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REVIEW

Direct analysis of biological samples using extractive electrospray ionization mass spectrometry (EESI-MS)

Haiwei Gu · Ning Xu · Huanwen Chen

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Abstract Mass spectrometry (MS) is one of the most widely used techniques for the analysis of biological samples. In the past decade, a novel improvement in MS was the invention of ambient ionization which stands out owing to its unique capability of direct analysis of complex samples with no or minimal pretreatment. In this review, extractive electrospray ionization (EESI), a representative ambient ionization technique, is introduced focusing on its mechanism, instrumentation, and applications in biological analysis. EESI uses a traditional ESI channel to produce primary ions which subsequently ionize neutral chemicals from the sample introduction channel through an online extraction process. When analyzing biological samples, EESI has advantages of rapid analysis, high matrix tolerance, and the ability to perform in vivo analysis. According to previous studies, EESI is able to directly analyze various chemicals in complex biological specimens in liquid, gas, and solid states. EESI can provide a sensitive and selective measurement of biological samples for both qualitative and quantitative purposes. Therefore, it is anticipated that EESI will have promising applications, especially in fields which require the fast and/or in vivo analysis of biological samples with complicated matrixes.

Keywords Extractive electrospray ionization (EESI) · Ambient mass spectrometry · Biological samples · Direct analysis

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H. Gu · N. Xu · H. Chen (✉)
Jiangxi Key Laboratory for Mass Spectrometry and Instrumentation, Department of Applied Chemistry, East China Institute of Technology,
Nanchang, Jiangxi Province 330013, China
e-mail: chw8868@gmail.com

Huanwen Chen completed his doctoral thesis in Analytical Chemistry at Jilin University, China, in 2001. During 2003–2005 he was a postdoc in the group of Prof. Graham Cooks at Purdue University, USA. He was a Simon Fellow in the group of Prof. Renato Zenobi at ETH Zurich in 2006–2007. Since 2008 he has been a full professor in the East China Institute of Technology, where he founded the Jiangxi Key Laboratory for Mass Spectrometry and Instrumentation and serves as the Laboratory Director. He received the First Class of Natural Science Award of Jiangxi Province as the Chief Contributor in 2010. His research interest is to develop new instruments and methods for directly obtaining structural information about molecules in complex samples.

Introduction

Mass spectrometry (MS) plays an important role in analyzing biological samples across multiple disciplines, such as systems biology and clinical testing [1–7]. In general, biological samples include urine, serum, saliva, tissue, cerebrospinal fluid, seminal fluid, gut aspirate, etc. The almost simultaneous introduction of matrix-assisted laser desorption ionization (MALDI) [8] and electrospray ionization (ESI) [9, 10], which are applicable to the analysis of solids and solutions respectively, led to enormous applications of MS in life sciences. The advantages of MS for the analysis of biological samples include high sensitivity, high specificity, quick response time, and being relatively universal. However, sample preparation when analyzing complex mixtures is still a key step to obtain high-quality MS spectra and ensure reliable outputs from MS analysis. For example, the sample preparation steps for chromatographically

coupled MS methods generally include extraction, drying, reconstitution, derivatization, etc., which are tedious and time-consuming even with assistance from automatic robotic systems [11–13]. To make matters worse, complicated sample pretreatment can cause reproducibility issues and untrackable experimental errors even when performed by an experienced researcher. Furthermore, direct, rapid, and in vivo MS analysis has recently gained more and more interest because the instruments are operated in the ambient environment so that biological specimens can be analyzed with high throughput in their original physical and chemical conditions. Therefore, it is hoped that novel ionization techniques could be further developed that would allow many new applications of the MS technique.

A breakthrough in the application of MS to high-throughput analysis was made by Cooks et al. [14, 15], who pioneered ambient MS with the invention of desorption electrospray ionization (DESI). Ambient MS takes advantage of sampling and ionizing under ambient conditions to carry out direct mass spectrometric analysis with no or minimal sample preparation [16]. Following the introduction of DESI, more than 20 ionization techniques were developed for ambient sample analysis, including direct analysis in real time (DART) [17, 18], surface desorption atmospheric pressure chemical ionization (DAPCI) [19–21], extractive electrospray ionization (EESI) [22–25], low temperature plasma (LTP) [26, 27], and easy ambient sonic ionization (EASI) [28, 29]. Ambient ionization techniques are attractive in biological analysis mainly owing to their high tolerance to complex matrixes. The analysis time of ambient MS for a biological sample is typically within a few minutes because sample preparation is minimized, which greatly increases extractable information per unit time in experiments. Ambient ionization was soon introduced into the biological field, e.g., EESI has been employed for the rapid characterization of living objects [30], native proteins [31], and metabolic biomarkers [32–34].

In this review, we aim to give a comprehensive introduction to EESI focusing on its applications in the direct analysis of biological samples. We start with a discussion of its ionization mechanism and then introduce EESI instrumentation. The analytical performance of EESI for analyzing various biological specimens is emphasized. Finally, we briefly discuss the scientific outlook for EESI and its future research perspectives in biological analysis.

EESI mechanism

EESI has a unique design utilizing two sprays and aligning them along a certain angle with respect to the mass spectrometer (Fig. 1). The first channel is an ESI channel generating primary ions, and the other one is the sample-infusing channel (to which no voltage is applied) where samples are nebulized towards the primary ion plume. This design is the key for EESI to tolerate complex sample matrixes, which makes it feasible to directly analyze biological samples with minimal pretreatment. Slightly acidified methanol/aqueous mixtures can be used in the ESI channel for positive-mode EESI experiments, and basic methanol/water solutions are used for the negative mode. After ionization, charged analytes are sampled through the ion optics system into the mass analyzer for further MS analysis.

A number of studies have investigated the ionization mechanism during EESI process in detail. Law et al. [35] performed a systematic study of the EESI mechanism using the fluorescence method. Rhodamine 6G emits fluorescence at a different wavelength in the liquid phase than that in the gas phase, and it turned out that EESI occurs in the liquid phase in most of the current instrumentation setups where EESI ion sources are placed very close (<20 mm) to the MS inlet. Another compound, H-acid (a pH sensitive fluorescent dye), was used to demonstrate that selective liquid-phase interaction is predominant during the EESI process. As shown in Fig. 2, KOH (sample channel) can enhance (compared with water) the fluorescence emission of H-acid (ESI channel), while the suppression effect caused by HCl (sample channel) is also clearly seen. The study also found that the performance of EESI is highly dependent on the analyte solubility in the solvents for primary ESI spray and sample spray, which further evidences that the selective extraction is occurring between the charged ESI droplets and the neutral analyte droplets.

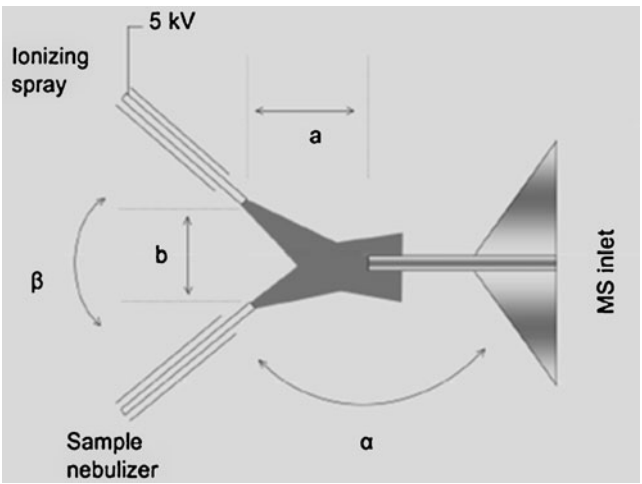

Fig. 1 Schematic of a traditional EESI ion source for direct liquid sample analysis. (Reprinted with permission from [22])

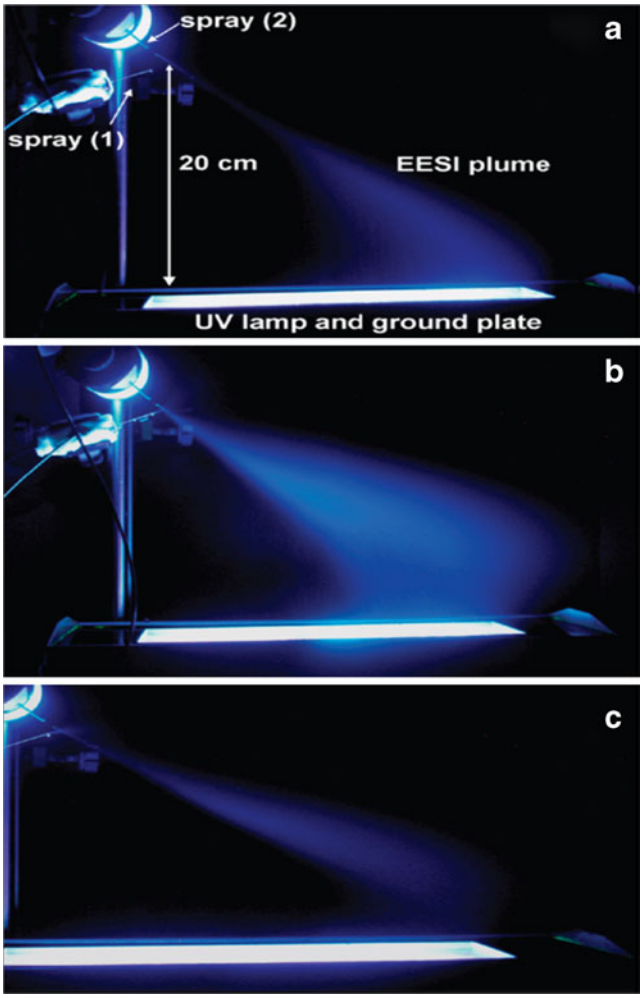

Fig. 2 Fluorescence images of H-acid sodium salt (ESI channel) confirming the selective extraction process in EESI. **a** The sample channel contains aqueous solution; **b** the sample channel contains aqueous KOH solution; **c** the sample channel contains aqueous HCl solution. (Reprinted with permission from [35])

Interestingly, ESI, DESI, and EESI have many similarities as well as differences. Nefliu et al. [36]. measured the internal energy distribution of typical ions generated by ESI, DESI, and EESI using the survival yield method. The ions from these three ambient ionization methods all have similar internal energy (1.7–1.9 eV) suggesting that similar processes are involved. Experimental parameters such as nebulizing gas pressure and solvent flow rate induced comparable trends in the survival yields in DESI and EESI experiments. In addition, Chen et al. [37, 38] found that EESI is a softer ionization method than ESI, at least for analyzing peptides and proteins. Biopolymers were detected by EESI-MS in a lower charge state compared with that by ESI-MS, implying the gentle charge deposition on biopolymers during EESI. Because the EESI technique is gentle, the biopolymers experienced less unfolding and therefore a lower charge state was obtained.

EESI apparatus

EESI was initially developed to directly analyze trace amounts of compounds in complex liquid matrixes such as urine and milk [22]. As a universal ambient ion source, EESI can be easily coupled to different kinds of mass spectrometers such as TOF and Ion Trap. The schematic illustration of a traditional EESI ion source is shown in Fig. 1. During EESI analysis, neutral samples from the sample channel and charged solvent droplets from the ESI channel are continuously introduced into the cross-section area in front of the MS inlet, such that neutral analytes are ionized under ambient conditions by primary ESI ions. EESI allows sample matrixes to be dispersed in a relatively large space formed among the ESI channel, the neutral sample introduction channel, and the ion inlet of MS spectrometer. Note that the matrix components can be further spread in this large three-dimensional space, and thus they have less opportunity to enter the MS inlet. The relatively clean solutions (e.g., a mixture of water, methanol, and acetic acid) are ESI sprayed and the samples of interest are introduced through the neutral channel, which eliminates the residue deposition issues caused by directly spraying complex matrixes under high electric voltages. Therefore, EESI tolerates extremely complex matrixes, so it is a promising technique for the direct and high-throughput analysis of biological samples with minimal pretreatment. Another merit of EESI is that samples are isolated from the direct bombardment by charged particles or energetically metastable atoms; thus EESI is a relatively soft ionization method.

EESI also has the outstanding ability to directly analyze volatile and nonvolatile compounds in gaseous samples [39–41]. Technically the instrument shown in Fig. 1 can be used for such analysis, and Fig. 3 shows a typical schematic of EESI-MS for the in vivo analysis of breath samples from human beings. A traditional ESI channel is used to generate primary ions. The sample channel can be easily configured by a piece of Teflon tube connected to a flow rate regulation valve which is recommended to ensure reproducible gas introduction into the EESI source. To further facilitate nebulizing exhaled gas and avoid breath condensation, the Teflon tube can be heated to 100 °C at the end that is close to the electrospray beam. In this way, many kinds of compounds in breath, both volatile and non/semivolatile, can be ionized in the cross section in front of the MS instrument.

The EESI ion source further evolved to allow analysis of viscous liquid samples, solid samples, and even tissue surfaces which cannot be directly sprayed in the sample

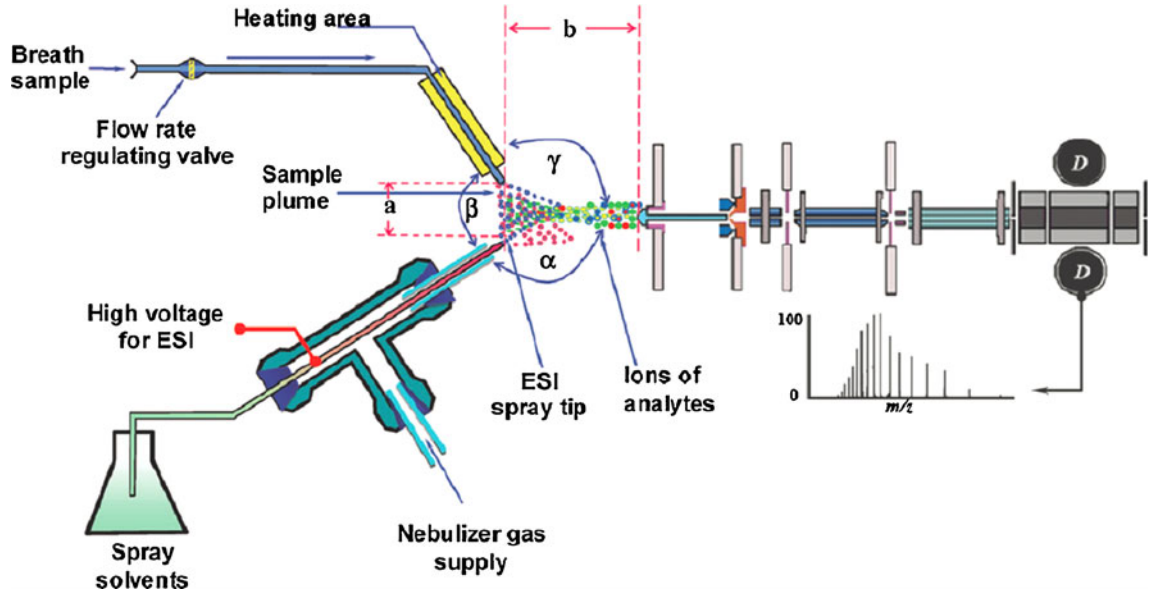

Fig. 3 Schematic of an EESI ion source for breath analysis. (Reprinted with permission from [39])

channel. Neutral desorption (ND) devices were coupled to EESI for the direct sampling of compounds within biological samples or from surfaces [42]. ND-EESI requires no sample pretreatment; it allows real-time chemical profiling of both volatile and nonvolatile analytes, which otherwise need extensive sample preparation prior to the traditional MS analysis. Figure 4 shows a schematic of an ND-EESI-MS device for the direct analysis of solid biological samples, in which the sampling gas beam (air or N_2) is used to impact sample surfaces [43]. This open-air ND device can be easily constructed from a gas emitter, a sample collector, and two gas delivery tubes. To improve sensitivity, open-air ND was soon updated to sealable ND devices as detailed in the previous publication [42]. A C-shaped glass cell is used to cover the sampling zone and ND is performed in an enclosed chamber. The improved sampling effectiveness can transfer most of the desorbed chemicals to the EESI source, which increases the detection sensitivity for those samples with medium viscosity. To liberate more analytes from liquid samples, the microjet ND device [42] was developed by inserting a microjet into bulk solution and bubbling neutral gas through the liquid. Microdroplets formed on surfaces are then transferred by neutral gas flow to the EESI source for further ionization.

EESI-MS applications

Monitoring regulated substances in biological samples

The detection of analytes such as pesticides and melamine in biological samples is of increasing importance in analytical chemistry driven by the potential threats that they pose to the environment and human society [44]. Reducing or removing biological matrixes is vitally important to performing a rapid, sensitive, and reliable analysis of pesticides in urine, which is commonly used to evaluate toxicological effects. Zhou et al. [32] evaluated the performance of EESI-MS for the direct detection of atrazine and its metabolites in undiluted human urine with minimal pretreatment. To increase selectivity, EESI was coupled to tandem MS. The limit of detection (LOD) of EESI-MS/MS is 0.4 fg for atrazine and 0.2 fg for 2-chloro-4,6-diamino-*s*-triazine (DACT). The linear dynamic range for both these two compounds is 4–5 orders of magnitude. As little as 4.3×10^{-14} g of atrazine can be detected and identified if spiked in raw urine. It is important to emphasize that the typical analysis time is about 1 min for each sample because all sample preparation is eliminated in EESI analysis. In addition, Zhu et al. [45] used EESI-MS to determine the presence of melamine in raw milk, wheat gluten, and milk powder. The linear dynamic range for the detection of melamine in milk is 3 orders of magnitude if using EESI-MS. The LOD is 500 ppb, which is well below the safety limit of melamine in milk [46]. The LOD is 200 ppb and 270 ppb in wheat gluten and milk powder, respectively. Obviously the rapid screening of melamine is of great importance to food security, especially when large batches of samples need to be examined in a short period of time. Considering the fast analysis time (ca. 1 min per sample), EESI is very promising for the rapid screening of regulated/toxic substances in biological samples.

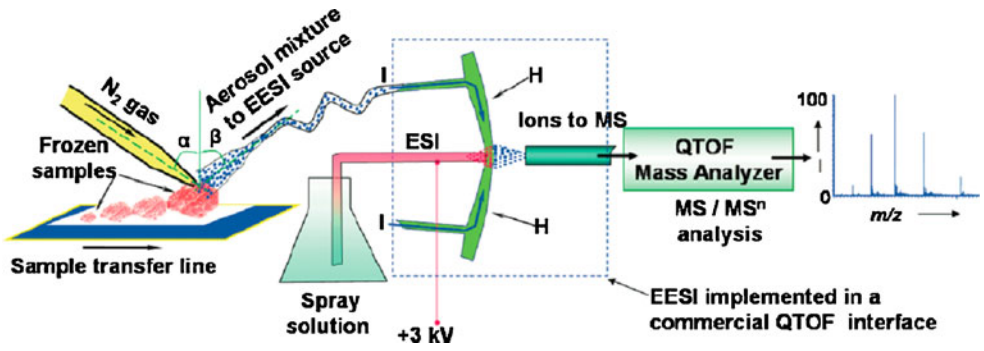

Fig. 4 Schematic illustration of an ND-EESI-MS device for surface and solid sample analysis. (Reprinted with permission from [43])

Systems biology analysis

Metabolomics, or metabolic profiling, is a relatively new area in systems biology, and it provides a novel approach and opportunity to investigate metabolic effects of intraneous or extraneous perturbations in biological systems. Metabolomics has been successfully applied in various research fields including toxicology, disease detection, functional genomics, pharmacology, etc. [3, 47–49]. Gu et al. [34] introduced EESI-MS to the field of metabolomics, and they measured the effect of diet on metabolites, in rat urine. Nuclear magnetic resonance (NMR) spectroscopy was used as well in this study; however, EESI-MS made it possible to perform targeted analysis by examining changes of metabolites in two specific metabolic pathways. Multivariate statistical analysis of EESI-MS data showed that rats with three different dietary regimens have distinct metabolic profiles depending on each individual metabolism pathway. They further concluded that by monitoring biochemical variations of particular pathways it is possible to decrease the number of statistical variables and to reduce the effect of diet in biofluid samples for such purposes as disease detection.

Proteomics is another important branch in systems biology, and it aims to study the structures and functions of proteins which are vital parts of living organisms [50–52]. The most promising developments to come from proteomics are biomarker discoveries that are useful for early disease diagnosis and personalized drugs. Chen et al. [37, 38] detected peptides and proteins in a lower charge state using EESI-MS than those by ESI-MS, which substantially improved the detection sensitivity for biopolymer analysis. Figure 5 compares MS spectra of a lysozyme solution from EESI-MS and ESI-MS experiments, and it can be clearly seen that at pH 5.3 EESI can detect the protein with only three charge states (+7 to +9). Ion/molecule reactions can happen between the multiply charged ions and the strong basic reagent, the concentration of which can be adjusted to manipulate the charge states of biopolymer ions. In ESI, the highest peak of melittin (MW 2846) is at *m/z* 569.8 (5+) and the intensity is 3.39×10^5. When a basic reagent (1,5-diazabicyclo[4,3,0]non-5-ene) is sprayed with an infusion rate of 0.1 μL/min, the base peak of melittin in EESI-MS spectra changes to that at *m/z* 1,423.8 (2+) with an intensity of 8.82×10^6. The sensitivity can be further improved as a peak intensity of 5.38×10^7 (at *m/z* 1,423.8) can be obtained if the infusion rate of the reagent is increased to 0.6 μL/min. As a result of the softness of the EESI mechanism, proteins can be ionized under ambient conditions with minimal conformation changes, which helps to maintain the bioactivity of proteins even during ionization. EESI is thus expected to have many applications in proteomics and clinical diagnosis involving protein analysis as it provides a sensitive and rapid tool to determine trace amounts of proteins in their native conditions.

Breath analysis

Breath analysis is a noninvasive method for gaining information on the clinical state of an individual by monitoring compounds present in the exhaled breath. A well-known example to many drivers is the blood alcohol testing. In 1971 Linus Pauling, a Nobel Prize laureate, demonstrated that human breath is a complex gas containing more than 200 different volatile organic compounds (VOCs) [53]. EESI-MS can provide a convenient way to probe dynamics of human bodies by fingerprinting both volatile and nonvolatile compounds in breath samples. Without hardware modification, Zenobi and Chen et al. coupled EESI to a Q-TOF mass spectrometer and

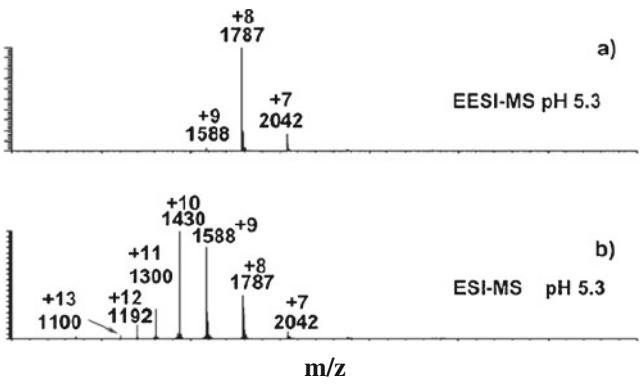

Fig. 5 MS spectra of a lysozyme solution. **a** EESI-MS, pH 5.3, charge states of +7 to +9; **b** ESI-MS, pH 5.3, charge states of +7 to +13. (Reprinted with permission from [37])

measured human breath with no sample preparation [40]. They detected nonvolatile compounds from breath samples associated with metabolic dynamics in human bodies, e.g., a higher level of urea after overnight fasting and a lower level of urea 2 h after eating cheese. Selective ion/molecule reactions in EESI were shown to be valuable for the medical diagnosis of diseases that alter chemical compositions and concentrations of human breath. Ding et al. [39] determined the concentration of nicotine to be 5.8 pg/mL in the breath sample from a male smoker. The LOD for nicotine is 0.05 fg/mL using EESI-MS; the relative standard deviation (RSD) of 10 measurements is 5.0 %; the linear dynamic range is 4 orders of magnitude. Interestingly, EESI has a great potential in personalized medical treatment and drug development by directly analyzing breath samples. Valproic acid (VPA) has long been used to treat epilepsy and other neuropsychiatric disorders owing to its modulatory effects in the γ-aminobutyric acid pathways and the sodium/calcium channels. Gamez et al. [41] utilized EESI-MS to monitor the drug metabolism of VPA in the human body. The VPA biomarkers observed in breath through EESI-MS are 4-hydroxy-VPA-γ-lactone and its related metabolites. By recording the intensities of biomarkers, EESI-MS is able to monitor the intake and clearance of VPA by examining the breath samples from a patient under the treatment of VPA for epilepsy. The EESI technique allows the real-time determination of pharmacokinetic profiles in humans in a noninvasive and painless manner, which may lead to a revolution in traditional therapy control and medical apparatus manufacturing.

Nutrition and food science

EESI was shown to have extensive applications in nutrition and food science as well, which can improve our quality of life in terms of providing food in a more secure and healthier way. For example, many people prefer fresh fish over frozen fish. EESI can provide a scientific approach at the molecular level to select fresh fish by monitoring odor-related compounds such as alkylamines [43]. Open-air ND-EESI was implemented to determine food quality online, differentiate meat spoilage in the frozen state, and distinguish plants contaminated by *E. coli* bacteria. Histamine is a molecular marker for meat spoilage resulting from the growth of microorganisms. The detection limit achieved by EESI is 10 fg/cm^2 (S/N=3) for histamine on the surface of frozen meat. Histamine was also detected at an increased level in fish that was exposed to room temperature for more than 1 day. In fact, virtually any surface can be directly analyzed with a gentle stream of transfer gas followed by the ionization of neutral molecules in ND-EESI. Microjet ND-EESI-MS is a useful tool for biofluidic samples with both low viscosity and high viscosity [42]. Law et al. [54] bubbled nitrogen through extra virgin olive oil (EVOO), and their approach enabled the direct extraction of molecules with different polarities and volatilities by modifying the composition of the primary ESI solvent. The chemical identification was confirmed on the basis of the tandem MS data; e.g., Fig. 6 shows the MS/MS spectra and molecular structures of (*E*)-2-hexenal, sinapic acid, coumaric acid, and tyrosol. It should be noticed that protonated (*E*)-2-hexenal (*m*/*z* 99) generates fragments at *m*/*z* 81 and 57 by loss of H_2O and CH_2CO, respectively. This fragmentation pattern is the same in EESI-MS/MS experiments with different primary ESI solvents. Because these primary ESI solvents have different polarity, EESI can differentially extract isobaric compounds in neutral samples. Therefore, it is quite possible that only protonated (*E*)-2-hexenal constitutes the peak at *m*/*z* 99 because this peak only generates a single fragmentation pattern in this study. Microjet ND-EESI is a simple and powerful method for obtaining mass spectra of EVOO without either liquid-liquid extraction or solid phase extraction, so microjet ND-EESI-MS, enables the rapid and 'green' investigation of various biological samples without any chemical contamination or sample pretreatment.

Cheese mainly consists of proteins and fat from milk. There are over 500 kinds of cheese in the world with different flavors, textures, forms, and quality. Wu et al. [55] developed sealable ND-EESI-MS to characterize various brands of cheese in the negative detection mode. The MS spectral data were reproducible and semiquantitative so that principal component analysis (PCA) was able to classify 49 cheese products into four different groups in the score plot as shown in Fig. 7. The PCA loading plots identified important metabolic features that were significant to the classification according to different distributors. By a similar approach, Zenobi and Chen et al. [33] utilized sealable ND-EESI-MS as a rapid and noninvasive tool for the differentiation of maturity and quality of fruits including bananas, grapes, and strawberries. The study showed that ND-EESI is a useful tool to many farmers for the maturity determination that is a key factor to decide storage life, final quality, and thus profits obtained from many fruits and vegetables.

Outlook and summary

With appropriate sampling devices, EESI has the capability for in vivo analysis and living object analysis. In vivo testing, including clinical trials and animal testing, is conducted within living organisms in their normal and intact state; therefore, it is often employed over in vitro analysis for a better observation of the overall effects of experiments on a living subject. In a broad sense, breath and skin analysis can be regarded as in vivo analysis as well. Chen et al. [30] successfully detected nicotine on the skin of a smoker by open-air ND-EESI, and they also found caffeine on the skin 30 min after the smoker drank three cups of strong

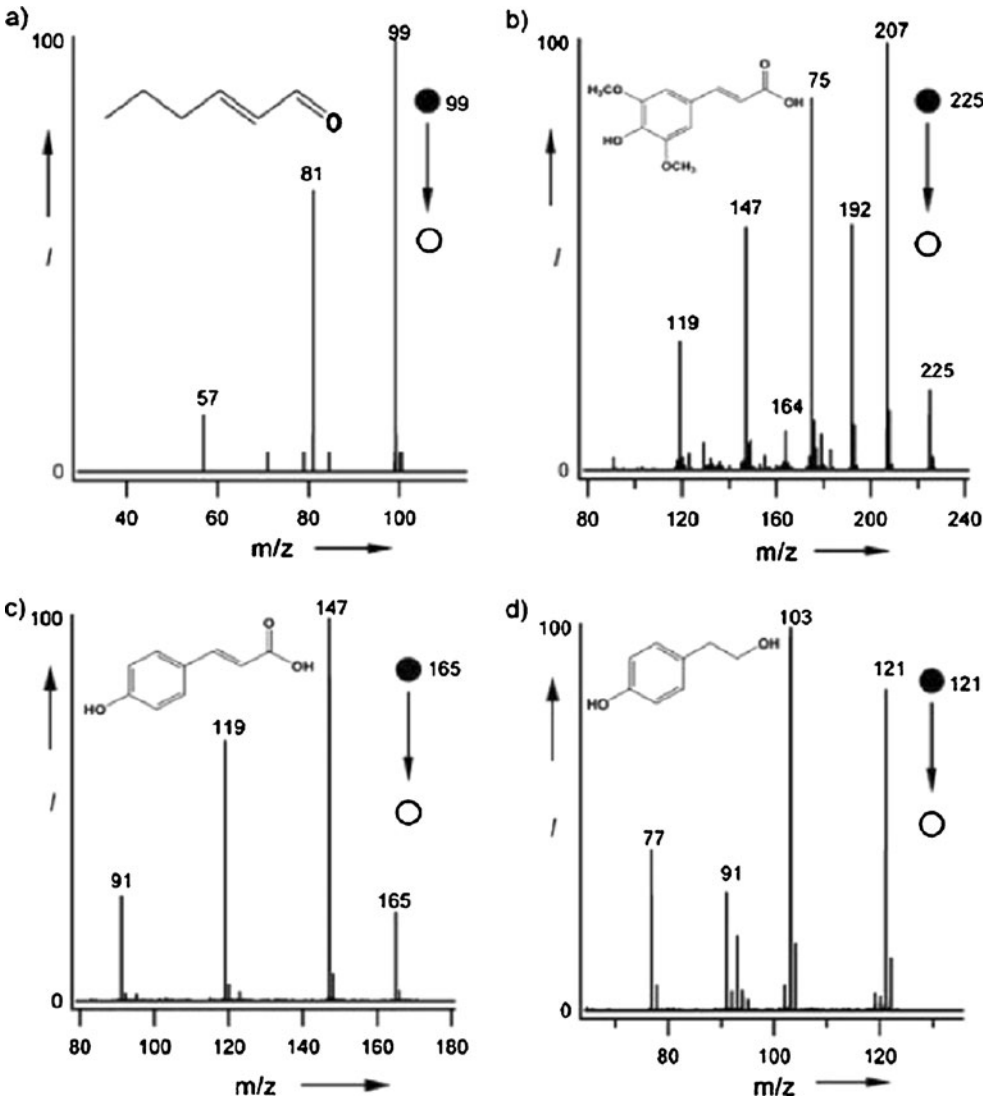

Fig. 6 EESI-MS/MS spectra and molecular structures of detected compounds from EVOO. **a** (*E*)-2-Hexenal; **b** sinapic acid; **c** coumaric acid; **d** tyrosol. (Reprinted with permission from [54])

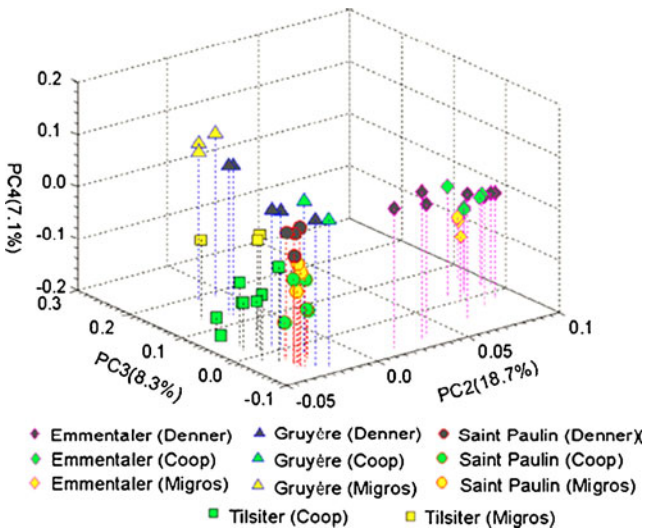

Fig. 7 PCA score plot based on EESI-MS data classifying cheese products into four different groups according to their types. (Reprinted with permission from [55])

black coffee. Theoretically, in vivo sampling systems for medical research and drug development can be coupled to EESI in the same way as that for ND devices. NanoEESI was recently developed, and it can perform direct EESI studies without sheath gas [56]. When coupled to miniature MS spectrometers nanoEESI can further facilitate field and in situ analysis with great convenience. It is thus expected that in the near future EESI will have wide in vivo analysis applications by providing a direct analytical tool to multiple areas including drug analysis and clinical diagnosis.

It is of equal importance to point out the disadvantages of using EESI for biological analysis. Because the configuration of EESI is relatively sophisticated, instrument optimization may take much time because the angles and distances shown in Fig. 1 all need careful adjustment. Much effort is required to optimize the experimental conditions for EESI-MS to reliably obtain quantitative information using a simple EESI source made of two sprayers. For example, every experimental parameter (e.g., the angles, the distances, and the flow rates)

shown in Fig. 1 has to be carefully optimized prior to measurement. The total time required for optimization varies from minutes to half an hour, which is largely depending on the operator's experience. Alternatively, employing an advanced EESI source, which is digitally controlled by a computer, significantly reduces the effort for source optimization and allows quantitative analysis with high confidence. Theoretically, EESI may encounter ion suppression effects, especially when an extremely complex sample is directly infused for a long time. Thorough cleaning and proper maintenance of the EESI are also recommended to minimize potential carryover effects. Without chromatographic separation, EESI lacks the retention information that might be valuable for compound identification. The interpretation of EESI-MS spectra from biological samples can be challenging because EESI tends to produce more ions which may have various molecular structures. In addition, because EESI is carried out under ambient conditions, in many studies (especially for biohazard samples) we need to design and implement specific interfaces which can cover the whole section between the EESI source and the MS inlet to avoid lab contamination and any safety threat to the instrument operators. In attempting to solve these issues, future work may include studying the accurate relationship between EESI configuration and its analytical performance, developing EESI sources with improved ruggedness, and commercializing an instrumentation with less adjustable parameters.

In summary, this review discussed the development and applications of EESI for the direct analysis of biological samples. The advantages of EESI-MS in biological analysis can be summarized as follows:

1. EESI-MS is sensitive, with LODs usually of subnanograms to subpicograms.
2. EESI-MS is relatively universal, and it has been and will continue to be applied to detect many kinds of chemicals with biological significance.
3. EESI has the capability of quantitative measurement with high reproducibility and wide dynamic ranges.
4. EESI-MS^n and ion/molecular reactions can exclude false positives to a maximum extent, ensuring the detection selectivity and reliability.
5. EESI-MS has the extraordinary advantage of rapid analysis with minimal sample preparation owing to its tolerance of biological matrixes. This advantage makes EESI-MS a promising tool in real-time and in situ analysis.
6. With a proper sampling device, EESI-MS allows the direct analysis of surfaces and samples in the gas, liquid, and even solid phases. EESI-MS shows a good performance for in vivo biological analysis.
7. Considering that EESI is a soft ionization method, it tends to cause less chemical and physical damage/contamination to samples of interest, which is important to areas such as biology and medical science.

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