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Rapid detection of sepsis in rats through volatile organic compounds in breath

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ABSTRACT

Background: Sepsis is one of the main causes of death in adult intensive care units. The major drawbacks of the different methods used for its diagnosis and monitoring are their inability to provide fast responses and unsuitability for bedside use. In this study, performed using a rat sepsis model, we evaluate breath analysis with Ion Mobility Spectrometry (IMS) as a fast, portable and non-invasive strategy.
Methods: This study was carried out on 20 Sprague-Dawley rats. Ten rats were injected with lipopolysaccharide from *Escherichia coli* and ten rats were IP injected with regular saline. After a 24-h period, the rats were anaesthetized and their exhaled breaths were collected and measured with IMS and SPME-gas chromatography/mass spectrometry (SPME-GC/MS) and the data were analyzed with multivariate data processing techniques.
Results: The SPME-GC/MS dataset processing showed 92% accuracy in the discrimination between the two groups, with a confidence interval of between 90.9% and 92.9%. Percentages for sensitivity and specificity were 98% (97.5–98.5%) and 85% (84.6–87.6%), respectively. The IMS database processing generated an accuracy of 99.8% (99.7–99.9%), a specificity of 99.6% (99.5–99.7%) and a sensitivity of 99.9% (99.8–100%).
Conclusions: IMS involving fast analysis times, minimum sample handling and portable instrumentation can be an alternative for continuous bedside monitoring. IMS spectra require data processing with proper statistical models for the technique to be used as an alternative to other methods. These animal model results suggest that exhaled breath can be used as a point-of-care tool for the diagnosis and monitoring of sepsis.

1. Introduction

Thanks to the pioneering work of Pauling L et al. [1], it has been known since the 1970s that human breath is a complex mixture of hundreds of compounds [1]. Gas Chromatography–Mass Spectrometry has made it possible to identify some of these compounds, revealing that exhaled breath includes traces of many volatile organic compounds (VOC), small inorganic molecules and non-volatile substances such as isoprostanes, cytokines or leukotrienes [2,3]. Accordingly, there is now a consensus on the diagnostic potential of breath, and there is considerable evidence available to support the use of breath analysis as a diagnostic tool for pulmonary diseases, liver diseases, gastric diseases, diabetes and inflammatory diseases such as sepsis [3–7]. The analysis of exhaled breath has a number of advantages compared with traditional diagnostic techniques: it is a non-invasive, painless procedure that does not require skilled medical staff [8,9].

Despite this undeniable interest, however, only a few breath tests, such as capnography and the urea breath test, are typically used in clinical routine. One reason for the continued reluctance to use breath analysis as a common diagnostic tool in clinical practice is lack of knowledge about the metabolic pathways of the compounds, although another factor is the lack of normalization and standardization methods [8,10]. Furthermore, bedside systems are not always compatible with the sophistication now required of analytical instruments. Gas chromatography–mass spectrometry (GC/MS), for example, is the most widely used instrument in breath analysis [11,12,7] but, although it offers very good sensitivity,

Abbreviations: P, intraperitonealy; LPS, lipopolysaccharide; VOCs, volatile organic compounds; IMS, ion mobility spectrometry; SPME-GC/MS, solid phase microextraction-gas chromatography/mass spectrometry; COPD, chronic obstructive pulmonary disease; CAR/PDMS, carboxen/polydimethylsiloxane; RIP, reactant ion peak; MCR-LASSO, multivariate curve resolution least absolute shrinkage and selection operator; SFFs, sequential floating forward selection; kNN, k nearest neighbours; PCA, principal component analysis; LDA, linear discriminant analysis; PCT, procalcitonin test; PCR, polymerase chain reaction; PFA, perfluoralkox; IL, interleukin; TNF, tumor necrosis factor.

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precision and resolution, the sampling procedures and subsequent interpretation of data can be demanding and time-consuming and require qualified personnel.

Alternative chemical sensing techniques, such as solid-state sensor arrays (e-noses) and ion mobility spectrometers (IMS), offer new approaches to this problem. E-noses have been applied for many preliminary studies [13] but their limited sensitivity and selectivity hinder their clinical application when the analytes of interest are at sub-ppm levels. Alternatively, IMS is a simple, portable and sensitive instrumental analytical technique that it is gradually expanding its range of applications, from security to food and environmental and clinical applications. IMS provides a response of less than a second to the trace levels of volatile organic compounds, based upon the mobility of gas phase ions in weak electric fields [14]. The mobility K of an ion depends on the ion's mass, charge, shape and size, but also on the measurement conditions, particularly pressure and temperature. An ion's mobility is usually reported by means of reduced mobility K0, where the effects of measurement conditions have been normalized to standard conditions. Moreover, an IMS can offer two different responses: the mobility spectra for both positive ions and for negative ions.

Promising results of breath analysis with IMS have been reported and its potential for application as a diagnostic instrument is huge. Lung cancer has been the main focus of attention [15,16] but interesting findings have also emerged with respect to COPD, sarcoidosis and vaginitis [17–19]. Other diseases such as sepsis have yet to be tested by IMS technology, however, even though the potential capacity of breath tests for diagnosings sepsis has been posited in some works [3].

Sepsis is a clinical condition characterized by systemic inflammation, aberrant immune response, and microcirculation/coagulation disorders generated by a host in combating an infection caused by bacterial toxins absorbed from infected wounds and passed into the bloodstream [20]. It is a common cause of morbidity and mortality in elderly, immuno-compromised and critically ill patients, and it is the commonest cause of death in adult intensive care units [21].

Several rodent models have been used for sepsis studies in various works [22–24] and, although the correlation between animal and human models is not perfect, rat models can make a positive contribution in many areas, such as the reduction and standardization of experimental variability and the simplification of the experimental set-up. One of the common sepsis models used in murine is the induction of sepsis through an exogenous bacterial infection. It is well known that mimicking bacterial infection by using lipopolysaccharide (LPS), which is a structural component of gram-negative bacteria, has been used as a model to activate the immune system, inducing fever, sepsis and multi-organ injury [25]. The main cytokines acting as endogenous pyrogens in response to LPS are interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α). Moreover, pulmonary dysfunction, including edema, is a well-recognized dysfunction in sepsis [26].

The analysis of exhaled breath in rats has been reported in some studies [27–29] and the analysis of rodent's breath with an IMS instrument has been reported in a recent feasibility study by Vautz et al. [30].

This work explores the viability of IMS instrumentation and chemometric techniques for generating a VOC discriminatory pattern of sepsis through breath sampling. This work has been performed in a rat model as a first step toward a possible future application in humans. Furthermore, although IMS is undoubtedly capable of providing fast VOC analysis, it also needs to be compared with a gold-standard technique in VOC analysis. In order to fill this gap, this study includes GC/MS measurements of the rat's breath as a reference technique, while also providing analyte identification capabilities using proper MS libraries.

2. Materials and methods

2.1. Animals

This study was carried out on 20 Sprague-Dawley male rats from Charles River (250–300 g) following an experimental protocol approved by the Ethical Committee of Animal Research at the University of Barcelona. One day before the experiment, 10 of these rats were intraperitoneally (IP) injected with LPS from *Escherichia coli* 055:B5 (Sigma Chemical Co., St. Louis, MO) at a concentration of 4 mg/kg and the other 10 rats were IP injected with saline solution as a control group. All the animals were housed in light–dark cycle-regulated air conditioned (23 °C) and air humidity (60%) animal quarters for 24 h. After this period, the animals were IP sedated and anaesthetized with a mixture solution containing Rompun (Bayer) in a concentration of 0.7 mL/kg and Imalgene 1000 (Merial Laboratories, Spain) in a concentration of 1 mL per kilogram of animal body weight. A tracheotomy was performed and one cannula (16GA BD Adsyte Pro, Becton Dickinson, Spain) was introduced into the trachea. The intratraqueal cannula was connected, by means of a T-piece, to the inspiratory and expiratory lines of a mechanical ventilator for rodents (model 683; Harvard Apparatus, USA). The rats were ventilated normally with a tidal volume of 5 mL, at a rate of 80 breaths/min using room air. We therefore obtained a discontinuous flow of 400 mL/min of expired gas for sampling. The ventilation period lasted for 20 min and at the end of this period the expiratory gas was sampled for further VOC analysis. Fig. 1 shows the sampling method used in this work. With GC/MS, the rat's breath was collected in a perfluoralkoxy (PFA) bag for subsequent analysis. With IMS, the breath sample was directly introduced into the instrument via the expiratory line and measured online.

2.2. Assessment of rat status

After expiratory gas sampling, the rats were sacrificed by aortic exsanguination. The rats' septic status as a result of the bacterial-LPS injection was investigated by assessing lung edema and systemic inflammation. To assess lung edema, the lungs of all the rats were excised, quickly weighed after removing the main airways, weighed (wet weight = W), dried at 70 °C for 48 h and weighed again (dry weight = D). The ratio W/D was computed as a conventional index of lung edema.

The systemic inflammatory status of the rats injected with LPS was determined by measuring the plasma concentrations of two representative inflammatory cytokines: IL1-β and TNF-α. To this

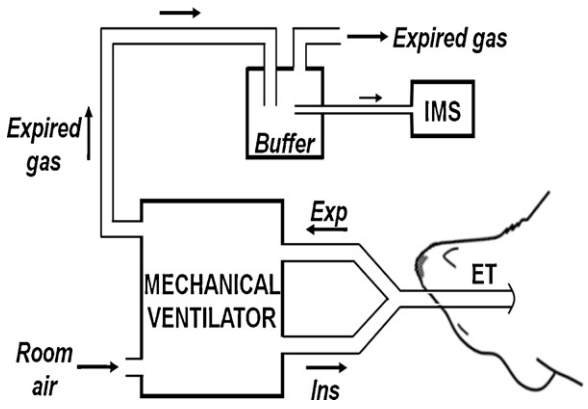

Fig. 1. Diagram of the experimental setup. The expired air from the ventilator was analyzed on-line by the Ion Mobility Spectrometer device, using a breath buffer of 50 mL. For the collection of expired air for Gas Chromatography/Mass Spectrometry, the exhaled air was obtained by directly connecting a collecting bag to the expiratory outlet of the ventilator.

end, the peripheral blood was processed to isolate the plasma (centrifugation at 3000 g using a vasculant rotor for 15 min at 4 °C). Enzyme-linked immunosorbent assays (ELISA) were performed for IL1-β and TNF-α (Quantikine, R&D Systems, Minneapolis, MN, USA).

2.3. Instrumentation and measurements

The IMS used in this study was the GDA2 device (Airsense Analytics, Germany) based on a 100MBeq Ni^{63} ionization source that works in both positive and negative modes. A hydrophobic membrane inlet was used to impede the entrance of most of the humidity in the ionization chamber. An electrostatic gate allowed the ions to travel at atmospheric pressure into the drift tube (length 6 cm), where they were accelerated by a constant electric field. The ions were neutralized in the collector at the end of the drift tube, thus causing an electric current. This made it possible to measure the time that the ions needed to reach the collector. The collector current was sampled at 33.3 kHz. Every 3 s, the IMS provided a different sample mobility spectrum (28 ms in length) with a response time of less than a minute. This spectrum corresponded to an average of 16 consecutive spectra for noise reduction. The default setting of the spectrum of the IMS based on the Ni^{63} ionization source included two reactant ion peaks corresponding to the different ion species formed from the nitrogen and from the humidity still present in the carrier air. In this work, the measurements were made at 50% internal dilution of sampling, with a continuous sampling flow of 200 mL/min. In order to avoid any conflict with the discontinuous flow obtained from the ventilator, 50 mL of exhaled air buffer was included between the IMS and the ventilator. All the samples were measured twice for up to 40 s after stabilizing the system and the IMS for 5 min.

Similarly, breath samples were taken to be analyzed with GC/MS. In this case, 1L of PFA bag was filled with breath using a discontinuous flow of 400 mL/min that was obtained from 80 breaths per minute and 5 mL of tidal volume. The PFA bag was filled in 2.5 min. This sampling process was carried out by directly connecting the outlet of the ventilator to the PFA bags. The GC/MS analyses were performed on a Focus GC-DSQ II (Thermo Scientific, USA) with a split/splitless injector. Breath was collected in 1L PFA bags and analyzed with SPME-GC–MS. A Carboxen/Polydimethylsiloxane (CAR/PDMS) 75-μm-thick fiber from Supelco was used for the preconcentration of the analytes. The sorption conditions were 30 min at room temperature. The desorption of volatiles from the fiber was undertaken at 250 °C for 5 min at the GC/MS injection port. A 60 m × 0.32 mm × 1.8 μm capillary column DB-624 (Agilent Technologies) was chosen for the chromatographic separation. Helium was used as carrier gas, with a flow rate of 1 mL/min. The MS analyses were carried out in a full scan mode (scan range 35–350 amu) with an ionization energy of 70 eV. The oven program temperature was as follows: initially, 40 °C held for 5 min, then ramped 10 °C/min to 180 °C; held for 1 min, then ramped 15 °C/min to 230 °C; and then held for 10 min.

At the beginning and end of each session of measurements, the blanks in the sampling system and the air of the laboratory were measured with IMS to ensure the reproducibility of the measurements. In order to eliminate the contribution of the anesthetic drugs to the ion mobility spectra and the chromatogram, these products were measured in a head-space mode by IMS and by SPME-GC–MS.

2.4. Signal Processing and statistical analysis

The signal processing strategies used in this work were based on multivariate signal processing and implemented in MATLAB 7.5 (Mathworks, USA), using the PLS Toolbox 5.8 (Eigenvector Research, USA). The signal processing applied to the IMS dataset was designed to find differences between healthy and diseased rats, and the signal processing applied to the GC/MS dataset focused on identifying compounds that could be potentially useful as sepsis biomarkers

The IMS dataset pre-processing includes a baseline correction by fitting a 4th order polynomial to specific spectral intervals devoid of peaks and a smoothing of the signal using a savitzky–golay [31] filter. The savitzky–golay filter performs a polynomial regression of order m ($m = 3$) and is fitted to the number of points (length = 15) to obtain the smoothed value of each point. The drift time of the IMS Reactant Ion Peak (RIP) was used as a reference for the spectra alignment and an area normalization procedure was applied to each spectrum. The multivariate signal processing strategy involved the use of the iterative algorithm MCR-LASSO [32] to estimate the pure contributions to the spectra, the Sequential Floating Feature Selection (SFFS) [33] to select the best subset of pure contribution for maximum discrimination between classes, and a kNN classifier [33] in the reduced space to evaluate the classification results under a bootstrap validation [34] strategy.

As regards the GC/MS dataset, the compounds were identified by comparison with mass spectra from the NIST 2005 library database available in the Thermo Xcalibur data system. The basic multivariate strategy involved using a combination of principal component analysis (PCA) [33] and linear discriminant analysis (LDA) [33], with a selection based on rank products [35,36]. This strategy made it possible to reduce dimensionality and order the identified compounds by their p-value. A kNN, SFFS and bootstrap validation were also used in the same way as in the IMS dataset analysis.

3. Results

3.1. Pathophysiological rat status

As expected, pulmonary edema was found only in the LPS-treated rat group. Indeed, W/D, a conventional index quantifying the magnitude of edema via the weight of water retained within the lungs, was 5.40 ± 0.28 (mean ± SEM) in the control rats and attained a higher value of 6.88 ± 0.58 in the septic rats. These values of W/D are in agreement with those previously reported in both healthy animals and rats with experimentally induced lung edema [37]. Moreover, concentrations of circulating inflammatory markers in plasma were significantly increased in LPS-infected mice compared to controls. Whereas in the control animals the concentration of IL1-β and TNF-α were 1.51 ± 1.01 pg/mL and 1.43 ± 0.14 pg/mL, respectively, in the LPS-injected animals these concentrations rose to 313.45 ± 81.80 pg/mL and 5.99 ± 0.30 pg/mL, respectively. All the differences observed between the controls and the animals with the bacterial endotoxin were statistically significant (t-test or Mann–Whitney rank sum test, as required): $p = 0.034$, $p = 0.002$ and $p < 0.001$ for lung edema, IL1-β and TNF-α, respectively.

3.2. Ion mobility spectrometry dataset

The IMS dataset featured 10 spectra from 40 breath samples (10 healthy rats + 10 LPS treated rats and an additional replicate of each one). In Fig. 2, spectra from a rat with sepsis are shown for both positive and negative modes. After preprocessing, MCR-LASSO was used to decompose IMS raw spectra into their pure contributions: pure spectra components, S, and their related concentration time evolution, C, were extracted. As a result, fourteen relevant pure components were obtained from negative and positive spectra. In Fig. 3, the plots show the components of the measured rat's breath in the positive and negative IMS modes.

The same procedure was applied separately to samples from anesthesia and laboratory ambient air measured in order to

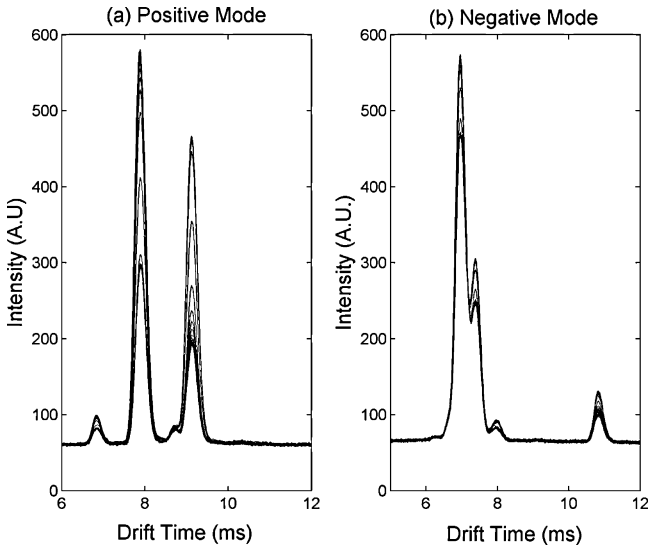

Fig. 2. Raw spectra for a rat with sepsis. (a) Positive mode. (b) Negative mode.

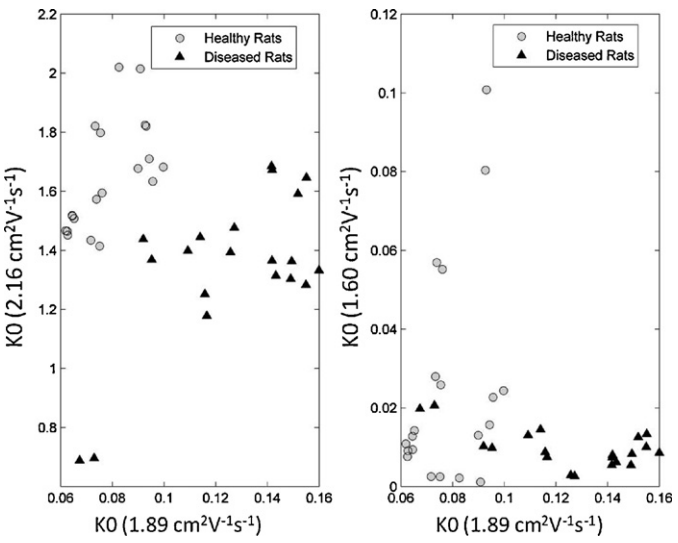

Fig. 4. Score plots of the final IMS pure component selection. Triangles correspond to 10 diseased rats (plus one replicate) and circles to 10 healthy rats (plus one replicate).

counteract their contribution. The resulting components were compared with the components of breath sample and were subsequently eliminated for further analysis. Undesirable contributions appeared at a drift time of 9.575 ms in positive mode and at a drift time of 8.99 ms in negative mode. Anesthesia (drift time = 12.48 ms in negative mode) as well as pure components related to the RIP peaks in positive mode (drift time = 8.06 ms and 9.03 ms) and negative mode (drift time 8.363 ms) were identified but were not considered for further evaluation. At the end of this process, eight pure components had been obtained.

As a result of the SFFS selection, the subset consisting of compounds with reduced mobility of $K0_1 = 1.89\,cm^2\,V^{-1}\,s^{-1}$ (positive spectra), $K0_2 = 2.16\,cm^2\,V^{-1}\,s^{-1}$ and $K0_3 = 1.60\,cm^2V^{-1}s^{-1}$ (negative spectra) were selected. Fig. 4 shows the distribution of rats in the space of the three selected compounds. For easier interpretation, two plots of $K0_1$ versus $K0_2$ and $K0_1$ versus $K0_3$ have been shown, as opposed to a three-dimensional plot. Bootstrap validation was applied to estimate the discrimination between healthy and LPS-treated rats and the final result was an accuracy of 99.8% (99.7–99.9%), a specificity of 99.6% (99.5–99.7%) and a sensitivity of 99.9% (99.8–100%). The confidence limits were calculated at a 95% confidence level.

3.3. GC/MS

Fig. 5 shows chromatograms obtained from diseased rats and healthy rats. Note the abundance of peaks and slight differences between both chromatograms.

Although not all the peaks of the samples can be identified, Table 1 lists nineteen compounds found and identified in breath samples from diseased and healthy rats. Three compounds were identified as related to a fiber induced by LPS and one compound was identified as linked to the anesthesia. All of these were discarded for the subsequent data evaluation study. In the end, fifteen compounds were selected as possible compounds associated with sepsis and the area under the peak was calculated for each one using MzMine2 [38].

The results of the application of PCA-LDA with rank products are shown in Table 1. Five compounds with a p-value of less than

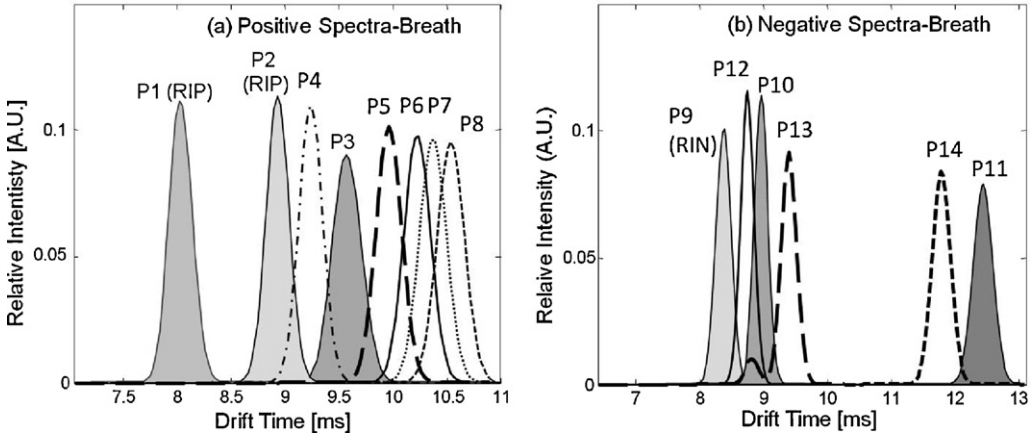

Fig. 3. Pure components peaks (P) from MCR-LASSO results for rat's breath. Every component from P1 to P14 has its Reduced Mobility $K0$ ($cm^2\,V^{-1}\,s^{-1}$) for positive and negative mode. Filled peaks correspond to anesthesia, air pollution and reactant ion peak from IMS, and the others are related to compounds from breath. Positive Spectra: P1 ($K0 = 2.35$): RIP comes from Nitrogen ion species, P2 ($K0 = 2.11$): RIP comes from water ion species, P3 ($K0 = 1.97$): a component from laboratory room air, P4 ($K0 = 2.04$), P5 ($K0 = 1.89$), P6 ($K0 = 1.84$), P7 ($K0 = 1.82$), P8 ($K0 = 1.79$). Negative Spectra: P9 ($K0 = 2.25$): RIN, P10 ($K0 = 2.11$): a component from laboratory room air, P11 ($K0 = 1.52$): anesthesia, P12 ($K0 = 2.16$), P13 ($K0 = 2.01$), P14 ($K0 = 1.60$).

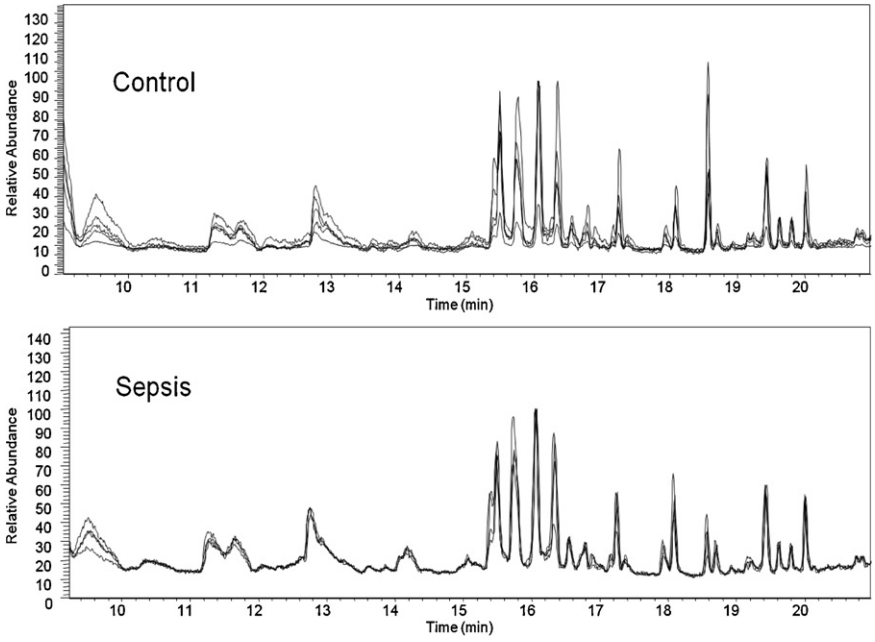

Fig. 5. Chromatograms of 10 rats with sepsis and 10 healthy rats.

0.001 were chosen by the algorithm as compounds possibly related to sepsis.

Fig. 6 shows the plot resulting from the discrimination model. Bootstrap validation was implemented for a strict validation of the discrimination model. The final results obtained with bootstrap validation have an accuracy of 85%, with a confidence interval between 84.6% and 85.9%. The results for sensitivity and specificity are 91% (89.7–92.2%) and 80% (79.3–80.7%), respectively. Again, the confidence limits were calculated to 95%.

4. Discussion

Despite the evolution of intensive care medicine and the broad range of clinical systems available nowadays, sepsis is still the first cause of death in non-coronary critical care units. Traditionally, sepsis diagnostics use culturing techniques of blood, urine, cerebrospinal fluid and bronchial fluid, among others. The major drawback of culturing techniques is the time needed to develop the culture, usually between 24 and 48 h. Although other techniques such as ELISA, ProCalcitonin Test (PCT) assays and DNA detection by Polymerase Chain Reaction (PCR) are faster, they need between 2 and 6 h to obtain a response and they are incapable of monitoring the dramatic changes found in sepsis [39]. In the absence of any real-time monitoring system for sepsis, breath analysis with IMS must be considered a promising alternative.

The potential capability of breath tests for the diagnosis of sepsis has been indicated in previous works [3,7] but, as far as we know, sepsis still remains untested by IMS technology. Other technologies such as GC/MS are also capable of offering a high performance in breath analysis but they are usually unable to match the portability and simplicity of the IMS measurements. IMS is more suited to the clinical trend of developing bedside patient systems but, unfortunately, it cannot easily identify unknown volatile compounds in a sample, so, in this respect, GC/MS measurements complement this

Table 1
Identification of compounds from GC dataset.

Compounds	Identification	Rank product (p-value)
1	Cyclohexane, methyl	0.000005
2	Acetone	0.000007
3	CO_2	0.00001
4	Pentafluoropropionamide	0.00003
5	Dimethylether	0.0002
6	Retention time (18.57) Mazas(42,48,56)	0.0010
7	o-Xylene	0.0191
8	Hexane, 2,3,4-trimethyl-	0.2676
9	Octane, 4-methyl-	0.5343
10	Decane	0.6611
11	2-Propanol, 1,3-dichloro-	0.8983
12	Toluene	0.9702
13	Acetic acid	1.6955
14	Propane, 2-ethoxy-2-methyl-	2.3828
15	Benzene	4.1241
Fiber	Silanediol, dimethyl- Cyclotrisiloxane, hexamethyl- Cyclotetrasiloxane, octamethyl-	
Anesthesia	Ketanone	

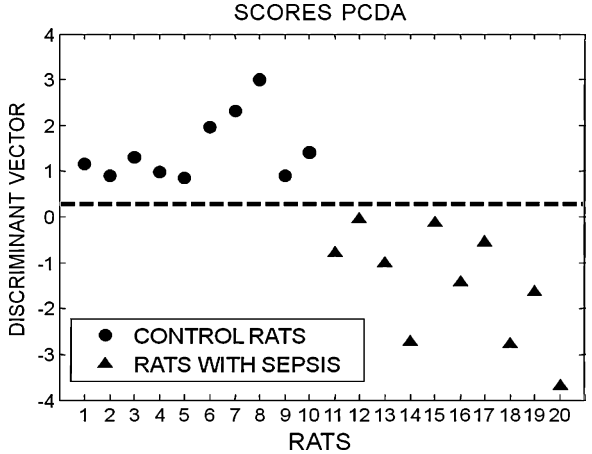

Fig. 6. Score plot of the final GC/MS discriminant vector. Triangles correspond to 10 diseased rats and circles to 10 healthy rats.

lack of knowledge as a reference technique. This study includes, for the first time, the measurement with IMS technology of rats' breath infused with LPS from *E. coli* as a sepsis animal model. This represents a first step in the potential applicability of IMS for the diagnosis of sepsis in human patients.

Although is well known that the injection of live bacteria and the injection of only LPS in an animal triggers different pathological effects, LPS is commonly used in sepsis models because the injected dose is completely controlled by the experimenter, while this is not the case when live bacteria are injected. Furthermore, although it has been demonstrated that LPS-induced models of endotoxic shock in rodents do not exactly reproduce septic complications in humans [40], they have been used to investigate endotoxin-dependent mechanisms in vivo. Thus, the LPS from the *E. coli* model used in rats in our study is suitable for examining LPS-dependent aspects of septic shock.

GC/MS measurements provided a list of compounds in the rat's breath. After the elimination of the compounds from the SPME-fiber and the anesthesia, fifteen compounds can be potentially used to separate healthy rats from treated rats. To obtain a subset of compounds related to sepsis, PCA-LDA and rank products were used as techniques that allow a maximum discrimination between classes and a ranking of compounds according to their discriminatory importance. Moreover, this methodology allows us to obtain a significance level for selected compounds considered as a *p*-value [36]. Thus, the *p*-value represents the probability of observing a compound at a certain rank, and compounds with the lowest rank are the most important in the separation. In this study we selected compounds with a *p*-value lower than 0.001. In the end, the first five compounds listed in Table 1 were selected as the most representative compounds in the discrimination between septic and healthy animals, and this could be considered a pattern correlated with sepsis. In this reduced space, a pattern recognition system provides promising rates of bootstrap validation: 85% of accuracy, 91% of specificity and 80% of sensitivity. These percentages must be understood in the light of the bootstrap validation procedure: they mean that, after 500 random selections of different sets of rats, overall 85% of the rats were well classified, and the same inference can be made from the figures for specificity and sensitivity.

Despite the good figures achieved with GC/MS measurements, the time, cost and infrastructure needed for the sampling and measurement make it impossible to use of these instruments in a bedside setting. The IMS alternative, however, does allow for this possibility because the sampling and measurement take only a few minutes. With respect to the IMS results, multivariate signal processing was able to detect the spectra of pure breath constituents. After discarding external pollutants and anesthesia, and after applying pattern recognition procedures, a pattern of three components was found. Although it is not possible to identify these compounds, they can be separated into two classes, with good levels of accuracy (99.8%), specificity (99.6%) and sensitivity (99.9%) under bootstrap validation. It must be stressed that bootstrap validation is designed to avoid over-optimistic results. It is interesting to note that even better results are achieved by processing the full IMS spectra instead of selected molecules. In this respect, we believe that sepsis produces a general alteration in the breath pattern and not just the secretion of a single or few biomarkers. Lack of knowledge about the metabolic pathway is therefore not a major issue, since the levels of many different VOCs are probably altered. However, the lack of identification of the resultant components might be solved by adding a multi-capillary column (MMC) before the IMS analysis, as described by Jünger et al. [41]. The addition of an MMC would not increase the time of the experimental analysis and it could be a solution for the identification and rapid diagnosis of breath samples. Finally, the outstanding results obtained are encouraging and open up the prospect of performing new experiments to validate the model developed for the diagnosis of sepsis and beginning carefully controlled studies with human patients.

In conclusion, breath analysis with IMS has been presented as an alternative for a rapid diagnosis of sepsis. The performance of this methodology in separating a healthy rat group from a diseased rat group is excellent and provides encouraging conceptual evidence at the experimental level. Therefore, the results obtained in the present animal study warrant further clinical studies in septic patients, in order to explore the routine capability of IMS as a non-invasive point-of-care diagnostic tool.

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References

[1] L. Pauling, A.B. Robinson, R. Teranishi, P. Cary, Proc. Natl. Acad. Sci. U.S.A. 68 (1971) 2374.
[2] D. Smith, C. Turnes, P. Spanel, J. Breath Res. 1 (2007) R1.
[3] W. Miekisch, J.K. Schubert, G.F.E. Noeldge-Schomburg, Clin. Chim. Acta 347 (2004) 25.
[4] R. Hyspler, S. Crhova, J. Gasparic, Z. Zadak, M. Cizkova, V. Balasova, J. Chromatogr. B 739 (2000) 183.
[5] G. Preti, E. Thaler, C.W. Hanson, M. Troy, J. Eades, A. Gelperin, J. Chromatogr. B 877 (2009) 2011.
[6] S. Svensson, M. Larstad, K. Broo, A.C. Olin, J. Chromatogr. B 860 (2007) 86.
[7] S. Van den Velde, F. Nevens, P. Van Hee, D. van Steenberghe, M. Quirynen, J. Chromatogr. B 875 (2008) 344.
[8] L. Buszewski, M. Kesy, T. Ligor, A. Amann, Biomed. Chromatogr. 21 (2007) 553.
[9] J.P. Spinhirne, J.A. Koziel, N.K. Chirase, Biosyst. Eng. 84 (2003) 239.
[10] W. Miekisch, J.K. Schubert, TrAC – Trends Anal. Chem. 25 (2006) 665.
[11] J. Van Berkel, J.W. Dallinga, G.M. Moller, R.W.L. Godschalk, E.J. Moonen, E.F.M. Wouters, F.J. Van Schooten, Respir. Med. 104 (2010) 557.
[12] M. Phillips, R.N. Cataneo, C. Saunders, P. Hope, P. Schmitt, J. Wai, J. Breath Res. 4 (2010) 8.
[13] C. Di Natale, A. Macagnano, E. Martinelli, R. Paolesse, G. D'Arcangelo, C. Roscioni, A. Finazzi-Agro, A. D'Amico, Biosens. Bioelectron. 18 (2003) 1209.
[14] G.A. Eiceman, Z. Karpas, Ion Mobility Spectrometry, CRC Press, Boca Raton, 1994.
[15] M. Westhoff, P. Litterst, L. Freitag,.W. Urfer, S. Bader, J.I. Baumbach, Thorax (2009) 744.
[16] J.I. Baumbach, M. Westhoff, Spectrosc. Eur. 18 (2006) 22.
[17] M. Westhoff, P. Litterst, L. Freitag, J.I. Baumbach, J. Physiol. Pharmacol. 58 (2007) 739.
[18] V. Bessa, K. Darwiche, H. Teschler, U. Sommerwerck, T. Rabis, J.I. Baumbach, L. Freitag, Int. J. Ion Mob. Spectrom. 14 (2011) 7.
[19] Z. Karpas, W. Chaim, R. Gdalevsky, B. Tilman, A. Lorber, Anal. Chim. Acta 474 (2002) 115.
[20] S.M. Opal, Int. J. Med. Microbiol. 297 (2007) 365.
[21] C. Brun-Buisson, F. Doyon, J. Carlet, P. Dellamonica, F. Gouin, A. Lepoutre, J.C. Mercier, G. Offenstadt, B. Regnier, JAMA 274 (1995) 968.
[22] S.J. Parker, P.E. Watkins, Br. J. Surg. 88 (2001) 22.
[23] M.J. Schultz, T. van der Poll, Ann. Med. 34 (2002) 573.
[24] A. Tarkowski, L.V. Collins, I. Gjertsson, O.H. Hultgren, I.M. Jonsson, E. Sakiniene, M. Verdrengh, Trends Microbiol. 9 (2001) 321.
[25] D.M. Soares, M.J. Figueiredo, J. Manvailer, R. Resende, C. Sorgi, L.H. Faciolli, J.C. Alves-Filho, F.Q. Cunha, G. Souza, Med. Microbiol. Immunol. (2011) (Published online).
[26] J. Lava, C.L. Rice, G.S. Moss, H. Levine, A. Rosen, L. Sehgal, S.A. Gould, J. Trauma. 22 (1982) 280.
[27] J.M. Sethi, A.M.K. Choi, W.J. Calhoun, B.T. Ameredes, Respir. Res. 9 (2008) 45.
[28] S. Weicker, T.A. Karachi, J.A. Scott, D.G. McCormack, S. Mehta, Am. J. Resp. Crit. Care 163 (2001) 1113.
[29] R.A. Dercho, K. Nakatsu, R.J. Wong, D.K. Stevenson, H.J. Vreman, J. Pharmacol. Toxicol. 54 (2006) 288.
[30] W. Vautz, J. Nolte, A. Bufe, J.I. Baumbach, M. Peters, J. Appl. Physiol. 108 (2010) 697.
[31] A. Savitzky, M.J.E. Golay, Anal. Chem. 36 (1964) 1627.
[32] V. Pomareda, D. Calvo, A. Pardo, S. Marco, Chemometr. Intell. Lab. 104 (2010) 318.
[33] A.R. Webb, Satatistical Pattern Recognition, 2nd Ed., Jhon Wiley & Sons, 2002.

[34] B. Efron, Ann. Stat. 7 (1979) 1.
[35] R. Breitling, P. Armengaud, A. Amtmann, P. Herzyk, FEBS Lett. 573 (2004) 83.
[36] S. Smit, M.J. van Breemen, H.C.J. Hoefsloot, A.K. Smilde, J.M.F.G. Aerts, C.G. de Koster, Anal. Chim. Acta 592 (2007) 210.
[37] I. Almendros, P.T. Gutierrez, D. Closa, D. Navajas, R. Farre, Respir. Physiol. Neurobiol. 162 (2008) 100.
[38] T. Pluskal, S. Castillo, A. Villar-Briones, M. Oresic, BMC Bioinformatics 11 (2010) 395.
[39] S.D. Carrigan, G. Scott, M. Tabrizian, Clin. Chem. 50 (2004) 1301.
[40] C.J. Gordon, Front. Biosci. 2 (2010) 293.
[41] M. Jünger, B. Böderek, J.I. Baumbach, Anal. Bioanal. Chem. 396 (2010) 471.