. 3, show product ions of *m/z* 120 and 104, respectively. Figure 4 shows a neutral loss (NL) spectrum of the AA mixture obtained by DESI and compared with that obtained by ESI-FIA, providing a further evaluation of specificity. The ESI-FIA experiment was performed on an equimolar mixture of Val, Leu, Met, Phe and Tyr (50 μM) prepared in water and pumped by a syringe into the mass spectrometer, while 4 μL of the same mixture were spotted onto a glass slide for the DESI analysis. Comparison of the 46 Da neutral loss spectra shows that different sensitivities were achieved for each AA. Because each AA needs different collision energy and cone voltage values, to provide an intense signal, the experiments on the AA mix have been performed using collision energy and cone voltage values that represent a compromise among the best values obtained from each single analysis. Comparing the spectra obtained by ESI-FIA and by DESI it is possible to recognize the same AA profile.

Quantitative AA study by neutral loss analysis

Amino acids evaluated in this study were selected because they are markers of four important IEM. Val and Leu are used for diagnosis of MSUD and Phe, Tyr and Met are used for diagnosis of phenylketonuria, tyrosinemia and

Table 1. Different solvent mixtures sprayed on 4 μL of spotted amino acid solution containing different amounts of Val, Leu, Met, Phe and Tyr. A flow rate of 2 μL/min was used and results are shown as percentage relative to the most intense signal obtained for each amino acid

Solvent	Formic acid %	Val % (300 μM)	Leu % (160 μM)	Met % (35 μM)	Phe % (70 μM)	Tyr % (100 μM)
H_2O/MeOH	2	100*	100*	100*	100*	100*
H_2O/MeOH	1	54	80	59	85	75
H_2O/MeOH	0.5	56	63	22	30	55
H_2O/MeOH	0.2	19	32	23	27	55
H_2O/ACN	2	15	16	15	14	22
H_2O/ACN	1	17	26	22	21	41
H_2O/ACN	0.5	16	24	19	19	37
H_2O/ACN	0.2	13	17	5	12	2

* The most intense signals obtained (100%) were 2220000 BPI for Val, 1130000 BPI for Leu, 168000 BPI for Met, 1080000 BPI for Phe, and 283000 BPI for Tyr.

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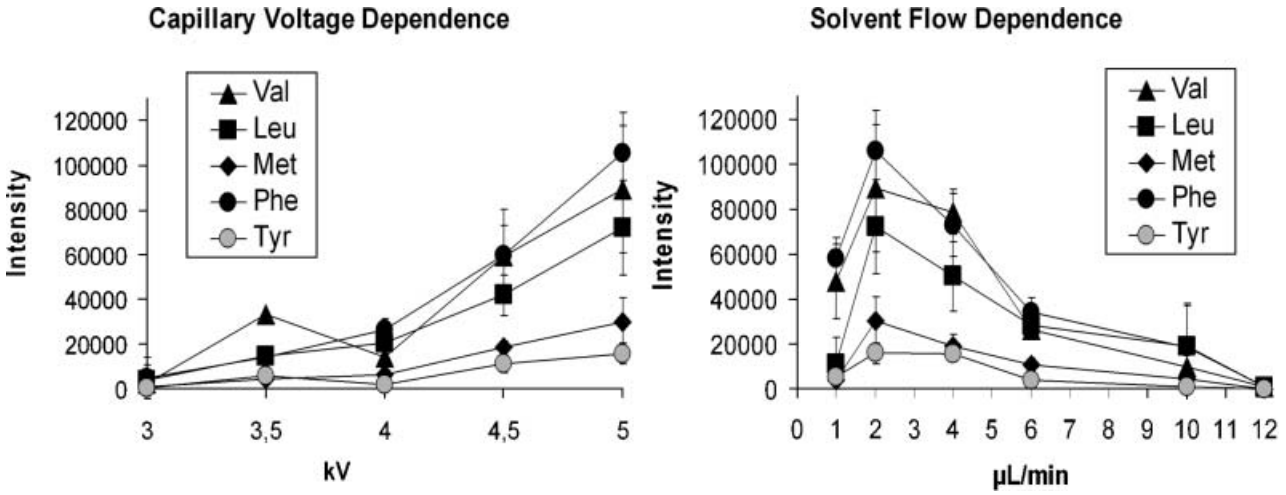

Figure 2. Effects of different capillary voltages on amino acid intensities (left) and effects of different flow rates using water/methanol (50:50) doped with formic acid (2%) as solvent spray (right).

homocystinuria, respectively. Calibration curves for standard AA were obtained by plotting the AA/internal standard peak ratio signal intensities versus the amount of AA deposited (pmol). Val, Leu, Met, Phe and Tyr in the NL scan show a linear response that ranged from 40 to 200 pmol for each calibration curve (Table 2). As reported in Table 2 the regression coefficients for Val and Leu display a high variability compared with those of Met, Phe, Tyr, which show good linearity. This variability could depend on various factors that should be further investigated, including some preanalytical variables such as the efficiency of extraction, solubility between analytes and internal standards, and their overlapping molecular weight (e.g. $^2H_2$-ornithine (134 Da), contained in the mixture of internal standards used in this study, has the same molecular weight as $^2H_3$-leucine). This aspect represents a limitation of the study and needs further investigations to better optimize the quantitative analysis of this method.

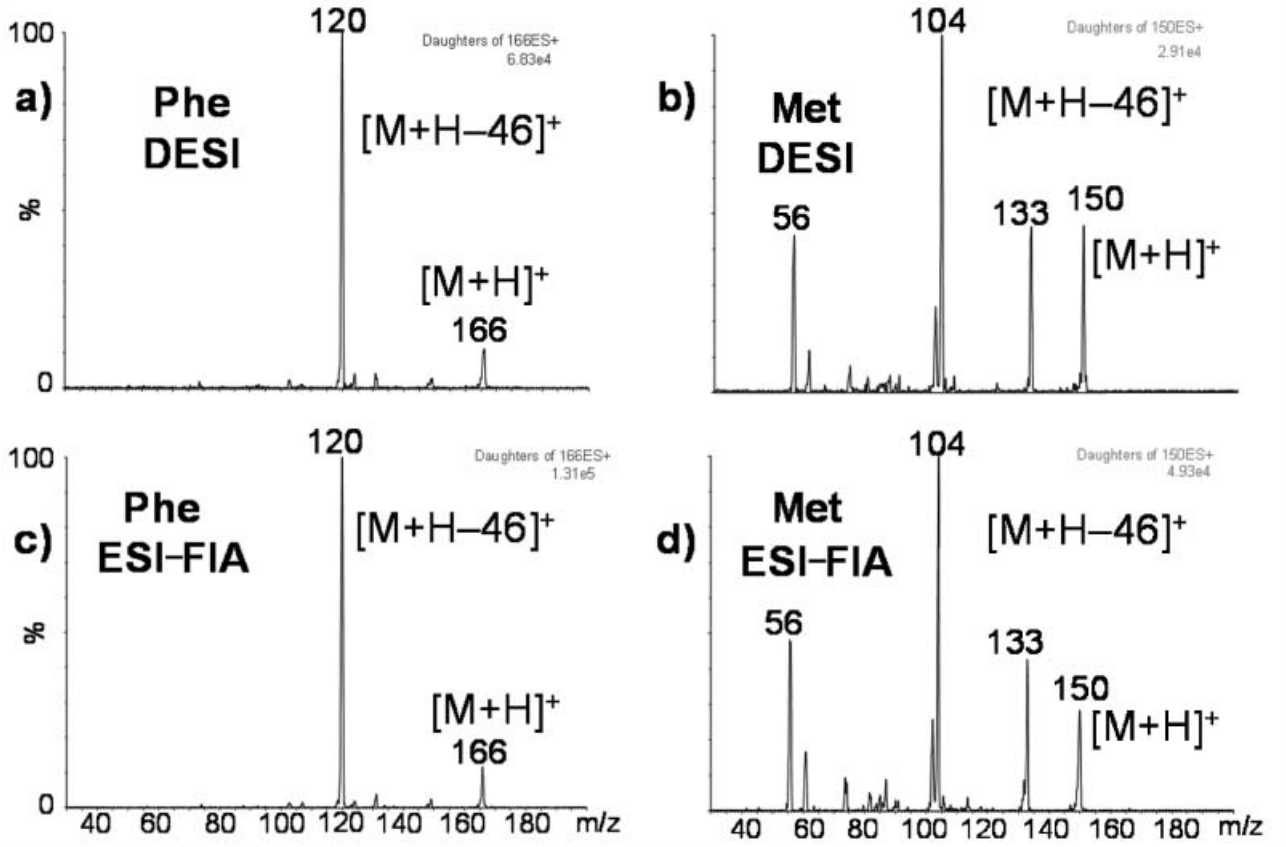

Figure 3. Product ion spectra by DESI of protonated Phe (a) and Met (b) compared with those obtained by ESI-FIA of protonated Phe (c) and Met (d).

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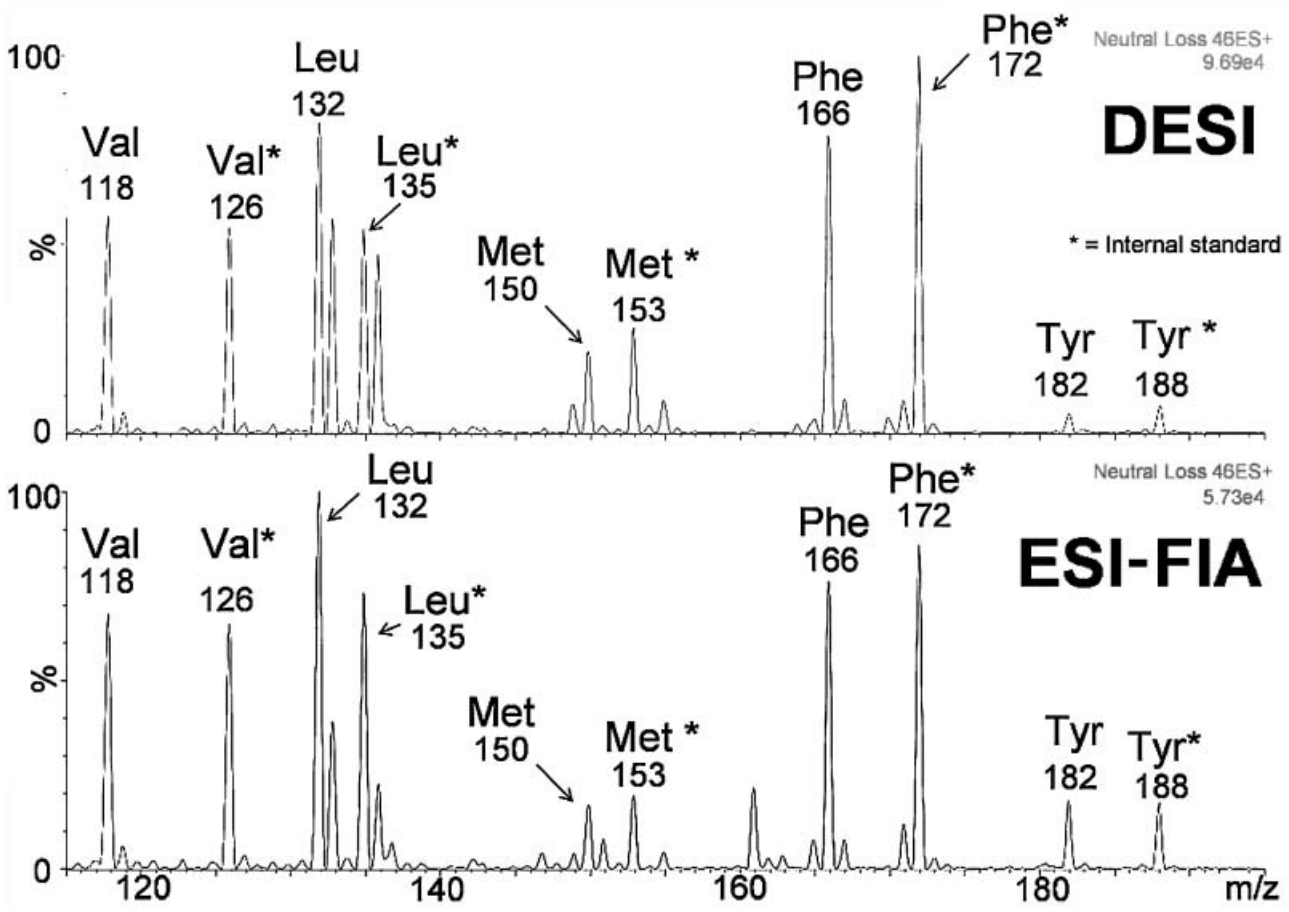

Figure 4. Comparison of Neutral Loss spectra obtained by DESI and by ESI-FIA. An equimolar mixture (50 μM) containing Val, Leu, Met, Phe, Tyr and relative internal labelled standards were used.

DESI analysis of AA was performed both directly on intact dried blood spots and on solution obtained after AA extraction from a dried blood spot. Analyses performed directly on a dried blood spot do not produce AA signals of adequate intensity and reproducibility, while by spotting 4 μL of the AA solution after extraction it is possible to obtain signals with good stability and intensity. Moreover, the extraction was performed using a solution containing a mixture of internal standards for the quantitative assay. In some experiments the solvent containing internal standards was also sprayed directly from the ESI probe onto the sample surface but this method does not produce accurate and reproducible results. Data from normal subjects and from patients affected by MSUD analyzed by DESI are shown in Fig. 5. The concentrations of Val and Leu in a healthy subject were on average (mean ± standard deviation (SD)) 137 ± 15 μmol/L (n = 5, coefficient of variance (CV) = 11%) and 178 ± 6 μmol/L (n = 5, CV = 3.2%), respectively. The inaccuracies of the Val and Leu measurements were −6% and −29%, respectively. These data suggest that the DESI method allows us to perform a 'semi-quantitative' analysis for these AA. Moreover, the Val and Leu concentrations in a MSUD patient were 522 ± 18 μmol/L (n = 3, CV = 3.5%) and 449 ± 69 μmol/L (n = 3, CV = 15%) and the inaccuracies were −29% and −31%, respectively. These values, even if underestimated compared with results from the reference method (ESI-FIA), are at least two times higher than the upper limit of our reference values and are clearly diagnostic of the disease.

In addition to what has been considered in this study, ions relating to other AA are also present and quantifiable in the NL scan spectrum, reported in Fig. 5, where the ion at *m/z* 116

Table 2. Slopes, intercepts and correlation coefficients as average values ± SD of four different calibration curves of investigated AA. The range of amounts applied was from 40 to 200 pmol for each amino acid. Data shown were calculated from signals of Neutral Loss scan spectra using the AA/internal standard peak ratio intensities versus the pmol of AA analyzed

Amino acid	n	Slope ± SD	Intercept ± SD	r ± SD
Val	4	0.0041 ± 0.0002	0.1606 ± 0.1142	0.9698 ± 0.0113
Leu	4	0.0046 ± 0.0010	0.2074 ± 0.0204	0.9114 ± 0.0466
Met	4	0.0044 ± 0.0004	0.0973 ± 0.0470	0.9941 ± 0.0043
Phe	4	0.0048 ± 0.0005	0.1129 ± 0.1855	0.9954 ± 0.0035
Tyr	4	0.0050 ± 0.0011	0.1201 ± 0.0427	0.9932 ± 0.0037

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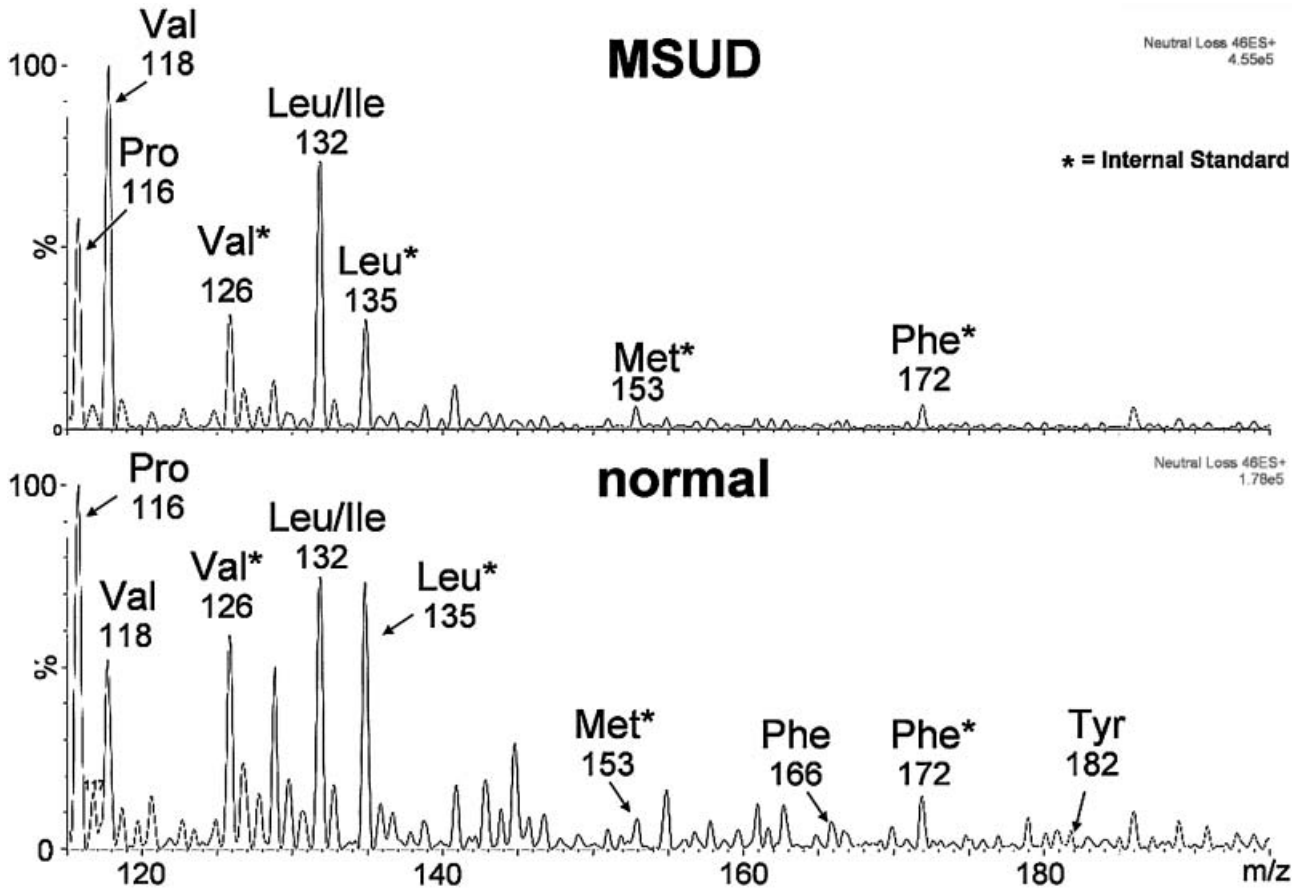

Figure 5. Comparison between normal and pathological patients samples (maple syrup urine disease, MSUD) analyzed by Neutral Loss DESI. The MSUD spectrum clearly displays an increase in Val and Leu signals. In addition, an appreciable peak for proline is also displayed in the figure.

is from proline. We have demonstrated, for the first time, in this study the acquisition of DESI NL spectra by an unmodified ESI-MS/MS instrument and without any modification of the ion source and selector. Our system works with a capillary ESI probe with an i.d. of 230 micron and with a nitrogen back-pressure of 80 psi, a value less than half that of other systems.[3,26] Our results also show a good MS specificity; in fact the spectra obtained by DESI in this study, both as product ions and as neutral loss, are comparable with those obtained using ESI-FIA. The qualitative results obtained in this work are very satisfactory and in agreement with previous work.[3–5,26] The calibration curves and samples analysis results display good quantitative response except for Val and Leu where the results could be influenced by some preanalytical factors and internal standard used in this study that affect their precision and accuracy.

CONCLUSIONS

According to others,[3,4] and as demonstrated in this method, the desorption electrospray ionization (DESI) of small and polar molecules from the solid state is easy and reproducible. The method presented here is able to distinguish pathological profiles from normal profiles although some qualitative/quantitative aspects should be improved and optimized for the screening of some inherited errors of metabolism. In our method, operating in manual mode, the turnaround time is about 12 min for 1 sample and 120 min for 50 samples and this should be improved by the introduction of an automated DESI device to obtain extremely high productivity. Finally, since the DESI device is lodged inside the source enclosure of our instrument, it prevents any risks during the analysis of biohazard materials.

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