Development of a simple and low-cost prototype probe fully-compatible with ASAP source for the analysis of human breath in real-time.

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Abstract

The interest of ambient ionization mass spectrometry in clinical and forensic analysis has increased in the last years as it allows the rapid and direct analysis of a wide variety of samples. Among the possible applications, the analysis in real-time of the exhaled breath has gained great attention, for the investigation of both endogenous compounds, as for example disease biomarkers, and exogenous compounds related to the consumption of certain products. Although commercial ionization sources are already available for breath analysis, they require an important economic investment and/or complex setups. In this article, we describe a new probe developed at our laboratory, fully compatible with the Waters Corp. atmospheric solids analysis probe (ASAP), and where manufacturing is neither expensive nor complex. This atmospheric breath analysis probe (ABAP) can be directly used in Waters mass spectrometry instruments without the need of modifying the ionization source, the instrument or the ASAP, being also compatible with the software provided by the manufacturer. The ionization of the compounds is based on atmospheric pressure chemical ionization. The prototype has been successfully used in a high-resolution mass spectrometry system, and applied to the analysis of compounds present in human breath, as well as to the tentative identification of different substances present in foods and reported in exhaled breath after food consumption. In addition, ABAP could also be compatible with other ionization techniques, such as photoionization.

Keywords: ambient ionization mass spectrometry; atmospheric solids analysis probe; breath analysis; ionization sources; high-resolution mass spectrometry.

1. Introduction

Mass spectrometry (MS) is becoming the gold standard in clinical and toxicological analysis, especially when coupled to chromatographic techniques, due to the excellent selectivity provided, high sensitivity and identification power [1,2]. Nevertheless, the interest in direct MS analysis using ambient ionization sources has increased in the last years, as it provides fast results with minimum (or even without) sample treatment.

This growing interest on direct MS analysis is reflected by the development of different commercially available ambient ionization sources, such as desorption electrospray ionization (DESI) and direct analysis in real time (DART) [3], together with other techniques such as rapid evaporative ionization mass spectrometry (REIMS) [4] or atmospheric solids analysis probe (ASAP) [5]. These ionization techniques have been used in several applied fields, such as food fraud [6], pesticide residue analysis [7] and clinical analysis [8]. In fact, clinical analysis is a hot topic of ambient ionization, as these techniques allow their integration in surgical systems [9,10], as well as their use in rapid clinical diagnosis, such as carcinogenic tissue [11,12].

An interesting application of direct MS in the clinical field, is breath analysis for biomarker detection [13]. MS-based breath analysis has been recently applied to the detection of gastric cancer [14], liver failure [15], lung cancer [16], cystic fibrosis and asthma [17]. Indeed, the interest in breath analysis by MS has led to the development and commercialization of specific systems for direct analysis in real time by the use of secondary electrospray ionization (SESI) [18,19]. This source has been successfully applied to the real-time determination of different markers in breath such as nonvolatile drugs [20], peppermint oil ingestion [21], and other compounds from the human metabolome [22].

Several ionization source prototypes for breath analysis have been developed based on different ionization principles, such as extractive electrospray for the analysis of supercharged proteins [23], atmospheric pressure chemical ionization (APCI) [24], and atmospheric pressure photoionization (APPI) [25,26]. However, some of these prototypes are complex in design, and could not be easily implemented by laboratories without an important financial investment.

On this basis, we set out the design and construction of a probe that could be easily implemented in MS instruments for real-time breath analysis with a minimum manufacturing complexity and investment (**Fig. 1a**), using the Waters Corp. (Manchester, UK) ASAP source (**Fig. 1b** and **c**) as a model for developing the APCI-based ionization probe. The probe was designed based on the fundamentals of the MS Nose APCI-based ionization sample introduction technique for the analysis of gaseous samples [27]. Our system has proven to be able to detect and identify compounds present in human breath that have been previously reported in the literature, as well as specific substances from foods in exhaled breath after food consumption. We decided to name this prototype as "atmospheric breath analysis probe" or ABAP.

2. Materials and methods

2.1. Reagents and chemicals

Ultrapure water was obtained by purifying demineralized water using an Ultramatic Plus GR from Wasserlab (Navarra, Spain). Acetonitrile (LC-MS grade), formic acid (LC-MS grade) and sodium hydroxide were purchased from Scharlau (Scharlab, Barcelona, Spain). Leucine enkephalin acetate salt hydrated (>95%) was purchased from Merck (Darmstadt, Germany).

2.2. Probe design

The ABAP prototype probe was designed in order to fit the following requirements: (i) easy to develop and implement, (ii) low cost, (iii) easy to use, (iv) fully-compatible with existing sources,

instruments and software, (v) APCI ionization with the option of using APPI, and (vi) applicable to real-time analysis of exhaled breath and other gaseous samples. The advantage of using an ASAP probe is that simplifies the device and provides a reproducible positioning of gas entering the source.

As detailed in **Fig. 1**, the ABAP prototype (**Fig. 1a**) was modelled on the commercial ASAP probe (**Fig. 1b**) for optimum compatibility with the ASAP holder (**Fig. 1c**) and the Waters Corp. ionization sources. As the ASAP holder has a switch that detects if the probe is installed, the ABAP has to activate this sensor for allowing instrument acquisition. For this purpose, the ABAP needs a bracket (**Fig. 1h**, piece 1) with a 25 mm OD, 21 mm ID and around 50 mm length, being in our case a PVC tube of the described characteristics (**Fig. 1d** shows the fitting of the probe and the holder). The ASAP probe is inserted into the holder through a stainless steel tube that should be fitted in order to prevent clearance that produces probe vibrations. For an optimum fit, the tube should be around 12 mm OD, and for our ABAP design, a stainless steel tube of 12.1 mm OD, 9.7 mm ID and 230 mm length was used (**Fig. 1h**, piece 2). It is important to know that the ASAP holder reduces its ID at the end of the probe holder, and the ASAP probe rests on this tightly. So, the distance between the PVC bracket and the end of the fitting stainless steel tube should be 142 mm for an adequate fitting as well as a correct ASAP holder switch activation.

For sample introduction in the ionization chamber, the tube used should pass through the ASAP holder end opening, and it must fit in order to prevent gas leaks in the source. In our design, a stainless steel tube of 4.3 mm ID, 6.4 mm OD and 280 mm length was used (**Fig. 1h**, piece 3). The distance between the end of the ASAP holder (the piece 3 of the ABAP) and the opening of the tube 3 was 44 mm for focusing the breath stream in the APCI corona pin discharge region (**Fig.**

1g). The full description of the materials used for the construction of the ABAP probe is shown in **Table 1**.

Pieces 2 and 3 were welded using TIG (tungsten inert gas) welding, and piece 2 was bonded to piece 1 using a thermostable adhesive. For sample introduction, a Tygon 2475 tube (**Fig. 1e**) was connected to the ABAP probe, using a 5-mL pipette tip as disposable mouthpiece (**Fig. 1f**). The whole setup was proved to be functional and fully compatible with the Waters Corp. instruments and software.

2.3. ABAP-HRMS analysis

The ABAP prototype was interfaced to a Xevo G2 QTOF hybrid quadrupole-time of flight mass spectrometer (Waters Corp, Manchester, UK) using the ASAP holder installed in a Z-Spray LockSpray ionization source, as shown in **Fig. 1** (Waters Corp, Manchester, UK). The corona pin current was operated in positive ionization mode at 1.6 μA. The source temperature was 120 ºC, and the desolvation temperature 200 ºC. The cone voltage was set to 20 V, using a cone gas flow of 20 L/h and a desolvation gas flow of 200 L/h (Nitrogen 99.995%). MS acquisition was performed in full-spectrum acquisition mode from *m/z* 50 to 1000. Mass-axis was daily calibrated from *m/z* 50 to 1000 using a 1:1 mixture of 0.05 M sodium hydroxide:5% formic acid, diluted 1:25 with acetonitrile:water (80:20) and using electrospray ionisation. For accurate-mass measurements, 2 μg/mL of leucine enkephalin solution in acetonitrile:water (50:50) with 0.1% formic acid was used as lock-mass, pumped at a flow rate of 15 μL/min, using the protonated molecule to recalibrate the mass axis. Further HRMS details can be found in literature [28]. MS data were acquired and processed using MassLynx data station operation software version 4.1 (Waters).

3. Results and discussion

3.1. Testing the probe with human exhaled breath

The ABAP probe prototype was tested by analyzing the exhaled breath of a healthy volunteer. The volunteer blew through the ABAP several times (**Fig. 2a**), observing an increment of the baseline of the total ion chromatogram (TIC) when the breath was introduced in the source. The accurate-mass full-range spectra of the exhaled breath (**Fig. 2b**) was obtained by combining the scans corresponding to the "breath peak" and subtracting the baseline. Several ions were observed in the APCI spectra, illustrating the ionization of some exhaled compounds. Additionally, a blank full-range spectrum was acquired flushing helium though the ABAP for identifying the ions coming from the prototype, the source, and the laboratory ambient (**Fig. 2c**). As observed in **Fig. 2c**, most of the ions observed in the exhaled breath were not present in the blank spectrum.

As the ABAP is based on APCI positive ionization, and due to the high-humidity level in exhaled breath (relative humidity 88-98%), it was expected that molecules were ionized based on proton-transfer mechanisms [29]. Some of the ions observed in breath spectrum were identified as compounds previously reported in this type of sample, being detected as protonated (or other adduct) molecule and with a mass error below 5 ppm for all the identified compounds.

Fig. 2d illustrates the detection of different compounds present in exhaled breath (spectrum focused in the 50-100 *m/z* range), and **Table 2** gives information about their tentative identification. Acetone (*m/z* 59) and 2-butanone (*m/z* 73) have been widely reported in human breath, as they are involved in different metabolic processes being eventually exhaled through the breath [25,26]. In the case of pyridine (*m/z* 80), this compound has been reported to be present in roasted coffee [30], as well as a marker for tobacco exposure [31]. The presence of these compounds in exhaled breath was assured after checking the blank spectrum (**Fig. 2e**) which did not show any of the studied ions.

Other examples are shown in **Fig. 3**, presenting the detection of different cyclomethicones, a group of liquid and highly volatile methyl siloxanes (silicones). Cyclomethicones are commonly used in personal care products such as antiperspirants, shampoos and skin creams [32,33], but can also be generated from different sources, such as residential oven use [34]. In the last years, the interest on cyclomethicones exposure assessment [35,36] has increased, and their presence in exhaled breath has been reported in literature [26,37]. In this work, up to three cyclomethicones were detected as $[M+H]^+$ and/or $[M+NH_4]^+$ in the healthy volunteer exhaled breath: dodecamethylcyclohexasiloxane (D6), tetradecamethylcycloheptasiloxane (D7) and hexadecamethylcyclooctasiloxane (D8), as shown in **Fig. 3** and **Table 2**.. Again, the no presence of these compounds in blank spectrum (laboratory ambient) was checked (please see **Fig. 2b** and **2c**).

3.2. Food consumption markers, long term detection and carry over evaluation

Another important feature of the ABAP probe to be tested was the identification of exogenous compounds as well as their evolution over time after food consumption. The possible carry-over of the probe from compounds sticking onto the inlet surface was also evaluated.

In a first experiment, the healthy volunteer consumed a mint candy, and blew through the ABAP-HRMS system several times in the next 30 min after consumption. As it was expected, menthone was detected at *m/z* 155 ($M+H^+$), as well as one fragment ion at *m/z* 137 in the exhaled breath (**Fig. 4a** and **Table 2**). Menthone is a natural compound present in peppermint, so it is not surprising the detection of this compound immediately after mint candy consumption [38] at high concentration, as it can be observed in the detector counts of the extracted ion chromatogram (EIC, mass extraction window of ±5 mDa) of menthone (**Fig. 4b**). Nevertheless, menthone presented also high analytical response 10 min after consuming the mint candy, as illustrated in **Fig. 4c**. The high

analytical response observed for this compound encouraged us to evaluate the possible carry-over effect, as exhale compounds could be adsorbed in the inner surface of the probe. For this purpose, helium was flushed through the ABAP 5 min after the last blew, observing a menthone EIC top intensity 100 times lower than the observed 10 min after consuming the candy (**Fig. 4d**). On the basis of these data, carry-over effects were considered negligible and should not be a crucial drawback of the ABAP-HRMS performance, but further studies for minimizing this effect should be performed. Finally, menthone was also detected in exhaled breath 25 min after taking the mint candy (**Fig. 4e** and **4f**, respectively), although its analytical response significantly decreased.

Another example is shown in **Fig. 5b**, where tetramethylpyrazine (*m/z* 137, $M+H^+$), a natural compound produced by the fermentation of cocoa [39], was detected in the volunteer exhaled breath 10 min after the consumption of a small piece of 85% cocoa chocolate.

3.3. Purge-and-analysis in real-time using ABAP-HRMS

Based on the results obtained in the chocolate experiment, a setup for detecting volatile compounds in solid samples was designed. This setup was performed considering the principle of purge-and-trap used for analyzing volatile compounds in solid and liquid samples. Briefly, purge-and-trap is based on heating the sample of interest inside a flask, flushing nitrogen or another gas though the headspace of the flask and placing an adsorbent in the gas exhaust.

On this basis, the purge-and-analysis in real-time setup was designed, connecting the exhaust of the beaker directly to the ABAP-HRMS system, as shown in **Fig. 5c**. In order to evaluate the suitability of this setup, crushed 85% cocoa chocolate was placed in a 5 mL glass vial, introduced in a purge-and-trap flask containing ultrapure water and heated at 60 ºC. Water was added to the flask for increasing the humidity of the air purge entering the ABAP-HRMS, as APCI protonation is based on proton transfer reactions in which water molecules are involved. The system

demonstrated its applicability for the real-time detection of volatile compounds in solid samples such as tetramethylpyrazine and vanillin (*m/z* 153, reported in cocoa products [40]) which were detected in the 85% cocoa chocolate sample (**Fig. 5a**). Interestingly, vanillin was not detected in exhaled breath analyzed 10 min after the consumption of the same chocolate product previously described.

3.4. ABAP limitations and future upgrades

The ABAP prototype has demonstrated its potential for the analysis in real time of exhaled breath, as well as of volatile compounds that can be determined using a purge-and-trap setup. Nevertheless, there are different issues that should be further investigated. First of all, the candy mint experiment demonstrated the presence of a slight carry-over effect, which should be minimized for preventing false positives in subsequent analyses. One possibility could be the use of a heated transfer line between the mouthpiece and the ABAP, plus a continuous stream of heated gas, which would maximize aerosol transport and prevent potential condensation and compound adsorption to the inner surface.

All the performed experiments were carried out using APCI ionization, but it is known that an important number of volatile compounds are ionized by APPI [26,41]. So, the ABAP prototype was also tested in the Waters Corp. APPI ionization source. Unfortunately, the APPI source did not recognize the ASAP holder and, although ABAP-APPI-HRMS and dual APPI/APCI data could be acquired, the desolvation gas flow and temperature could not be set, producing an undesirable ion signal decrease. Therefore, if the APPI would be used, an external gas source and heating system should be employed. Additionally, the Waters APPI ion source is not equipped with a lockmass delivery system, being necessary the continuous introduction of a reference compound through the ABAP to recalibrate mass axis. An in-house lockmass for the ABAP-APPI-

HRMS system was designed using a peristaltic pump delivering 0.5 mL/min of a 10 µg/mL perfluorotributylamine solution in acetone:toluene 1:1. Toluene was added as doping agent for promoting charge transfer reactions in APPI. Unfortunately, the lockmass solution was not efficiently evaporated due to the lack of a heated gas source, producing a low intense and unstable perfluorotributylamine signal.

Finally, the ABAP-HRMS was operated in accurate-mass full-range acquisition, obtaining complex spectra. In the case of exhaled breath, cleaner spectra were obtained after combining breath "peak" and subtracting background ions, as commented in **Fig. 2**. If compatible, different acquisition modes could be used for obtaining MS/MS spectra or cleaner data. For example, data-dependent acquisition (DDA) would be an interesting working mode, providing accurate-mass full-range spectra and accurate-mass MS/MS data. Nevertheless, the software configuration of the Waters ASAP did not allow the use of DDA acquisition when using a HRMS instrument, unlike MS/MS instruments in which ASAP-DDA is fully-compatible [5]. Another interesting approach is the use of ASAP in HRMS equipped with an ion mobility separation and using data-independent acquisition (DIA). In this case, cleaner fragmentation spectra would be obtained, as fragment ions would be drift-aligned with precursor ion, as well as measuring the collision cross section (CCS) value of the compounds of interest that can be used as additional identification parameter [42].

4. Conclusions

Breath analysis is a topic of increasing interest, especially when ambient ionization techniques that allow the analysis of exhaled breath in real time are used. In this work, we present a prototype probe, named ABAP, which can be adapted, at low cost, to Waters Corp. instruments using the commercially available ASAP probe. This new source is based on APCI ionization, producing mainly protonated molecules due to the high humidity of exhaled breath.

The described prototype has proven its functionality for detecting compounds naturally present in breath, such as acetone and 2-butatone, as well as markers of exposure, such as pyridine (exposure to tobacco and/or coffee) and cyclomethicones (present in personal care products). Additionally, compounds typically found in food were also detected in breath after its consumption. This was the case of menthone, which was detected even 25 min after consuming a mint candy. ABAP-HRMS was also shown to be useful for real-time monitoring of volatile compounds associated with different processes, like chemical reactions or biological processes, among others.

Future ABAP upgrades will be focused on the minimization of carry-over, compatibility of APPI with lockmass system for detecting volatile compounds that could not be ionized by APCI, and the use of additional acquisition modes such as DDA or ion mobility-DIA.

Author Contributions

D. Fabregat-Safont and J.V. Sancho conceived the work. D. Fabregat-Safont designed and developed the prototype probe. D. Fabregat-Safont, M. Ibáñez and J.V. Sancho evaluated the probe performance. M. Ibáñez and F. Hernández obtained financial support. D. Fabregat-Safont and J.V. Sancho wrote the first version of the manuscript. M. Ibáñez and F. Hernández provided feedback and useful comments.

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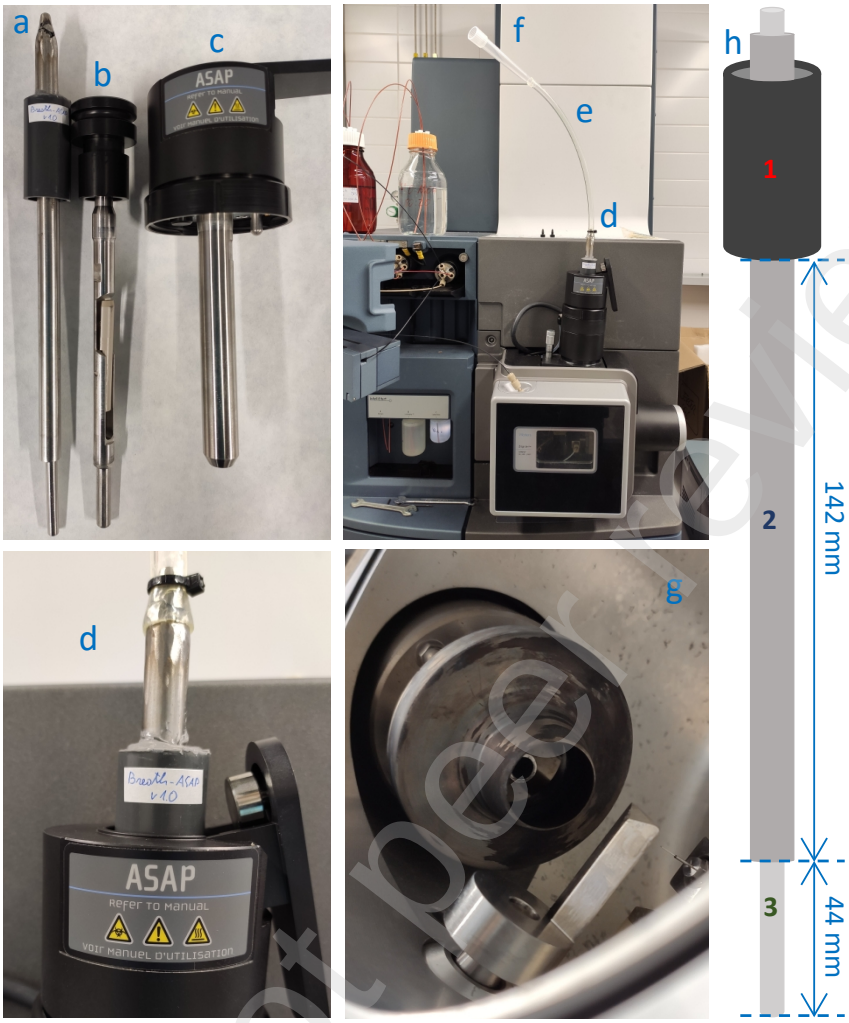

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413 **Fig. 1.** Prototype of the ABAP probe. (a) ABAP probe designed and used at our laboratory. (b)
414 Waters Corp. ASAP probe used as a model for ABAP. (c) ASAP holder for introducing the probe
415 into the ionization chamber. (d) ABAP installed in a HRMS instrument (Xevo G2 QTOF from
416 Waters Corp.). (e) Tygon 2475 tube. (f) Pipette tip used as disposable mouthpiece. (g) Detail of
417 the probe in the ionization chamber. (h) Scheme of the ABAP probe.

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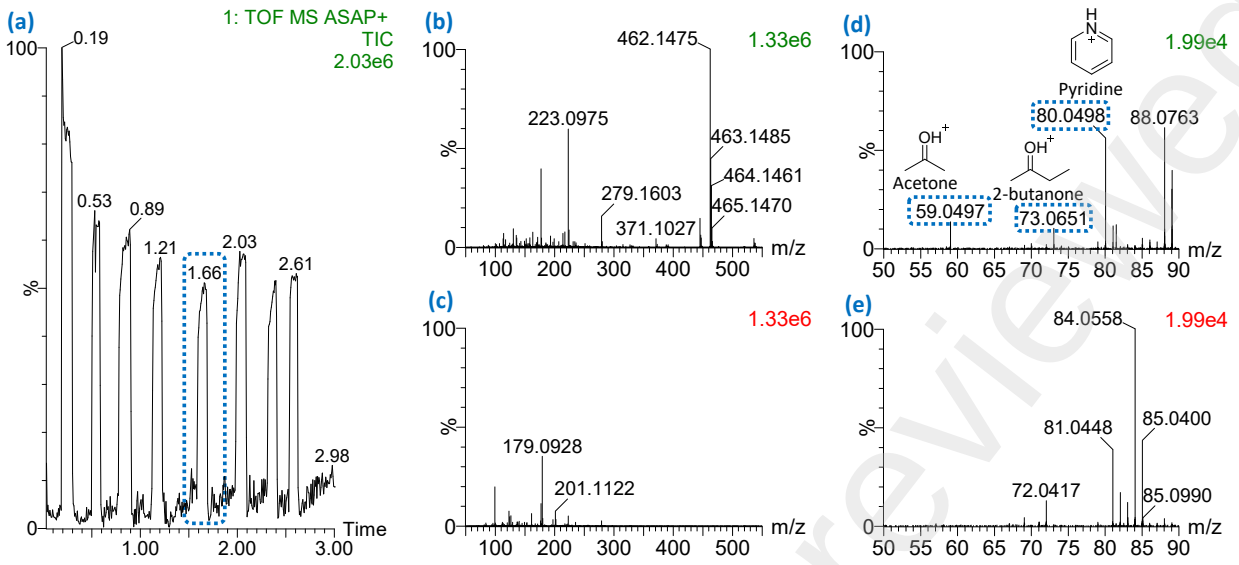

419

420 **Fig. 2.** Examples of the analysis of exhaled breath using the ABAP-HRMS system. (a) Signal
421 obtained when the volunteer blew into the ABAP-HRMS system. (b) Accurate-mass full-range
422 spectra of the exhaled breath. (c) Accurate-mass full-range spectra of the helium blank. (d)
423 Detection of acetone, 2-butanone and pyridine in breath. (e) Helium blank of the studied mass
424 range.

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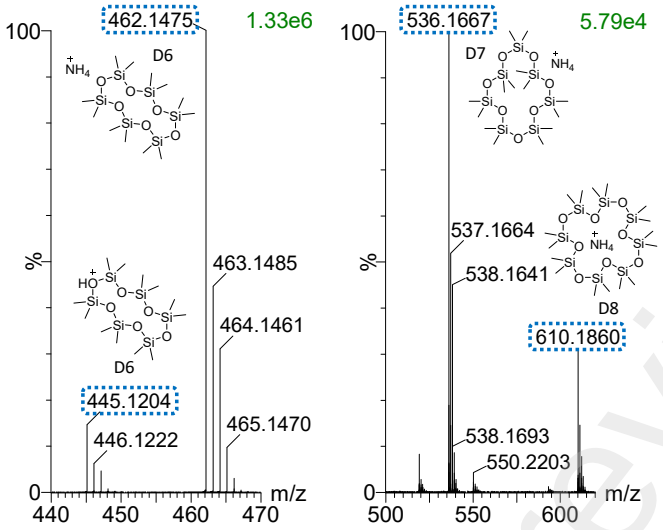

426

427 **Fig. 3.** Detection of D6, D7 and D8 in breath.

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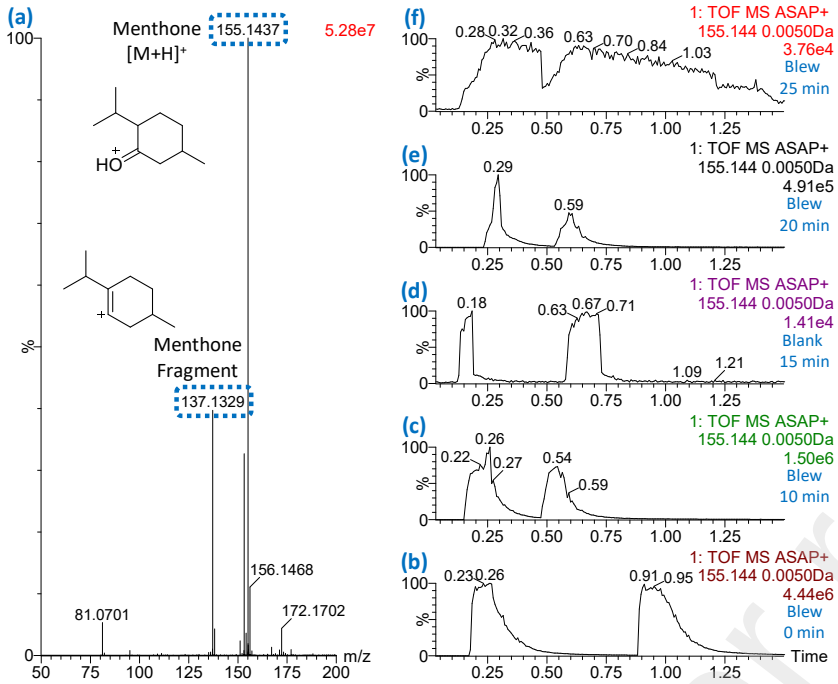

Fig. 4. Detection of menthone in breath after the consumption of a mint candy. (a) Accurate-mass spectrum obtained 0 min after consumption. (b) EIC of menthone in breath 0 min after consumption. (c) EIC of menthone in breath 10 min after consumption. (d) EIC of menthone in a helium blank 5 min after the last blew. (e) EIC of menthone in breath 20 min after consumption. (f) EIC of menthone in breath 25 min after consumption.

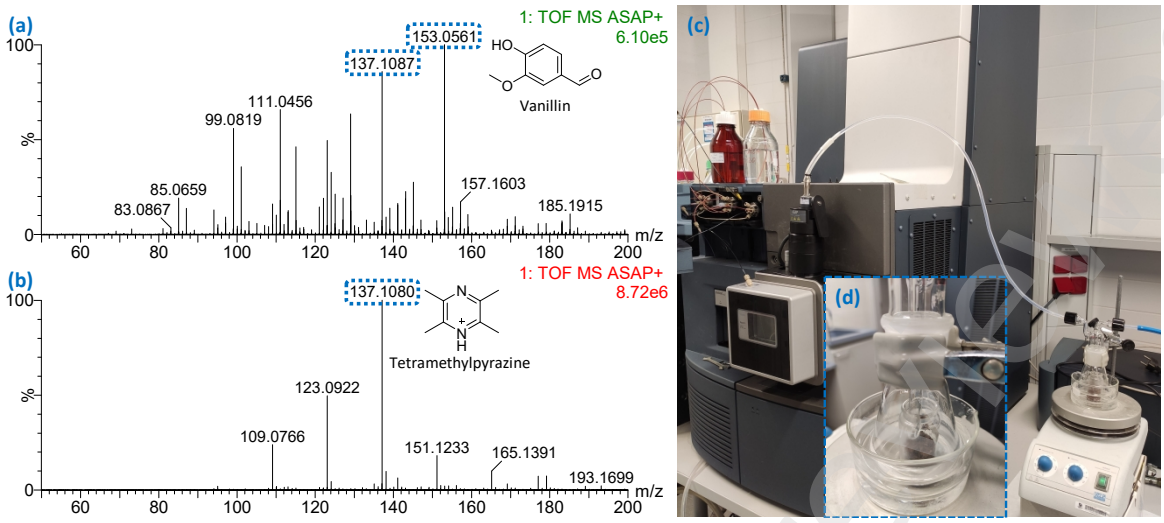

Fig. 5. Experiments performed with 85% cocoa chocolate. (a) Detection of tetramethylpyrazine and vanillin in chocolate using the purge-and-analysis in real-time ABAP-HRMS setup. (b) Detection of tetramethylpyrazine in exhaled breath 10 min after consuming a small piece of chocolate. (c) Picture of the purge-and-analysis in real-time ABAP-HRMS setup, illustrating how the air purge is directly introduced in the prototype probe. (d) Detail of the chocolate sample inside the flask containing water, heated at 60 ºC.

446 **Table 1.** Materials and components used for the ABAP probe prototype.

Piece	Material	Description	Value
1	PVC	Bracket	Length: 50 mm ID: 21 mm OD: 25 mm
2	Stainless steel	ASAP holder fitting	Length: 230 mm ID: 9.7 mm OD: 12.1 mm
3	Stainless steel	Entrance to ionization chamber	Length: 280 mm ID: 4.3 mm OD: 6.4 mm

447

448 **Table 2.** Tentative identification by ABAP-HRMS of the compounds detected in exhaled human

449 breath, and in exhaled human breath after the consumption of food products.

Compound	m/z	Elemental Composition	Ion	Mass error mDa (ppm)	Reference reported in breath/product
Acetone	59.0497	$C_3H_7O^+$	$[M+H]^+$	0.0 (0.0)	[25,26]
2-butanone	73.0651	$C_4H_9O^+$	$[M+H]^+$	-0.2 (-2.7)	[25]
Pyridine	80.0498	$C_5H_6N^+$	$[M+H]^+$	-0.2 (-2.5)	[25,31]
D6	445.1204	$C_{12}H_{37}O_6Si_6^+$	$[M+H]^+$	-0.1 (-0.2)	[26,37]
	462.1475	$C_{12}H_{40}NO_6Si_6^+$	$[M+NH_4]^+$	0.4 (0.9)	
D7	519.1377	$C_{14}H_{43}O_7Si_7^+$	$[M+H]^+$	-1.7 (-3.3)	[26]
	536.1667	$C_{14}H_{46}NO_7Si_7^+$	$[M+NH_4]^+$	0.8 (1.5)	
D8	610.1860	$C_{16}H_{52}NO_8Si_8^+$	$[M+NH_4]^+$	1.3 (2.1)	[26]
Menthone	155.1437	$C_{10}H_{19}O^+$	$[M+H]^+$	0.1 (0.6)	[38]
	137.1329	$C_{10}H_{17}^+$	$[M+H-H_2O]^+$	-0.1 (-0.7)	
Tetramethylpyrazine	137.1080	$C_8H_{13}N_2^+$	$[M+H]^+$	0.1 (0.7)	[39]
Vanillin	153.0561	$C_8H_9O_3^+$	$[M+H]^+$	0.9 (5.9)	[40]

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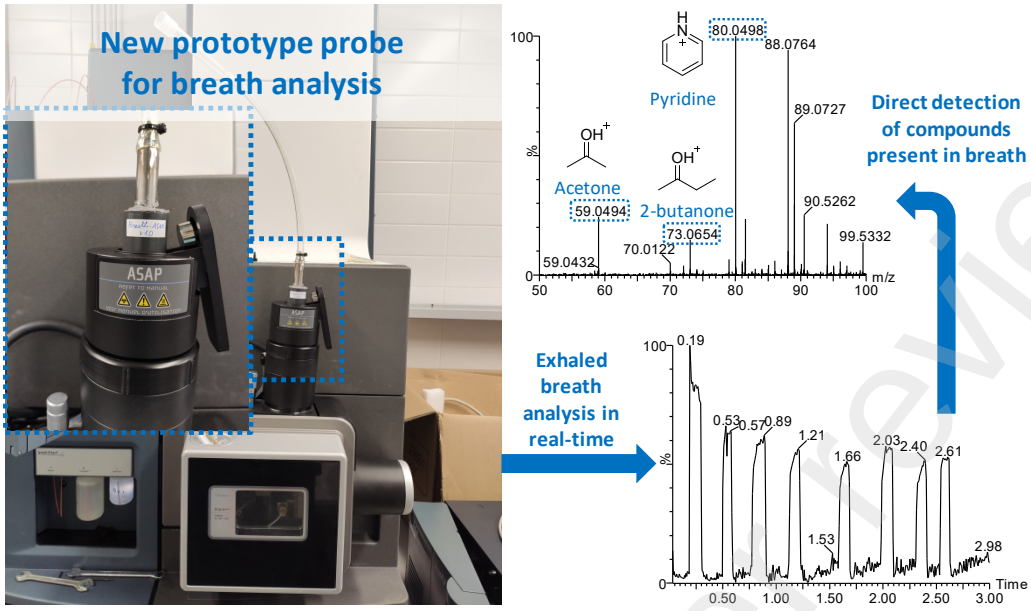
452 **Graphical Abstract**



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