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Article *in* Nature Protocol · February 2008

DOI: 10.1038/nprot.2008.109 · Source: PubMed

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Neutral desorption sampling of biological surfaces for rapid chemical characterization by extractive electrospray ionization mass spectrometry

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Published online 28 August 2008; doi:10.1038/nprot.2008.109

Extractive electrospray ionization mass spectrometry (EESI-MS) allows the real-time, direct analysis of complex gaseous and liquid samples without any sample pretreatment under ambient conditions. By using a neutral desorption (ND) sampling gas beam to gently impact a surface, the analyte present on a surface can be efficiently sampled and then transported to the EESI source for soft ionization without any previous work-up. By separating the sampling process and the ionization process in both space and time, ion suppression effects are significantly decreased in ND-EESI-MS. Therefore, virtually any surface can be gently sampled in real time, allowing *in vivo* analysis of living objects while maintaining the native conditions of the sample. This greatly simplifies the procedure for the characterization of complex surfaces (e.g., plants, food, skin) and prevents potential chemical contamination, providing a unique platform for wide applications in multiple disciplines such as chemistry, biology and the life sciences. Here we present a complete description of a protocol for ND-EESI-MS for *in vivo* analysis of living objects, including equipment setup, reagent preparation, data acquisition and analysis steps. The data collection can be completed within a few minutes even though the time required for the entire analytical process, which largely depends on the preparation of samples and materials, takes slightly longer.

INTRODUCTION

Mass spectrometry provides high sensitivity and high specificity for most measurements and is currently widely used as a powerful tool for measurements in life sciences[1], biological engineering[2,3], clinical diagnosis[4–7] and in the pharmaceutical industry[8–12]. One of the trends in MS involves creating sample ions at ambient conditions outside the vacuum system, facilitating high-throughput analysis[13–18]. A number of novel methods, including desorption electrospray ionization pioneered by Cooks[13,14,19], direct analysis in real time[15] and desorption atmospheric pressure chemical ionization[16–18], allow the direct analysis of complex surfaces by ambient MS[20]. These new desorption/ionization techniques have been demonstrated to be useful tools for high-throughput analyses of surfaces without sample pretreatment. In general, these methods are for the analysis of the molecular composition of solid surfaces, although volatile samples that are adsorbed to the surface of solids or embedded in the bulk can also be detected.

Motivated by the need for the direct analysis of liquid samples in complex matrices, EESI[21] was introduced in 2006. In EESI, neutral samples that may include a complex matrix are directly injected into an electrospray plume. Analyte ions are created by interactions and collisions occurring between the neutral molecules and the primary ions produced by electrospraying a solvent (e.g., an acetic acid/methanol water solution). Although EESI is a variant of electrospray ionization (ESI), ion suppression effects are drastically reduced because the matrix is dispersed over a relatively large volume. Complex samples such as urine or milk can be directly infused in neutral form into an EESI source. It is possible to obtain a constant signal for more than 7 h (refs. 21–23), with a sensitivity similar to that of ESI. This is only possible in ESI with clean samples[21]. In addition to its analytical applications, EESI has also been used to reduce the charge state of biopolymers either by ion/molecule reactions[24] or ion–ion reactions[25] at ambient pressure. Another merit of EESI is that the neutral sample (e.g., biological material) is safely isolated from high voltage or direct bombardment by charged particles. Furthermore, EESI can be implemented on commercially available instruments without any hardware modification. EESI has been used for *in vivo* chemical fingerprinting of volatile and nonvolatile compounds in breath[26], successfully demonstrating that metabolic changes can be easily tracked.

Most biological samples are complex mixtures of fluids supported by a solid structure such as tissue, skin, blood vessels and so on. It is highly desirable to perform direct *in vivo* mass spectrometric analysis of biological samples, especially the rapid characterization of the molecular composition on the surface of living objects. To perform *in vivo* EESI mass spectrometric analysis without changing the native conditions of a biological sample, a neutral gas beam was used to gently desorb compounds from biological surfaces such as human skin, animal tissues (e.g., fish meat, pork, lamb and so on), or plant leaves. The analytes were then transported to an EESI source for ionization. This is thus a novel platform on the basis of ND sampling EESI-MS, where the desorption process is separated from the extractive electrospray ionization process in both space and time, resulting in the minimization of ion suppression effects and the possibility of remote monitoring of samples (e.g., explosives) under extreme conditions (e.g., biohazards)[27]. ND-EESI analysis on living objects can be performed while maintaining the integrity of the sample completely.

Human skin, frozen meat and vegetable leaves have been successfully analyzed by ND-EESI-MS implemented on a commercial electrospray ionization–quadrupole time-of-flight–mass spectrometry (ESI-QTOF-MS) instrument[28,29]. Ion formation in ND-EESI does not result in serious matrix and memory effects, allowing for the sensitive detection of both volatile and nonvolatile, fairly high molecular weight ($>1{,}000$ Da) compounds in biological

samples[29]. Recently, ND-EESI-MS was applied to investigate pharmaceutical compounds in commercial drugs using a hybrid quadrupole traveling-wave-based ion mobility TOF mass spectrometer[30]. ND-EESI-MS has also been carried out using a homemade EESI source coupled to a commercial ion trap mass spectrometer (LTQ, Finnigan). This method of mixture analysis with tandem mass spectrometry was used for the direct sensitive detection of various explosives on skin surfaces[27].

Many promising applications of ND-EESI can be anticipated in the near future. A complete description of the protocol for the ND-EESI-MS for the rapid analysis of living objects, including the experimental setup, reagent preparation, data acquisition and analysis steps, is presented in this contribution.

Protocol

This protocol details the application of ND-EESI-MS for gentle sampling of biological surfaces in the rapid characterization of living objects, with an emphasis on small molecules.

Theoretically, virtually any neutral gas can be used for desorption. However, air or nitrogen gas is preferably used to prevent chemical contamination, which may cause a biological response of the living object and disturb its native physiological/pathogenic state. Depending on the specific application, chemically selective reagents instead of an airflow can also be used. This is useful for the selective detection of compounds of interest in samples where the native conditions of the sample do not have to be maintained.

Using the protocol provided here, it is possible to obtain mass spectrometric fingerprints of a living object. It is also possible to perform semiquantitative analysis of specific compounds from a living object, although this is not demonstrated in this protocol. In addition, information related to molecular structures can also be obtained by coupling the ND-EESI to a tandem mass analyzer (e.g., an ion trap or a multiple-stage quadrupole mass filter). By adding reactive reagents into the electrospray solution, selective ion/molecule reactions can also be implemented in the EESI process to improve the specificity. This has been demonstrated for selective ionization of nonpolar species in human breath[26] and the selective detection of explosives on biological surfaces with complex matrices[27]. However, in the examples described, the protocol is based on the general procedure that involves neutral desorption by nitrogen and extractive ionization using an ESI plume obtained by spraying slightly acidified water.

Extractive electrospray ionization has already been coupled to many types of instruments, including ion trap mass spectrometers. Its sensitivity is determined both by the EESI source and the mass spectrometer used. To date, the best sensitivity, comparable with that of ESI for most compounds tested[21–23], was achieved on Finnigan LTQ (ion trap) mass spectrometers. Another advantage of ion traps is that multistage tandem mass spectrometry can be easily performed with a single mass analyzer. The MS^n capability is required to eliminate false-positive signals, particularly in cases where unknown samples with complex matrices are directly analyzed by this protocol. However, similar to other ambient techniques[13–18], authentic compounds are generally required to confirm the identity of analytes in EESI-MS. In positive ion mode, the EESI process produces and protonates analyte molecules that are then detected. Compounds with low proton affinities will therefore not be detectable with high sensitivity. Alternative strategies such as selective cationization[26] can be employed for ionizing nonpolar compounds. In other words, EESI-MS is not a universal method that detects all possible compounds on biological surfaces. Also, the reproducibility of ND-EESI-MS for analysis of living objects has not been systematically tested. On the basis of what has been done, the EESI-MS holds promise for quantitative analysis, but reliable quantitative results can be obtained only when the stability of the ND-EESI source is further improved.

Experimental design

Our main goal was to use EESI for rapid, *in situ* qualitative analysis of a wide range of biological surfaces, e.g., for the detection of metabolites on skin or products from bacterial infestation on meat and vegetables. This could in principle be achieved by seeking easily detectable marker compounds and proving their identity by comparison with reference compounds in tandem-MS experiments.

However, it turned out that a lot more information is available in the ND-EESI data. Therefore, pattern recognition and chemometric analysis of the data was preferred in many cases, and only a small subset of the detected compounds were identified[28]. The reproducibility and sensitivity of the experiments are important for this chemometric approach. Reproducible results were fairly easily obtained when the experimental conditions were well controlled. In terms of sensitivity, biogenic amines spiked onto the frozen surface of meat were detectable at the sub-nanogram level with a signal-to-noise ratio ≥ 3 (see ref. 28). Mass spectral scans were typically averaged for 1–2 min. About 10–15 measurements were carried out per sample as each data point required at least five replicates for statistically meaningful results. To date, this protocol has only been used for qualitative analysis. Calibration can be done with external standards or by adding internal standards to the samples for quantitative analysis. Accurate quantitative analysis of analytes in biological samples should become possible once the procedure is properly validated against other methods.

MATERIALS

REAGENTS

! CAUTION For your safety, please read the Material Safety Data Sheet (MSDS) for every chemical reagent before use!

- Skin samples: the skin of healthy, nonsmoking, male volunteers were analyzed directly from a part of the body (e.g., the arm; no tissue had to be removed for the analysis). The sampled area of skin was about 10–25 mm^2
- Meat samples: St Peters Fish, fresh and packaged, bought from a local store
- Water (Ultrapure; 18 MΩ cm^{-1})
- Methanol (HPLC grade) **! CAUTION** Flammable; causes eye and skin irritation; harmful if swallowed; avoid contact with the eyes, skin and clothes.
- Acetic acid (HPLC grade) **! CAUTION** Poisonous and corrosive. Liquid and mist cause severe burns to all body tissue. May be fatal if swallowed. Harmful if inhaled. Inhalation may cause lung and tooth damage. Flammable liquid and vapor. Contact with eyes may cause irritation.
- Nicotine (analytical reagent grade) **! CAUTION** Poisonous—may be fatal if inhaled, swallowed or absorbed through the skin. Readily absorbed through the skin. Typical permissible exposure limit is 0.08 p.p.m. Skin-rabbit lethal dose (LD50) is 50 mg kg^{-1}.
- Caffeine (analytical reagent grade) **! CAUTION** Harmful if swallowed. Experimental teratogen. Irritant in humans.
- Ethanol (75% (vol/vol), Medical grade): bought from a local hospital **! CAUTION** Flammable. Contact with eyes may cause irritation.
- Cotton tampon (Medical grade): bought from a local hospital.

- Dry ice: bought from departmental stockroom ! CAUTION Dry ice is extremely cold (−109 °F, −79 °C) and can cause severe frostbite within seconds of direct contact. Avoid contact with skin and eyes. Never handle dry ice with your bare hands. Do not put dry ice in your mouth or ingest it.
- Compressed nitrogen (99.995%) ! CAUTION May cause asphyxiation in high concentrations.
- *E. coli* strains: *E. coli TG1* strains. This material is only toxic in large quantities or if open wounds are exposed to it. More information on bacteria in risk group 2 can be found on the following websites: http://www.nih.gov/ and http://www.ncbi.nlm.nih.gov/
- Spinach leaves: bought from a local supermarket

EQUIPMENT

- Centrifuge (Allegra X-22 Series bench-top centrifuge, F0850 Fixed-Angle Rotor, part number 364640)
- Freezer: the temperature should go down to −20 °C
- Syringes (Hamilton, cat. nos. 81165 and 81265)
- Syringe pumps (Harvard)
- Water bath and controller: laboratory equipment. Temperature adjustable from room temperature (22 °C) to 90 °C
- Vegetable container: Teflon plastic boxes with a volume of about 1.5 liters, bought from the local supermarket
- Dry ice container: Teflon plastic box with a large volume of about 3 liters, bought from the local supermarket
- Fish meat sample container: Teflon plastic boxes with a volume of about 500 ml, bought from the local supermarket
- Temperature meters: working range from −40 to 60 °C
- Mass spectrometer (ESI-QTOF Ultima, Waters Micromass)
- ND device (homemade device consisting of the sharp jet tube and the sample collector). See EQUIPMENT SETUP.
- EESI source (homemade by implementing EESI on a commercial TOF mass spectrometer). See EQUIPMENT SETUP.

REAGENT SETUP

Meat sample Fish meat samples were cut into pieces (25–50 g each) to facilitate handling during the ND process. Before use, all fish meat samples were kept frozen at −20 °C in sealed Teflon bags. Different spoilage stages of the fish meat samples were generated by exposing the samples to air at room temperature (22 °C) for 0, 1 or 2 d. All meat samples were maintained at −20 °C (using an air bath controlled by dry ice) during measurements. It should be noted that while still frozen, there was no difference in smell between the different fish meat samples.

Skin sample: depending on the purpose of the application, cleaning of skin before analysis might be required For example, to sample metabolites excreted from the skin, it is highly recommended to clean the skin surface to eliminate previously accumulated contamination. Ethanol (75% vol/vol) aqueous solution can be used to clean the skin samples. Although the skin can be directly analyzed by ND after cleaning, it is desirable to wait for 15–30 min after wiping the skin to allow for the metabolites to be excreted again. Note that exposure of the cleaned skin surface to any chemicals (e.g., air containing chemical aerosols) should be avoided before analysis.

▲ CRITICAL The skin surface should not be cleaned by any chemical solution if one is attempting to detect unnatural contaminants (e.g., explosives, pesticides and so on) accumulated on the skin. Although the method is completely safe and does not involve any chemical contact other than a stream of air, special permission may still be required by some institutions or in some countries before testing human subjects.

***E. coli* strains** All the *E. coli* cells used were of the *E. coli TG1* strain, but in principle, any type of *E. coli* strain can be used. All the *E. coli* strains should be separated from their medium by centrifugation at 50–500*g* (5–10 min for the centrifuging process) at room temperature followed by gentle washing with water (three times; 5–10 min for the entire washing process). No cell lysis was observed.

Spinach leaves Spinach samples were purchased from a local food store and separated into an experimental and a control group (150 g each). The control group was kept at 5 °C before use to keep it fresh. The experimental group was sprayed with a suspension of *E. coli* (e.g., 10 mg in 5 ml of water), making sure that the *E. coli* cells were evenly distributed on all the leaves. ▲ CRITICAL The *E. coli* should be highly metabolically active and the sample for examination should be well contaminated by the *E. coli* strains. Contaminated samples were kept at 30 °C for 6–10 h before analysis. The incubation time is heavily dependent on the activity of the *E. coli* strains and the environmental conditions (e.g., moisture, temperature and so on). Although all the spinach sample surfaces were still wet when analyzed, they were directly used without further treatment.

EQUIPMENT SETUP

Sharp jet tube (custom-built device) A gas emitter, a cone-shaped tube with a sharp tip, made from either Teflon or stainless steel, is connected to a Teflon gas tube, which supplies a nitrogen gas beam. The inner diameter of the gas emitter tip of the stainless steel ND device was 600 μm and the outer diameter was 1.2 mm. The gas emitter can be designed to be even sharper than the ones described here; however, such delicate parts are more difficult to fabricate.

! CAUTION The stainless steel gas emitter should be used with caution to avoid any potential injury caused by the sharp stainless steel edges. To simplify the machining of the required equipment, a commercially available micropipette (10 μl) can also be used as a gas emitter. The above-mentioned homemade plastic gas emitter, together with the sample collector in a V-shaped configuration for sampling the skin of a hand is shown in **Figure 1a**.

Sample collector (custom-built device) For neutral desorption sampling, a neutral gas beam from the sharp gas emitter impacts the sample surface such that neutral molecules are released from the sample surface. The molecules are then collected by a sample collector, of about 5-cm length, that tapers from a big opening (inner diameter 7.8 mm, outer diameter 9 mm) to a narrow opening (inner diameter 3.8 mm, outer diameter 5 mm). As shown in **Figure 1a**, the sample collector can simply consist of a commercially available Teflon pipette tube (1 ml). The narrow end of the sample collector was connected to the desolvation gas line (Teflon tube, inner diameter 5 mm; length 50 cm) of the QTOF-MS instrument. This allows the sample plume generated by the ND process to enter the wide end of the sample collector before reaching the EESI source through the desolvation gas line of the instrument. Note that the inner diameter of the wide end of the sample collector can vary from 8 to 12 mm, whereas the outer diameter may vary from 9 to 13 mm. No serious signal variation was detected in our experiments with these dimensions. The sample collector can also be made from stainless steel. A homemade stainless steel sample collector is shown in **Figure 1b**. All the parameters for optimization of the ND sampling can be precisely adjusted using the prototype, and an improved long term stability of ND-EESI-MS can be anticipated. However, the use of a Teflon pipette simplifies the development of the neutral sample collector, as such pipettes are widely available and no precise machining is required. Therefore, it is advised to use a commercial micropipette (1 ml) as the sample collector for the first tries. All the data shown in this protocol was obtained using a plastic ND device like the one shown in **Figure 1a**. ▲ CRITICAL Before starting a measurement, ensure that the ND device is properly connected to the gas supplier and the desolvation gas line.

Implementing EESI on a commercial ESI interface The EESI can be implemented on a commercial ESI-TOF-MS instrument without any hardware modification (shown in **Fig. 2**). Breath samples or analytes liberated from a surface are directly introduced in gaseous or aerosol form into the ESI source (maintained at 80 °C) through the desolvation gas line, which has an outlet placed orthogonally to the ESI spray. A 10% (vol/vol) acetic acid water mixture infused at 5 μl min^{-1} was used as the electrospray solution to generate protons. Analytes in the gaseous sample stream undergoing collisions with charged droplets from the ESI spray are ionized in the source, and their mass

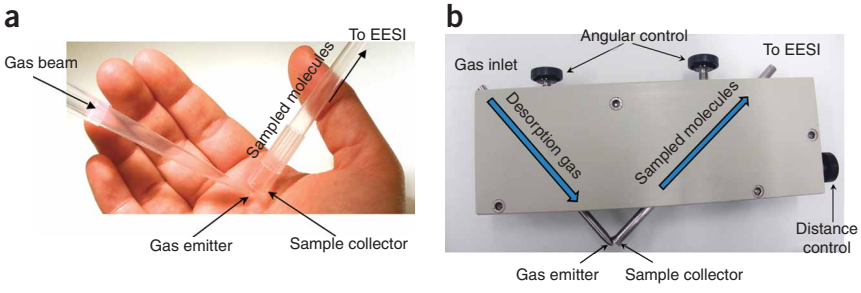

Figure 1 | Photographs of ND-EESI sampling devices. (**a**) An easily available plastic ND device for *in vivo* sampling of hand skin, consisting of a pipette tip as a gas nozzle and a plastic sleeve connected to a plastic tubing for collection of the desorbed material. (**b**) Adjustable gas emitter/collector device fabricated from stainless steel, with better control of the operating parameters.

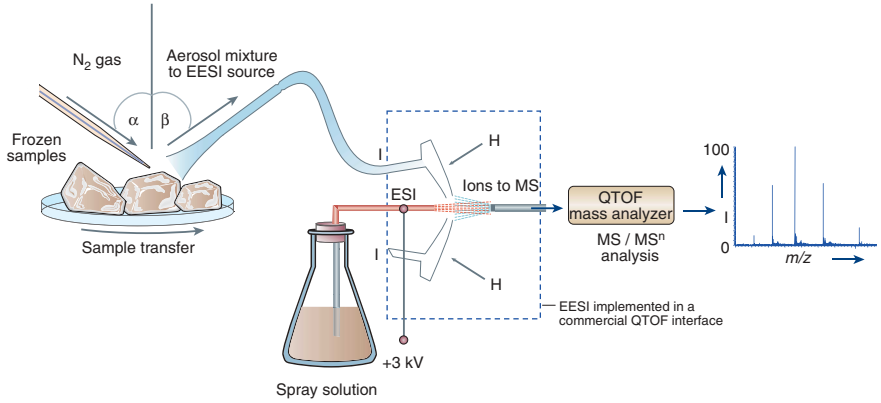

Figure 2 | Schematic diagram of atmospheric pressure ND-EESI-MS. The compounds from the surface of biological samples such as frozen meat are desorbed without sample pretreatment by nitrogen gas flow at room temperature. This creates a neutral aerosol mixture containing molecular metabolites. The aerosol was transported to the EESI source through the desolvation gas inlet (I). The aerosol passed through the heated region (H), which is maintained at 80 °C. The distance between the desorption gas flow tip and the sample surface was 2–3 mm and the desorption (α) and collecting (β) angles were set to be 30° and 45°, respectively. The aerosol transfer line used was a flexible Teflon tube (inner diameter 5 mm; length 120 cm), demonstrating the possibility for remote analysis.

spectrum is recorded by the TOF analyzer. The sensitivity of EESI could be further improved if the EESI source could be precisely adjusted (e.g., with hardware modifications).

Extractive electrospray ionization works at atmospheric pressure. In theory, any type of MS instrument with an ESI/atmospheric pressure chemical ionization source can be used as an EESI source. For example, a homemade EESI source was successfully installed on an LTQ MS instrument. EESI has also been implemented in an orbitrap mass spectrometer. EESI is a source that fits many types of mass spectrometers and is not limited to a particular kind of mass analyzer.

ND-EESI interface for surface sampling As illustrated in **Figure 2**, the ND-EESI can be carried out using the sharp gas emitter (see EQUIPMENT), the sample collector (see EQUIPMENT) and the ESI interface of the QTOF-MS instrument. A dry nitrogen gas beam at room temperature (22 °C) was used to impact the biological sample surfaces (e.g., the icy surface of frozen meat). Part of the ice on the meat surface melted owing to the impact of the warm gas flow, generating an aerosol from the liquid on the sample surface. The aerosol contains molecules like metabolites produced by the microorganisms on the meat surface, which are supplied into the EESI source through the desolvation gas line. ▲ CRITICAL The glass protection around the ESI source should be properly installed on the MS instrument to prevent any gas leakage, and the differential pumping system of the TOF-MS instrument should be properly maintained. The velocity of a typical neutral desorption gas beam is estimated to be about 10 m s^{-1}, which can be created using a relatively low nitrogen gas flow rate such as 1–3 liter min^{-1}. However, for a dry surface not covered by ice, a stronger desorption gas beam is needed for high efficiency. The sample collecting tube was tightly mounted in front of the desolvation gas line so that aerosols can be efficiently collected. The angles between the sample surface and the axes of the desorption gas flow or the sample collecting tube were optimized within the following limits: $\alpha = 20–60°$, $\beta = 60–20°$ (as shown in **Fig. 2**). $\alpha = 30°$ and $\beta = 45°$ represents a typical combination. The distance between the gas jet tip and the surface was 2–3 mm. The optimization was done to ensure that the analytes desorbed from the sample surface provide a stable high signal level of the analytes. Note that the time for optimizing the ND source varies from 1 min to 1 h and is largely dependent on the experience of the operator. It is recommended that the operator uses a standard compound for the first trial to ease the optimization process.

Samples kept under ambient conditions like spinach are directly sampled by ND in open air and at room temperature. Similar to the case of the icy meat sample, the aerosol generated in the ND process was directed to the EESI source for ionization.

EESI-TOF mass analysis An ESI-QTOF-MS instrument (QTOF Ultima, Waters Micromass) controlled by the software Mass Lynx software (version 4.0) was used. Technically, ND and EESI can be carried out easily on any mass spectrometer or ion mobility instrument equipped with an ESI source. Many other instruments (e.g., hybrid quadrupole traveling-wave-based ion mobility TOF mass spectrometers, linear ion trap mass spectrometers or similar instruments with an atmospheric pressure interface) can also be used, but some source modification may be necessary, depending on the original configuration of the source and interface of the instrument.

Recalibrating the TOF-MS instrument before EESI analysis by following the standard procedure provided by the instrument manufacturer is strongly recommended but not compulsory. A sodium formate solution (10 mg $liter^{-1}$) in 90/10 (vol/vol) 2-propanol/H_2O can be used as the standard for calibrating the low mass range. A mass accuracy of 10 p.p.m. should be achieved after the calibration. Source conditions should be similar to normal ESI operation. The electrospray was operated with a voltage of +3 kV. The solvent was introduced with an infusion rate at 2 μl min^{-1}. The source block was heated to 80 °C. The cone and first ion tunnel RF1 voltages were set to 50 and 45 V, respectively, for maximum signal intensity. Full-scan spectra were recorded without collision gas. Ions were detected with a multichannel plate detector assembly operated at 2,300 V.

Extractive electrospray ionization has been implemented on this commercially available ESI-QTOF-MS instrument without hardware modification. Collision-induced dissociation (CID) can be performed with 10–25 U of collision energy. For ionization with higher selectivity, reactive chemicals can also be added into the electrospray solvent. A successful demonstration of this variation of EESI involved using an $AgNO_3$ solution as the spray solvent for the selective ionization of sulfur-containing molecules in breath[26]. Similarly, selective desorption can be implemented during the ND process with different chemicals such as acetone. However, to ensure a 'green' analysis process for the neutral desorption sampling of living objects and to avoid contamination by toxic agents, either nitrogen or air would be recommended as the gas beam.

PROCEDURE

1| Prepare the meat, skin or vegetable samples as described in options A–C.

(A) Preparation of fish meat samples

(i) Separate the fresh fish meat into three sample groups, each containing 2–3 fish slices (20–50 g). Place the first sample into a Teflon bag and then into a freezer at −20 °C. This is marked as the fresh ('0-days') sample, indicating that it has been kept sealed in a Teflon bag at −20 °C until use and not exposed to air at room temperature.
▲ CRITICAL STEP The '0 days' fish sample should be kept truly fresh to ensure that the biomarkers of fresh fish smell can be detected.

(ii) Place the other two groups of fish samples in ambient air at room temperature (22–25 °C), noting the time at which they were taken out of the freezer.

Figure 3 | Pictures of three fish samples. (**a**) Fish meat sample exposed to room temperature for 0 d; (**b**) fish meat sample exposed to room temperature for 1 d; (**c**) fish meat sample exposed to room temperature for 2 d.

(iii) After 24 h, collect one group of fish samples and place them into a sealable Teflon bag. The fish sample is then marked with '1 day', indicating that the sample had been exposed to air at room temperature for 1 d. The sealed sample is then put into the freezer for storage at −20 °C until use.

(iv) Collect the last group of the fish samples after 48 h into the experiment and place them into a sealable Teflon bag. This sample is then marked with '2-days', indicating that the sample had been exposed to air at room temperature for 2 d. The sealed sample is then put into the freezer for storage at −20 °C until use. **Figure 3** shows photographs of the three different fish samples. Note that the three different samples could not be differentiated by their smell or color when frozen, but ND-EESI-MS could provide a good differentiation of the fish meat samples.

(B) Preparation of skin samples

(i) The skin surface was cleaned using a cotton tampon saturated with 75% ethanol (vol/vol) aqueous solution (Medical grade).

(ii) The skin surface should be kept clean and naturally warm. Exposure to a polluted environment, to cold air or to strong wind should be avoided before the experiment. A waiting time of 15–30 min is recommended after the cleaning and before the experiment to allow for the metabolites to be excreted from the body.

▲ **CRITICAL STEP** The skin surface should not be cleaned if the aim of the experiment is to detect contaminants accumulated on the skin.

(C) Preparation of vegetable samples

(i) *Preparation of E. coli for spraying on vegetable samples*. *E. coli* strains were cultivated in a microbiological laboratory by experienced specialists. Separate all the *E. coli* strains from the medium by a centrifuge operated at 50–500*g* for 10 min at room temperature. After cleaning, remove the supernatant and discard it, and flush the *E. coli* gently with water thrice. More than 20 mg of *E. coli* should be harvested for the next step. Although 20 mg represents a large excess of *E. coli*, the minimal amount of *E. coli* cells required to produce detectable signals has yet to be evaluated.

(ii) *Preparation of vegetable samples*. Separate the fresh spinach leaves into an experimental and a control group (150 g each). The control group is kept at 5 °C in a refrigerator before use.

(iii) Spray a suspension of *E. coli* (e.g., 10 mg dry cells in 5 ml of water) onto the experimental group of spinach leaves, ensuring that the *E. coli* cells are evenly distributed on all the leaves.

▲ **CRITICAL STEP** The *E. coli* cells should be highly active! The activity of E. coli cells can be tested by specialists in a biology lab.

(iv) Incubate the spinach leaves with the *E. coli* solution at 30 °C for 6–10 h before analysis. Note that the incubation time is heavily dependent on the activity of the *E. coli* strains and the environmental conditions (e.g., moisture, temperature, etc.). The spinach samples in the control group were directly used for EESI analysis without further treatment.

ND-EESI-MS experiments

2| *Optimization of the ESI source*. We recommend recalibration of the mass spectrometer before performing the ND-EESI-MS experiments (see EESI-TOF-MS analysis section for details). After calibration, clean the ESI source, cone area, capillary, syringe and other parts of the ESI source using a methanol:water (1:1) solution to remove any possible contamination incurred during the calibration process.

3| *Optimization of the EESI source*. The EESI source should work properly after proper calibration of the ESI-TOF-MS instrument. Further cleaning is usually necessary should problems arise. A standard sample (e.g., 0.001 M nicotine in methanol:water (1:1, vol/vol) solution) is suitable for EESI source optimization. A sealable flask containing 200 ml of nicotine solution should be prepared in such a way that the liquid is 5–10 cm from the top of the flask. A gas supply tube (inner diameter 3 mm, outer diameter 5 mm) should be inserted into the liquid such that the distance from the bottom of the flask to the end of the gas supply tube is about 5–10 mm (shown in **Fig. 4**). A constant gas flow (e.g., 20 ml min^{-1} from a nitrogen gas tank) should be gently bubbled through the liquid. The gas bubbles carrying nicotine molecules should be transported through another gas introduction tube (inner diameter 6 mm, outer diameter 8 mm) to the desolvation gas line of the TOF-MS instrument. Consequently,

the nicotine molecules should be introduced into the EESI source for ionization. Although the gas flow rate is kept constant, source parameters such as positions, voltages and electrospray solvent flow rate can be optimized, with the target of optimizing the stable signal level of protonated nicotine (*m/z* 163).

4| *Optimization of the ND device*. The ND should work properly under the conditions described above (see ND-EESI interface for surface sampling section for details). If the ND device is to be optimized, a standard solution of nicotine or caffeine (0.001 M, ethanol water (1:1) solution) can be used. A volume of 10 μl of the standard solution should be deposited on a surface (e.g., skin) on a spot less than 1 cm^2. The spot should then be directly placed under the sampling tip for neutral desorption. The neutral molecular species sampled from the surface should be collected by the sample collector and transferred along the desolvation gas line to the EESI source for ionization.

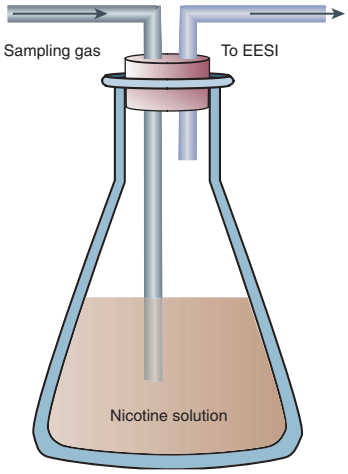

Figure 4 | Setup for introduction of nicotine as a standard compound for the optimization of EESI.

5| Keeping the EESI source parameters and the TOF-MS instrumental parameter constant, the incident angle, collecting angle, tip-to-sample distance and gas flow rates of the ND device can be further optimized if necessary, with the aim of optimizing the stable signal of protonated nicotine (*m/z* 163) or caffeine (*m/z* 195) to the highest level. The ND parameters of the examples shown are given as follows: nitrogen gas flow rate, 30 arbitrary units (directly read from the QTOF-MS instrument); incident angle (α), 30–45°; collection angle (β), 45–30°; desorption tip-to-sample distance, 2 mm; tip-to-tip distance, 1–2 mm; collecting tip-to-sample distance, 0–2 mm; length of sample transfer line (including the desolvation gas line), 30 cm.
▲ **CRITICAL STEP** It should be ensured that the source region is properly sealed and airtight before enabling the ND process. If a commercial ESI interface such as the one of the QTOF-MS is used, the glass cover of the source region should be tightly fixed to the instrument and the differential pumping system should work properly.

6| Frozen meat, skin or vegetable samples can be obtained as described in options A–C, respectively.

(A) Sampling frozen meat by ND for EESI-MS analysis

(i) The fish meat sample should be placed under the sampling tips of the ND device, and ND should work properly under the optimized experimental conditions. No further optimization is necessary as long as the experimental parameters are maintained after optimization.
▲ **CRITICAL STEP** To maintain the fish sample at −20 °C throughout the experiment, the fish sample was kept at close proximity to dry ice kept in a Teflon container supported by a lab jack. A piece of plastic foam (20 cm × 10 cm × 15 cm) was used as a spacer to allow the flow of cool air supplied by the dry ice placed under the frozen fish meat. However, the experiment can also be run if the frozen fish is directly placed on top of the dry ice.

(B) Sampling skin by ND for EESI-MS analysis

(i) The skin surface should be placed under the sampling tips of the ND device, and ND should work properly under optimized experimental conditions. No further optimization is necessary as long as the experimental parameters are maintained after optimization.

(C) Sampling vegetable leaves by ND for EESI-MS analysis

(i) Place the wet vegetable leaves directly under the sampling tips of the ND device, and ND should work properly under the optimized experimental conditions. To prolong the ND process, it is recommended that the leaves be exposed evenly to the device.

Data acquisition

7| Acquire data using the mass spectrometer's acquisition software (Mass Lynx, version 4.0, for the QTOF-MS instrument and Xcalibur 2.0 for the LTQ-MS instrument, respectively). All necessary steps and standard procedures to record a mass spectrum are described fully in the instrument manuals and should be followed exactly without any special alteration. Background subtraction was done on all spectra by acquiring 60 s of scan time with and without introducing a sample. The background subtraction algorithm from Mass Lynx software (version 4.0) was used.

Background subtraction procedure

8| For detection of trace amounts of analytes, it is necessary to subtract the spectral background. If the instrument was not cleaned properly to ensure the elimination of any possible chemical background, proper background subtraction is also necessary even for the analysis of relatively concentrated samples. The signal for background subtraction can be obtained from the background of the ESI source without introduction of the ND nitrogen gas.

The appropriate software should be used to subtract the mass spectral background. The Mass Lynx software was used for the QTOF-MS, and the Xcalibur software was used for the LTQ-MS instrument. The time course of the total ion current (TIC) should be

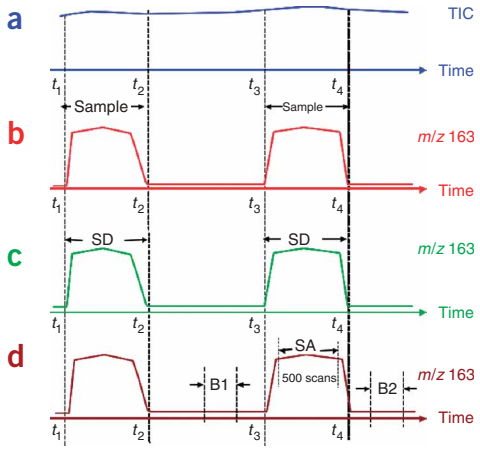

Figure 5 | Schematic diagrams to illustrate the procedure for background subtraction. SD, sample duration; SA, signal averaged; B1, background recorded before sample introduction; B2, background recorded after sample introduction; the time for B1 is equal to the time for B2, and the time for SA (e.g., 500 scans) is equal to the sum of the time for B1 (e.g., 250 scans) and B2 (e.g., 250 scans). The sample was introduced twice with equal duration, from t_1 to t_2 and from t_3 to t_4, respectively.

displayed by following the instructions detailed in the user manuals. For both the TIC trace and the mass spectrum recorded, background subtraction should be performed using the standard commands implemented in the commercial software. A typical TIC trace is shown schematically in **Figure 5a**. Note that for every time point of the TIC trace, the corresponding mass spectrum can be displayed in the MS window of the software.

To perform a background subtraction, first display a mass spectrum by choosing a single time point of the TIC trace during a period when the sample is being supplied. Numerous mass peaks should be displayed in the mass spectral window.

9| Then, locate at least one analyte signal in the mass spectrum (e.g., *m/z* 163) and display the TIC trace of an intense analyte peak (e.g., *m/z* 163). If the TIC trace corresponds well to the time of sample introduction (as shown in **Fig. 5b**), it can be concluded that the time duration of the signal is well defined (as shown in **Fig. 5c**).

10| Subtract the spectral background manually. This can be easily done using the standard commands of the software. For example, the signal of *m/z* 163 was averaged for 1 min (e.g., 500 scans), and so the background should be subtracted for the total same duration. This is usually done with 250 scans from the background recorded before the sample introduction and another 250 scans from the background recorded after the sample introduction. This is schematically shown in **Figure 5d**.
▲ **CRITICAL STEP** The time duration for the background subtracted should be exactly the same for the signal averaged! It is also recommended to subtract the background equally before and after the sample introduction.
? TROUBLESHOOTING

● TIMING

Step 1A, preparing the fish samples in various stages of spoilage: 3 d
Step 1B, preparing the skin samples: 5 min
Step 1C(i), preparing the *E. coli* samples: 2–3 d
Step 1C(ii–v), preparing the vegetable leaves contaminated by *E. coli* samples: 6–10 h
Step 2, optimizing the ESI source plus instrument calibration: 30–60 min
Step 3, optimizing the EESI source: 5–30 min
Step 4, optimizing the ND device: 5–30 min

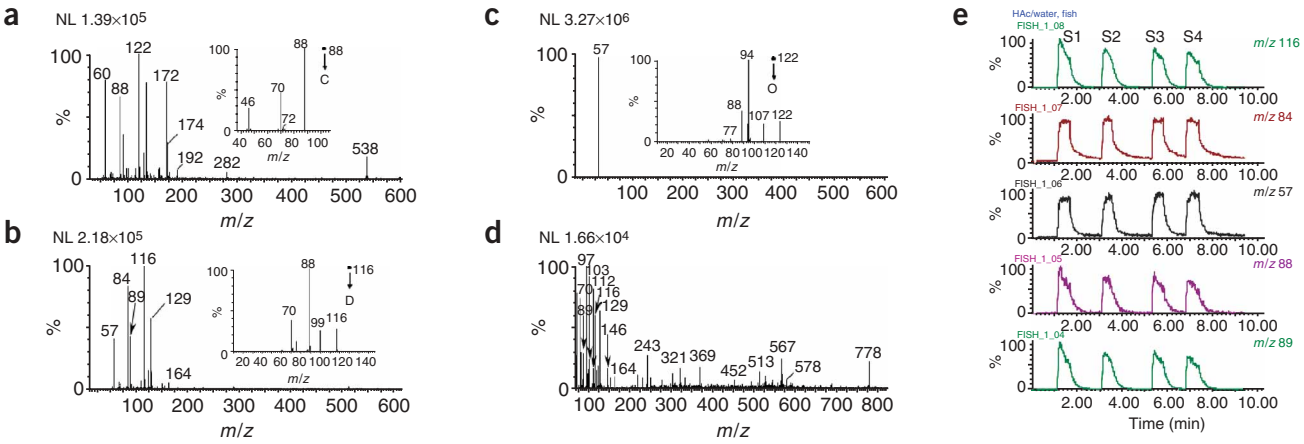

Figure 6 | Mass spectra and TIC traces of signals of frozen fish meat samples recorded by ND-EESI-MS. (**a**) Fish meat sample exposed to room temperature for 0 d; (**b**) fish meat sample exposed to room temperature for 1 d; (**c**) fish meat sample exposed to room temperature for 2 d; (**d**) zoomed view of the mass range >60 Da of the spectrum shown in **c**. Insets show the CID spectra of ions of interest. (Copyright John Wiley & Sons Limited. Reproduced with permission from ref. 29.) (**e**) TIC traces of signals detected at various *m/z* values by ND-EESI-MS using the fish sample exposed to room temperature for 1 d. (Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from ref. 28.)

Step 6, sampling of a surface by ND-EESI-MS: only a few seconds are required to record one spectrum. The time required is not dependent on the sample surface but is related to the experience of the operator
Steps 9 and 10, background subtraction: 1–3 min

? TROUBLESHOOTING

No signal obtained from the EESI source using a standard solution

- Double-check the ESI source to ensure that the ESI source works properly.
- Make sure that a proper gas stream is supplied.
- Make sure that the standard compounds can be protonated in ESI source.
- Make sure that the standard compounds are delivered to the EESI source through the sample transfer line and desolvation gas line.

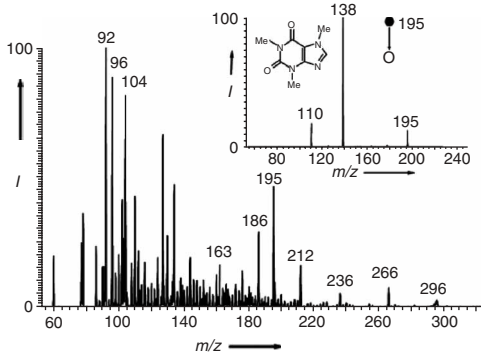

Figure 7 | Mass spectra recorded from the skin of a regular smoker after coffee consumption showing the presence of nicotine (*m/z* 163) and caffeine (*m/z* 195). Inset shows the CID spectrum of protonated caffeine (*m/z* 195). (Copyright John Wiley & Sons Limited. Reproduced with permission from ref. 29.)

No signal obtained from the ND-EESI source using a standard solution

- Double-check the EESI source as described above, to ensure that the EESI source works properly.
- Check if the glass shell of the TOF-MS instrument is tight enough and is able to seal the source region properly without any gas leakage.
- Check the sample collecting tube to ensure that the transfer line is not blocked.
- Check the differential pumping system, to ensure that all the pumps are working properly to provide a good vacuum for the instrument.

For further questions

Please direct all questions directly to one of the email addresses given below for further questions and comments: zenobi@org.chem.ethz.ch or chw8868@gmail.com.

ANTICIPATED RESULTS

The protocol presented here allows one to analyze living objects such as human skin and plant tissue in just a few seconds. **Figures 6–9** show typical mass spectra obtained after performing ND-EESI-MS on various samples such as frozen meat, human skin and plant leaves. As shown in **Figure 6**, the freshness of frozen meat products could be easily determined by the mass spectral fingerprinting of the metabolites generated by the microorganisms on the meat surfaces.

While still frozen, the meat samples could not be differentiated by smell or color. In contrast, meat samples at different ages of spoilage could be easily distinguished by ND-EESI-MS. It was not even necessary to defrost the meat samples to record ND-EESO-MS data; sufficient material was released by the impact of the ND gas jet for differentiation.

Figure 6e shows the TIC of signals detected at various *m/z* values by ND-EESI-MS using the same fish sample as that for **Figure 6b**. **Figure 6e** demonstrates that the ion signals detected from the meat samples are distinguishable from the background signals. The multiple TIC traces represents the reproducibility of the measurements. Furthermore, as the signal drops quickly when the sample is removed, this high-throughput method would be a highly anticipated analysis technique for the meat industry.

Further rapid sample analyses were carried out at room temperature on the surface of human skin. The largest organ of the human body is the skin. Sensitive detection of trace analytes on human skin can provide information about the metabolism and the external living environment. For instance, nicotine and caffeine can be seen in the mass spectra (**Fig. 7**) taken by ND-EESI of skin from a smoker after coffee consumption. Similarly, external compounds such as explosives were detected from the skin exposed to air contaminated by the explosives (shown in **Fig. 9**).

Neutral desorption–extractive electrospray ionization mass spectrometry is a gentle method that can also be used for online, real-time monitoring of living plants. An example is shown in **Figure 8**, where iceberg lettuce samples are differentiated by

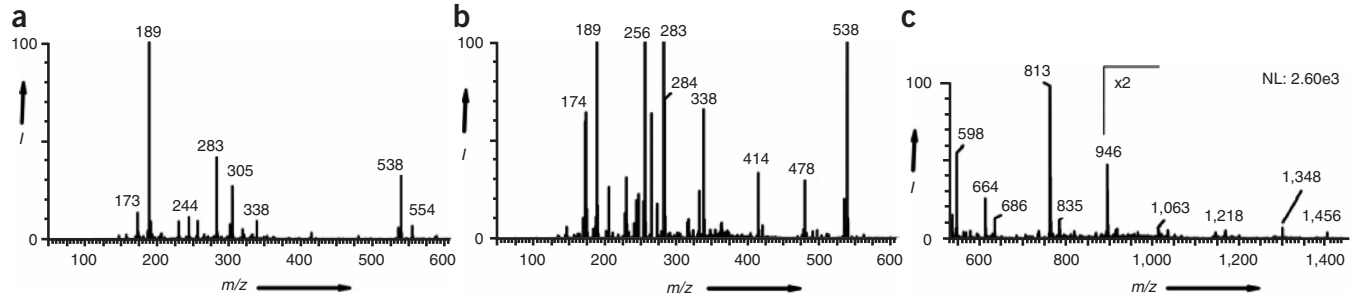

Figure 8 | Mass spectra recorded from vegetable leaves. (**a**) Fresh green leaves; (**b**) fresh leaves contaminated by *E. coli* strain TG1. (**c**) Multiple peaks were detected in the relatively high mass range from sample (**b**). (Copyright John Wiley & Sons Limited. Reproduced with permission from ref. 29.)

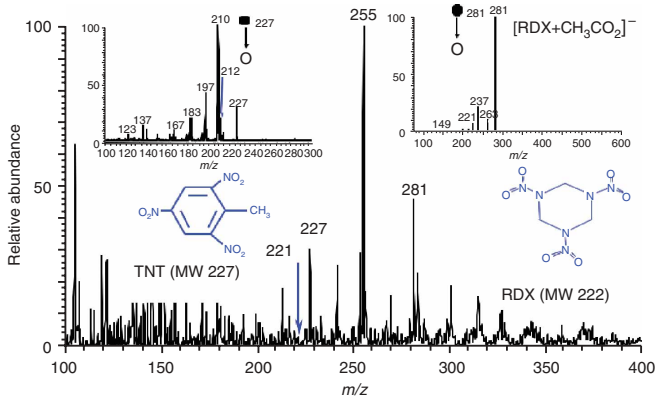

Figure 9 | Detection of trinitrotoluene (*m/z* 227) and RDX (*m/z* 222) by ND-EESI-MS on human skin. The instrument was operated in negative ion detection mode. Acetic acid aqueous solution (1:10) was used as the electrospray solvent. Insets show the CID spectra of ions of interest. It should be noted that these data were recorded with a homemade ND-EESI source coupled to an LTQ linear ion trap mass spectrometer. RDX was detected at $m/z = 281$ in the form of a noncovalent cluster with acetate, $RDX \cdot CH_3COO^-$.

mass spectral fingerprints recorded by ND-EESI-MS. More interestingly, plant leaves that were contaminated by *E. coli* cells for a few hours produce a significant difference in the mass spectra: more peaks were detectable in the relatively low mass range, probably due to the metabolites released from the *E. coli* cells. Multiple dominant signals in the relatively high mass range (e.g., *m/z* 600–1,500) were also detectable from the plant contaminated by *E. coli* cells, whereas no signal was found from the control samples.

As EESI does not require the separation of the analytes from the matrix, no sample pretreatment is required in EESI-MS experiments. Neutral desorption is a soft sampling method that allows the native conditions of the sample to be maintained. Thus, this process presents a gentle and 'green' method exceptionally suitable for the *in vivo* analysis of biological samples, which require real-time monitoring. More importantly, the sample can be located far away from the instrument. This is particularly interesting for the remote analysis of biological samples, online monitoring in industry or analysis of samples under extreme conditions such as hazardous environments. For example, explosives such as trinitrotoluene, cyclotrimethylenetrinitroamine (RDX) and triacetonetriperoxide have been successfully detected from a place located several meters away from the EESI source[27].

There are many other potential applications of ND-EESI-MS, and the general optimization steps of EESI and ND described in this protocol can be used in other applications involving ND and/or EESI.

ACKNOWLEDGMENTS We acknowledge funding from the National Natural Science Foundation of China (grant no. 205005). We also acknowledge the valuable help of Dr. Shuiping Yang and Dr. Arno Wortmann in performing some of the EESI experiments shown here, the assistance of Dr. Chunmei Li for providing the *E. coli* samples and helping to clean the *E. coli* medium, and help from Jia Hui Grace Leong with the editing.

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1. Dhingra, V., Gupta, M., Andacht, T. & Fu, Z.F. New frontiers in proteomics research: a perspective. *Int. J. Pharm.* **299**, 1–18 (2005).
2. Kumar, S., Mohan, A. & Guleria, R. Biomarkers in cancer screening, research and detection: present and future: a review. *Biomarkers* **11**, 385–405 (2006).
3. Nordhoff, E., Kirpekar, F. & Roepstorff, P. Mass spectrometry of nucleic acids. *Mass Spectrom. Rev.* **15**, 67–138 (1996).
4. Downes, M.R. *et al.* Application of proteomic strategies to the identification of urinary biomarkers for prostate cancer: a review. *Biomarkers* **11**, 406–416 (2006).
5. Smith, L., Lind, M.J., Welham, K.J. & Cawkwell, L. Cancer proteomics and its application to discovery of therapy response markers in human cancer. *Cancer* **107**, 232–241 (2006).
6. Pusch, W., Flocco, M.T., Leung, S.M., Thiele, H. & Kostrzewa, M. Mass spectrometry-based clinical proteomics. *Pharmacogenomics* **4**, 463–476 (2003).
7. Pandor, A., Eastham, J., Beverley, C., Chilcott, J. & Paisley, S. Clinical effectiveness and cost-effectiveness of neonatal screening for inborn errors of metabolism using tandem mass spectrometry: a systematic review. *Health Technol. Assess.* **8**, 1–13 (2004).
8. Rubin, A.E., Tummala, S., Both, D.A., Wang, C.C. & Delaney, E.J. Emerging technologies supporting chemical process R&D and their increasing impact on productivity in the pharmaceutical industry. *Chem. Rev.* **106**, 2794–2810 (2006).
9. Hsieh, Y., Fukuda, E., Wingate, J. & Korfmacher, W.A. Fast mass spectrometry-based methodologies for pharmaceutical analyses. *Comb. Chem. High T Scr.* **9**, 3–8 (2006).
10. Geoghegan, K.F. & Kelly, M.A. Biochemical applications of mass spectrometry in pharmaceutical drug discovery. *Mass Spectrom. Rev.* **24**, 347–366 (2005).
11. Corona, G. & Toffoli, G. High throughput screening of genetic polymorphisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Comb. Chem. High T Scr.* **7**, 707–725 (2004).
12. Gelpi, E. Contributions of liquid chromatography-mass spectrometry to 'highlights' of biomedical research. *J. Chromatogr. A* **1000**, 567–581 (2003).
13. Cooks, R.G., Ouyang, Z., Takats, Z. & Wiseman, J.M. Ambient mass spectrometry. *Science* **311**, 1566–1570 (2006).
14. Takats, Z., Wiseman, J.M., Gologan, B. & Cooks, R.G. Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science* **306**, 471–473 (2004).
15. Cody, R.B., Laramee, J.A. & Durst, H.D. Versatile new ion source for the analysis of materials in open air under ambient conditions. *Anal. Chem.* **77**, 2297–2302 (2005).
16. Chen, H.W. *et al.* Surface desorption atmospheric pressure chemical ionization mass spectrometry for direct ambient sample analysis without toxic chemical contamination. *J. Mass Spectrom.* **42**, 1045–1056 (2007).
17. Na, N. *et al.* Direct detection of explosives on solid surfaces by mass spectrometry with an ambient ion source based on dielectric barrier discharge. *J. Mass Spectrom.* **42**, 1079–1085 (2007).
18. Chen, H.W. *et al.* Instrumentation and characterization of surface desorption atmospheric pressure chemical ionization mass spectrometry. *Chinese J. Anal. Chem.* **35**, 1233–1240 (2007).
19. Chen, H.W., Pan, Z.Z., Talaty, N., Raftery, D. & Cooks, R.G. Combining desorption electrospray ionization mass spectrometry and nuclear magnetic resonance for differential metabolomics without sample preparation. *Rapid Commun. Mass Spectrom.* **20**, 1577–1584 (2006).
20. Mulligan, C.C. *et al.* Direct monitoring of toxic compounds in air using a portable mass spectrometer. *Analyst* **131**, 556–567 (2006).
21. Chen, H.W., Venter, A. & Cooks, R.G. Extractive electrospray ionization for direct analysis of undiluted urine, milk and other complex mixtures without sample preparation. *Chem. Commun.* **19**, 2042–2044 (2006).
22. Gu, H.W. *et al.* Monitoring diet effects via biofluids and their implications for metabolomics studies. *Anal. Chem.* **79**, 89–97 (2007).
23. Zhou, Z.Q. *et al.* Rapid detection of atrazine and its metabolite in raw urine by extractive electrospray ionization mass spectrometry. *Metabolomics* **3**, 101–104 (2007).
24. Chen, H. *et al.* Manipulation of charge states of biopolymer ions by atmospheric pressure ion/molecule reactions implemented in an extractive electrospray ionization source. *Eur. J. Mass Spectrom.* **13**, 273–279 (2007).
25. Zhou, Y.M., Ding, J.H., Zhang, X. & Chen, H.W. Ion-ion reactions for charge reduction of biopolymer at atmospheric pressure ambient. *Chin. Chem. Lett.* **18**, 115–117 (2007).
26. Chen, H.W., Wortmann, A., Zhang, W.H. & Zenobi, R. Rapid *in vivo* fingerprinting of non-volatile compounds in breath by extractive electrospray ionization quadrupole time-of-flight mass spectrometry. *Angew. Chem. Int. Ed.* **46**, 580–583 (2007).
27. Chen, H.W. *et al.* Rapid detection of explosives on human skin by neutral desorption extractive electrospray ionization mass spectrometry. *J. Amer. Soc. Mass Spectrom.* (in press).
28. Chen, H.W., Wortmann, A. & Zenobi, R. Neutral desorption sampling coupled to extractive electrospray ionization mass spectrometry for rapid differentiation of biosamples by metabolomic fingerprinting. *J. Mass Spectrom.* **42**, 1123–1135 (2007).
29. Chen, H., Yang, S., Wortmann, A. & Zenobi, R. Neutral desorption sampling of living objects for rapid analysis by extractive electrospray ionization mass spectrometry. *Angew. Chem. Int. Edit.* **46**, 7591–7594 (2007).
30. Williams, J.P. & Scrivens, J.H. Coupling desorption electrospray ionization and neutral desorption/extractive electrospray ionization with a travelling-wave based ion mobility mass spectrometer for the analysis of drugs. *Rapid Commun. Mass Spectrom.* **22**, 187–196 (2008).