**Video Article**

# Real-time Breath Analysis by Using Secondary Nanoelectrospray Ionization Coupled to High Resolution Mass Spectrometry

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URL: https://www.jove.com/video/56465
DOI: doi:10.3791/56465

Keywords: Chemistry, Issue 133, volatile organic compounds, breath analysis, secondary nanoelectrospray ionization, high resolution mass spectrometry, real time

Date Published: 3/9/2018

Citation: Li, X., Huang, D.D., Du, R., Zhang, Z.J., Chan, C.K., Huang, Z.X., Zhou, Z. Real-time Breath Analysis by Using Secondary Nanoelectrospray Ionization Coupled to High Resolution Mass Spectrometry. *J. Vis. Exp.* (133), e56465, doi:10.3791/56465 (2018).

## Abstract

Exhaled volatile organic compounds (VOCs) have aroused considerable interest, since they can serve as biomarkers for disease diagnosis and environmental exposure in a non-invasive manner. In this work, we present a protocol to characterize the exhaled VOCs in real time by using secondary nanoelectrospray ionization coupled to high resolution mass spectrometry (Sec-nanoESI-HRMS). The homemade Sec-nanoESI source was readily set up based on a commercial nanoESI source. Hundreds of peaks were observed in the background-subtracted mass spectra of exhaled breath, and the mass accuracy values are -4.0-13.5 ppm and -20.3-1.3 ppm in the positive and negative ion detection modes, respectively. The peaks were assigned with accurate elemental composition according to the accurate mass and isotopic pattern. Less than 30 s is used for one exhalation measurement, and it takes approximately 7 min for six replicated measurements.

## Video Link

The video component of this article can be found at https://www.jove.com/video/56465/

## Introduction

With fast development of modern analytical techniques, hundreds of volatile organic compounds (VOCs) have been identified in human exhaled breath[1]. These VOCs mostly result from alveolar air (~350 mL for a healthy adult) and anatomical dead space air (~150 mL)[2], which are affected by body metabolism[3,4,5,6,7,8] and environmental pollution[9], respectively. As a result, if identified, these VOCs are promising to be used as biomarkers for disease diagnosis and environmental exposure in a non-invasive manner.

Though gas chromatography mass spectrometry (GC-MS) is the most widely used technique for qualitative and quantitative analysis of exhaled VOCs[2], direct MS techniques, which have been developed for real-time breath analysis, have the advantages of high time resolution and simple sample pre-preparation. Direct MS techniques, such as proton transfer reaction MS (PTR-MS)[10], selected ion flow tube MS (SIFT-MS)[11], secondary electrospray ionization MS (SESI-MS)[12,13] (also named as extractive electrospray ionization MS, EESI-MS[14,15]), trace atmospheric gas analyzer (TAGA)[16] and plasma ionization MS (PI-MS)[17] have been investigated in recent years.

Among all the direct MS techniques, SESI is well-known as a universal soft ionization technique[19,20,21]; and the source is easy to be customized and coupled to different types of mass spectrometers, *e.g.*, time of flight mass spectrometer[8,15], ion trap mass spectrometer[14] and orbitrap mass spectrometer[12,18]. Up to now, SESI-MS has been successfully used in diagnosing respiratory diseases[22], gauging circadian rhythm[3,6,23], pharmacokinetics[7,8], and revealing metabolic pathways[4], *etc*. Most recently, a commercial SESI source has become available.

In this study, a facile and compact secondary nanoelectrospray ionization source (Sec-nanoESI) was set up and coupled to a high-resolution mass spectrometer. Real-time measurements of exhaled VOCs in breath were presented.

## Protocol

Caution: Please consult all relevant material safety data sheets (MSDS) before use. Please use appropriate personal protective equipment, *e.g.*, lab coat, gloves, goggles, full length pants and closed-toe shoes).

## 1. Set up the Sec-nanoESI source

1. Set up a Sec-nanoESI source according to the SESI process, *i.e.*, the breath gas is introduced to intersect an electrospray plume and ionized by the charged droplets (**Figure 1**). The sources built in individual labs depend on the interface of the mass spectrometer used[24,25]. Here, set up the Sec-nanoESI source based on a commercial nanoESI source (**Figure 2**) and implement to a benchtop quadrupole orbitrap mass spectrometer.
   NOTE: The main body of the source is a cubic stainless-steel chamber (Length 25 mm, I.D. 13 mm) (**Figure 2b**) with an inlet (I.D. 4 mm) to introduce the nanoESI capillary into the chamber. Thus, the chamber is not completely sealed (**Figure 2b**).
2. Install two stainless-steel tubes (Length 8 mm, O.D. 5 mm, I.D. 3 mm) on each side of the chamber for gas delivery.
3. Equip two quartz windows (I.D. 14 mm) on the top and bottom of the chamber to check the position of the tip of the nanoESI capillary and nanoESI spray either by eyes or a digital microscope.
4. Weld the chamber to the sweep cone of the mass spectrometer.
   NOTE: The design may change depending on the particular geometry of the atmospheric pressure interface of the mass spectrometer used in individual labs.

## 2. Instrument optimization

1. Calibrate the mass spectrometer in both positive and negative ion detection modes according to manufacturer's instructions. By applying calibration, mass spectrometer parameters, such as lens potentials and detection conditions, are optimized to give good sensitivity and peak shape at a specified resolution value. The mass resolution of 70000 is used here.
   1. Perform complete Q Exactive calibrations by using the commercial ESI source; however, mass calibration can be performed with any compatible sources, including customized ones.

2. Set the temperature of the ion transfer tube (ITT) of the mass spectrometer > 100 °C. Though the highest temperature can be set at 350 °C, it may result in the decomposition of some compounds. Thus, 150°C is used in this experiment.
   NOTE: For the mass spectrometers featuring a sampling orifice instead of ITT, the temperature of the sampling orifice is set > 100 °C.
3. For the ESI solvent and flow rate, select the appropriate ESI solvent on the basis of properties of solvent (*e.g.*, polarity and volatility) and targeted compounds (*e.g.*, proton affinity). A mixture of water and methanol in various ratios has been commonly used as ESI solvent[25]. In this experiment, use water containing 0.1% (v/v) formic acid, for high ionization efficiency has been reported this solvent[13,19,23]. Set the flow rate of ESI solvent in the range of 0-1.5 µL/min and 200 nL/min.
   NOTE: Degas ESI solvent for 30 min before use.
4. Optimize Sec-nanoESI source parameters, mainly nanoESI voltage and nanoESI capillary tip position. The voltage commonly ranges from 2.0 to 4.5 kV. Use 2.5 kV here.
   NOTE: Higher ESI voltage is applied as the flow rate increases. The distance between the tip and the mass spectrometer orifice can be adjusted from 1 to 5 mm. After optimization, the normalized intensity level (NL) observed in a mass spectrum should be > $1x10^6$ and the variation of total ion chromatogram (TIC) should be < 10% in both positive and negative ion detection modes. The mass spectrum and TIC are obtained in the mass range of *m/z* 50-750.
5. Apply pure gas to the source. This is an optional step, aiming at reducing the influence of VOCs from indoor air. High purity nitrogen gas ($N_2$, 99.99%) or pure air can be used. With the presence of pure gas, the NL observed in a mass spectrum should be > $1x10^5$ and the variation of TIC should be < 10% in both positive and negative ion detection modes. High purity $N_2$ is used here and delivered at 0.8 L/min.
   NOTE: The total flow rate of pure gas and breath gas should be higher than the flow rate through the orifice of the mass spectrometer.

## 3. Measurement of exhaled breath

1. Inhale the indoor air and perform a normal exhalation to breathe out all the air in the lungs at a constant flow rate. Monitor the exhalation flow rate either by a manometer or a flow meter visible to the subject. Use Teflon (PTFE) tubing to deliver the breath gas[23].
   1. To prevent the condensation of water vapor inside the tubing, heat the tubing at a temperature of 80-100 °C[7,23,27] or use a Nafion dryer[28,29]. In this experiment, the subject exhaled at 0.4 L/min controlled by a flow meter.
   2. Connect the inlet of the flow meter to a Nafion tubing (Length 60 cm) to remove the water vapor in the exhaled breath and connect the outlet of the flow meter to a PTFE tubing (length 13 cm, I.D. 4 mm). It takes < 30 s for one exhalation measurement.
   3. Perform 4-6 replicated measurements[28,29].
   4. To minimize confounding effects, have participants from eating, drinking, and brushing their teeth at least 30 min prior to the measurements[23].
      NOTE: To minimize the influence of VOCs from indoor air, it has been reported to inhale pure gas instead of indoor air[26]. When a Nafion tubing is used, some polar compounds may be lost.
   5. During measurement, keep checking if the ion intensity exceeds the linear detection limit of the instrument or not. The saturation of signal can lead to an artifact peak that does not practically result from the compound in the sample. By inhaling through the nose, part of ambient VOCs and particles would be removed; however, it is noteworthy that compounds in the nasal passages may also be detected.

## 4. Obtain a breath fingerprint and a time trace of a compound

1. Obtain chromatograms and mass spectra. Use software (e.g., Xcalibur) to record chromatograms and mass spectra. Because this is direct MS analysis and no chromatographic separation is performed, the total ion chromatogram (TIC) actually indicates the time trace of all the signals detected in the mass spectra and the extracted ion chromatogram (EIC) shows the time trace of a specified compound.

NOTE: For other commercial mass spectrometers, the chromatograms and mass spectra can be obtained by the corresponding data acquisition software.

2. Obtain an exhaled breath fingerprint by selecting a number of scans in the TIC when exhaled breath is measured. Obtain a mass spectrum representing an average of these scans by the software.
    1. To eliminate background peaks from the breath fingerprint, use the Subtract Background tool in the software. Please refer to the user guide provided by the manufacturer. In brief, select the same number of scans when no breath sample is introduced, and subtract the background mass spectrum from the breath fingerprint.
    NOTE: In this method, the threshold to identify the features in the breath fingerprint is defined as three times the standard deviation of the background signal. For other commercial mass spectrometers, background subtraction can be performed by the corresponding data acquisition software.

3. Obtain a time trace of a specified compound. Select the peak of a targeted compound in the breath fingerprint, and the time trace of the compound is acquired subsequently by the software.

## Representative Results

**Figure 3** shows the breath fingerprints in the mass range of *m/z* 50-750 recorded under both positive and negative ion detection modes. 291 peaks (peak intensity > $5.0 \times 10^4$) and 173 peaks (peak intensity > $3.0 \times 10^4$) have been observed in background-subtracted breath fingerprints in the positive and negative ion detection modes, respectively. To identify peaks in the mass spectra, please refer to prior publications for details[12,18,24,29]. In brief, both volatile metabolites and VOCs from indoor air have been detected. For example, the peak at *m/z* 74.0606 (**Figure 3a**) results from exhaled N,N-dimethylformamide or aminoactone according to the Human Metabolome Database (HMDB); peaks at *m/z* 462.1447 and *m/z* 536.1638 (**Figure 3a**) are from the adducts of exhaled ammonia and polysiloxanes (laboratory contaminants)[12]. The typical mass accuracy values in positive and negative ion detection modes are -4.0-13.5 ppm and -20.3-1.3 ppm, respectively. **Figure 4** presents the time trace of indole, a typical endogenous compound, which is detected by six replicated measurements of exhaled breath from one subject. It takes less than 7 min for all six measurements.

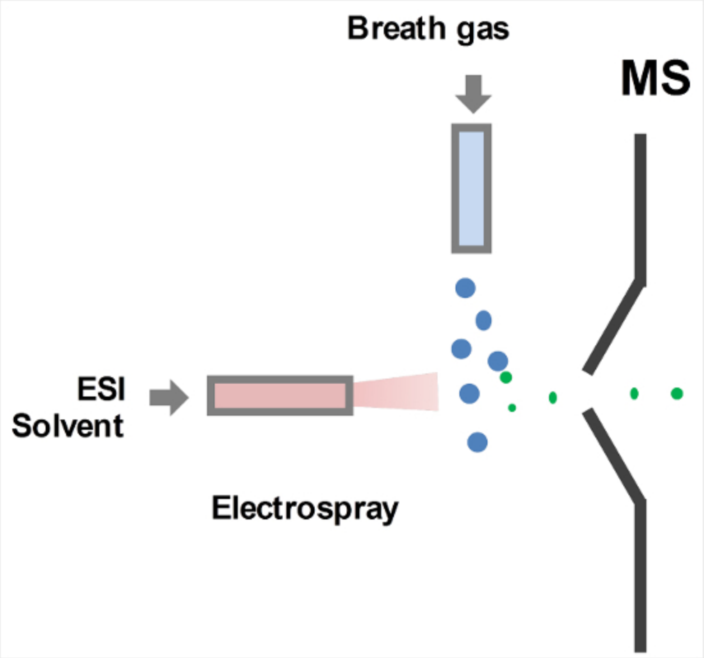

**Figure 1.** Schematic for SESI-MS analysis of VOCs in exhaled breath. Please click here to view a larger version of this figure.

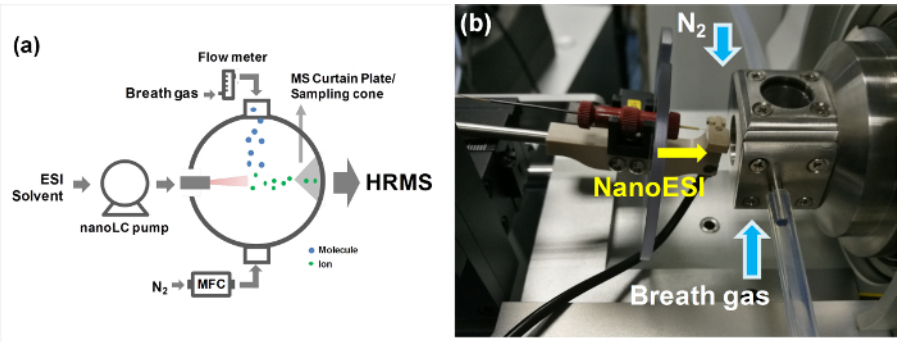

**Figure 2.** (a) A schematic diagram and (b) a photo of the Sec-nanoESI source used in this experiment. Please click here to view a larger version of this figure.

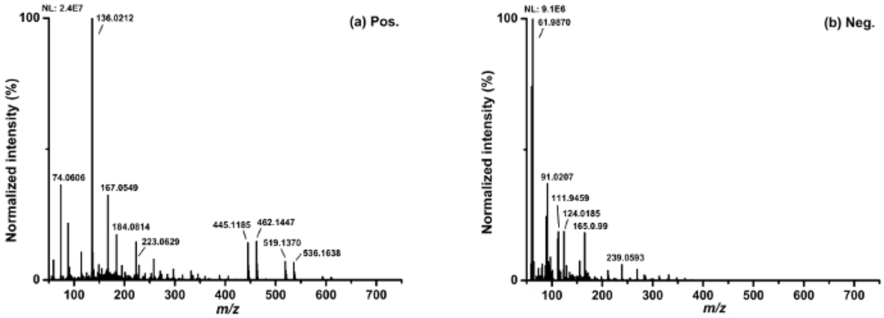

**Figure 3.** Background-subtracted breath fingerprints obtained in (a) positive and (b) negative ion detection modes in the mass range of *m/z* 50-750. Please click here to view a larger version of this figure.

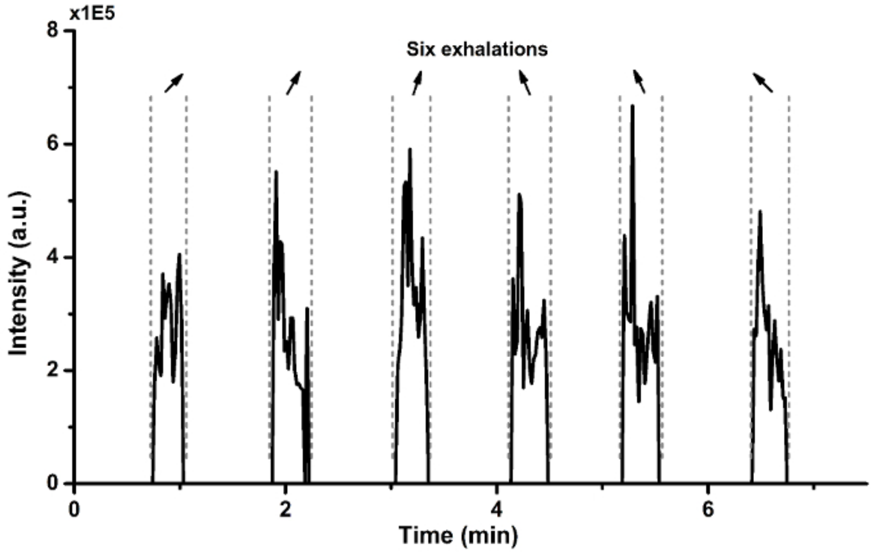

**Figure 4.** Time trace of indole detected by six replicated measurements of exhaled breath from one subject. Please click here to view a larger version of this figure.

## Discussion

Constructing the Sec-nanoESI source based on a commercial nanoESI source, the ionization efficiency is higher than that of using an ESI source[30]. In addition, the ionization efficiency is further improved in a closed chamber, as it isolates the process from the ambient background air, and at the same time facilitates the mixing between the gas sample and the spray plume. By using a Sec-nanoESI, less parameters need to be optimized compared to an ESI source, making it easier for installation, application and maintenance.

If no signal is observed or sensitivity decreases significantly when performing breath analysis by Sec-nanoESI-MS, one should check the position of the spray capillary tip and also the formation of droplets at the tip of the capillary. Align the tip with the orifice of the mass spectrometer. Change the spray capillary to a new one if the spray capillary is blocked or the tip is contaminated. Otherwise, check whether the

ITT of the instrument is blocked or contaminated. Replace or clean the ITT if necessary. Turn off ESI voltage before checking the spray capillary. Set the temperature of ITT at room temperature and wait until the temperature drops down.

SESI-HRMS has been demonstrated to be a sensitive and selective technique for real-time breath analysis[4,6,12]. In the past few years, this technique has been successfully applied to gauging circadian variation[3,6], monitoring pharmacokinetics[7,8], identifying metabolic pathways[5], *etc.* Lately, amino acids in human breath have been successfully quantified by SESI-MS for the first time, which is remarkable progress in quantitative analysis[5]. With further investigations, SESI-HRMS could establish itself as a useful and efficient noninvasive clinical method.

## Disclosures

The authors have nothing to disclose.

## Acknowledgements

This work has been financially supported by National Natural Science Foundation of China (No. 91543117).

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