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Proton transfer reaction time-of-flight mass spectrometric measurements of volatile compounds contained in peppermint oil capsules of relevance to real-time pharmacokinetic breath studies

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Abstract
With the growing interest in the use of breath volatiles in the health sciences, the lack of standardization for the sampling and analysis of exhaled breath is becoming a major issue leading to an absence of conformity, reproducibility and reliability in spectrometric measurements. Through the creation of a worldwide ‘peppermint consortium’, the International Association of Breath Research has set up a task force to deal with this problem. Pharmacokinetic studies are proposed, and a real-time analytical technique that is being used is proton transfer reaction-time-of-flight-mass spectrometry (PTR-ToF-MS). This paper presents details on how the volatile compounds contained in a peppermint oil capsule, and hence on breath, appear in a PTR-ToF-MS. To aid that study, the key volatiles in the headspace of peppermint oil were first identified using gas chromatography-mass spectrometry, notably: menthol, menthone, 1,8-cineole, menthofuran, limonene, α-pinene and β-pinene. A PTR-ToF-MS analysis of these compounds has been undertaken, divorced from the complexity of the peppermint oil matrix using ‘normal’ and ‘saturated’ humidity drift-tube conditions, with the latter used to mimic breath samples, and over a range of reduced electric fields. There are no characteristic product ions that can distinguish monoterpenes and 1,8-cineole, and hence, without pre-separation, a combined washout for these volatiles can only be provided. By operating the drift tube above about 130 Td, there are characteristic product ions for menthone, menthofuran and menthol, namely m/z 155.14 (protonated menthol), m/z 151.11 (protonated menthofuran), m/z 139.15 (loss of H2O from protonated menthol) and m/z 83.09 (a fragment ion, C6H11+ from menthol). These have been used to monitor, with a high specificity, the temporal profile of these three compounds in breath following the ingestion of a peppermint oil capsule. To aid in the analyses, the proton affinities and gas-phase basicities for the key volatiles investigated have been determined using density functional theory.

1. Introduction
Real-time analysis of exogenous volatiles contained in breath is particularly suited for pharmacokinetic studies. There are a number of real-time highly sensitive soft chemical ionization mass spectrometric methods to determine the endogenous and exogenous volatile compounds contained in a person’s breath, including proton transfer reaction mass spectrometry (PTR-MS) [1–5], selected ion flow tube-mass spectrometry (SIFT-MS) [6, 7], electrospray ionization-mass spectrometry (ESI-MS) [8, 9], and ion mobility spectrometry (IMS)
These techniques are particularly ideal for breath-to-breath analysis and pharmacokinetic breath studies [13]. However, there are no agreed sampling and analysis protocols for real-time (and off-line) breath studies. This results from the large variety of volatiles present in breath, the many different diseases being investigated, the diversity of breath sampling and collection methods and the diverse array of analytical instruments. But standardization is a high priority issue for the breath research community if independent studies are to be compared and in order to establish reliable and reproducible approaches and results if breath tests are to be of any use in a clinical environment [14, 15].

During the International Association of Breath Research (IABR) 2016 breath summit in Zurich, Switzerland [16], a task force was created with the aim to collect benchmark data that could be used to compare different breath sampling, analytical instruments and analyses techniques. The founding members are Paul Thomas (Loughborough University, UK), Simona Cris- tescu (Radboud University, Netherlands), Jonathan Beauchamp (Fraunhofer Institute for Process Engineering and Packaging IVV, Germany) and Stephen Fowler (University of Manchester, UK). The primary aim established by this task force is to ascertain and compare the breath washout (temporal) profile of certain exogenous compound coming from ingested peppermint oil capsules using different analytical techniques, with a focus on menthone. Post ingestion, the gel of the capsule dissolves in the stomach, releasing the chemicals contained in the capsule into the gut. From there the volatile compounds contained in the oil enter the blood circulation stream. Then, through the interchange of gases between blood and lungs, these volatiles can be detected in trace quantities in exhaled breath.

This use of a peppermint oil capsule for standardization is a challenging task, given that there will be a number of known (and unknown) variables in such a study. An important one is the consistency (thickness) of the gelatine forming the capsules, which will lead to variations in the time for dissolving in the gut. Another issue is the variation of the concentration of the compounds contained in the capsules. By using capsules coming from the same manufactured batch, any variations in gel thickness and compound concentrations should be minimized, but there may well be some variation. In addition, there will be inter-individual and intra-individual variations on how the capsules are dissolved in the stomach, and hence the time release will vary person-to-person, and on different days for the same person. Then there are issues of biological variations in metabolism, absorption and excretion (both inter-individual and intra-individual), dependences on a person’s body mass index, age, ethnicity, etc. All of these will lead to substantial exogenous volatile breath profile variations, and hence these complexities will in themselves result in problems for any standardization.

The peppermint task force at present includes fifteen research groups across the world, which will be referred to in this paper as the ‘peppermint consortium’, and they are using several analytical instruments to detect volatiles in the breath samples following the ingestion of peppermint oil. However, before any detailed breath washout measurements on any analytical device are presented, it is important to ascertain what volatile components need to be monitored in breath. This is a key aim of the work presented in this paper. The next step is then to determine how these volatiles are detected for specific analytical instruments under specific operating conditions. An important analytical technique being used within the consortium for real-time breath-to-breath measurements is proton transfer reaction-time-of-flight-mass spectrometry (PTR-ToF-MS). In this paper, we present details of PTR-ToF-MS investigations on the key individual volatile compounds that have been identified by gas chromatography-mass spectrometry (GC-MS) to be contained in the specific peppermint oil capsules being used by the consortium.

A key output of the results presented is to specify which product ions (m/z values) should be monitored for these volatiles with any PTR-MS instruments at any given reduced electric fields. The reduced electric field is the ratio of the electric field strength (E) to the total molecular number density (N) in the drift (reaction) tube of a PTR-MS. These m/z values can then be used to monitor the peppermint oil volatiles in breath following ingestion of a peppermint oil capsule. To provide values for the intensities of the product ions, we present results over a wide reduced electric field range, namely 80–210 Td, which covers the typical values used in PTR-MS studies, and at two different absolute humidity levels corresponding to ‘normal’ and ‘humid’ operational conditions, with the latter approximately corresponding to that of exhaled breath. This is needed if volatiles are to be monitored as precisely as possible during real-time breath sampling.

Finally, the proton affinities (PA) and gas-phase basicities (GB) for many of the volatiles of interest in this study are not available in the literature. Therefore, in order to aid in the interpretation of the PTR-MS results presented in this paper, density functional theory (DFT) calculations have been undertaken to determine these values. The reported PA and GB are not only of use to PTR-MS studies, but also to other analytical techniques which rely on proton transfer reactions, such as SIFT-MS, IMS and ESI-MS.

2. Methods

2.1. Peppermint oil capsules

Peppermint oil capsules (Boots Pharmaceuticals Digestive Health Peppermint Oil Capsules, UK, https://boots.com/boots-peppermint-oil-200mg-60-capsules-10115320) are being used by the peppermint oil consortium. These capsules are readily purchased over the counter as health supplements,
but for the peppermint consortium a large number of bottles containing 60 capsules each were purchased in bulk. This means that all capsules used by any research group come from the same batch (batch number 200207). The capsules’ shell consists of beef gelatine and glycerol. However, the use of beef gelatine restricts the type of volunteer. There will be people who cannot participate in the study either for religious reasons or dietary concerns e.g. vegetarians and vegans.

2.2. Experimental
2.2.1. Gas chromatography-mass spectrometry (GC-MS)
PTR-MS does not unambiguously identify volatile compounds, because often there is no pre-separation, all it provides is the compounds, because often there is no pre-separation, PTR-MS does not unambiguously identify volatile compounds. Therefore, a GC-MS analysis of the vapours of peppermint oil will be performed to correctly identify the volatile compounds contained in the peppermint oil. A GC-MS analysis of the vapours above this oil was initially performed by us. The procedure for the GC-MS measurements has been described in detail elsewhere [17]. Hence only the more technical details are presented here.

Three different oil samples originating from three different capsules were analysed. From each capsule 0.4 ml peppermint oil was extracted using a 1 ml syringe (BBraun, Melsungen, Germany) and transferred to an evacuated headspace glass bottle of 50 ml volume (VWR International, Vienna, Austria) closed by a Teflon® septum. After equilibrating the pressure in the vial, by adding nitrogen (99.9999% purity), 15–20 ml headspace sample was collected by piercing the septum using a 20 ml glass syringe (Socorex, Ecublens, Switzerland). The needle of the syringe was then changed to a Luer Lock adapter containing a membrane (VWR International, Vienna, Austria). Extraction of volatiles was performed using a needle trap device (NTD) containing 2 cm Carbopack X and 1 cm Carboxen 1000 (PAS Technology, Magdala, Germany). The NTD was then connected to the 20 ml glass syringe filled with the sample through piercing the membrane-containing adapter, and was placed into an incubator at 40 °C to ensure a stable temperature during the extraction process. The other end of the NTD was connected to an electronic mass flow controller (model F-201DVRAD-11-V, Bronkhorst, Ruurlo, Netherlands) via a Teflon tube. For the generation of the sample flow, a pump (Vacuubrand, Wertheim, Germany) was placed at the end of the sampling system. A steady flow of 8 ml min⁻¹ through the NTD was used during adsorption.

VOCs were released from NTDs by thermal desorption at 290 °C in the injector of the gas chromatograph operating in split-less mode for 1 min. Chromatographic analyses were performed using an Agilent 7890A gas chromatograph equipped with a 5975C Inert XL mass selective detector (Agilent, USA). A capillary column RXT-624 30 m × 0.32 mm × 1.8 μm (Restek Corporation, US, Bellefonte, PA, USA) was used to separate species of interest. The column temperature program was as follows: 40 °C raised at 5 °C min⁻¹ to 150 °C, constant temperature of 150 °C for 2 min, increased at a rate of 10 °C min⁻¹ to 240 °C, and then maintained at a constant temperature of 240 °C for 5 min.

MS analyses were performed in full scan mode, with a m/z scan range from 20 to 200 Th. Ionization of the separated compounds was done by electron impact at 70 eV. Chromatographic data was acquired using the Agilent Chemstation Software (GC-MS Data Analysis from Agilent, Waldbronn, Germany). For data analyses and for identification of the compounds the software AMDIS and the mass spectrum library NIST 2008 (Gatesburg, USA) was applied.

2.2.2. Proton transfer reaction-mass spectrometry (PTR-MS)
PTR-MS is a popular analytical instrument used for a wide range of applications, including environmental analysis, food science, atmospheric chemistry, health science, homeland security, and breath analysis [1, 3, 5, 14, 17–23]. Thorough descriptions of the PTR-MS operating principles and its applications are provided in a text book by Ellis and Mayhew [1]. In brief, PTR-MS detects volatile molecular species at ultra-low levels (ppt to ppb) on the basis of chemical ionization within a drift (reaction) tube. More specifically, it exploits the proton transfer reaction of H₂O⁺ and, depending on the reduced electric field used and the humidity within the drift tube, also protonated water clusters, originating from a hollow cathode discharge or being produced in the drift tube through three-body association processes, with molecules of interest M:

\[ \text{H}_2\text{O}^+.(\text{H}_2\text{O})_n + \text{M} \rightarrow \text{MH}^+ + (n+1)\text{H}_2\text{O}, \]  

where \( n = 0 \) and 1 are the most important for our operational conditions (see results), but also (in low concentrations and only at low E/N (less than approximately 100 Td)) \( n = 2 \). A consequence of the employment of this ionization mechanism is the fact that the bulk components of breath gas O₂, N₂, and CO₂ do not readily react with H₂O⁺.(H₂O)ₙ, and consequently proton transfer from H₂O⁺.(H₂O)ₙ is almost entirely selective to volatiles with PA greater than that of (H₂O)ₙ+1. However, very low signals of m/z 29.01 and m/z 45.00 are observed in PTR-MS spectra, corresponding to the molecular ions N₃H⁺ and CO₂H⁺. These ions are considered to be formed after the exit from the drift tube, because otherwise they would react with water in the drift tube [1]. Although observed with very low signal intensities, the temporal change in the CO₂H⁺ intensity can be used to accurately determine the transitional (changeover) phase from dead space to end-tidal phases of
exhalation. Thus, basically, PTR-MS users have an in-built fast response calibrator.

Although \( \text{H}_2\text{O}^+ \) (and associated protonated water clusters - depending on the value of the reduced electric field) dominate the reagent ion signal, other reagent ions are always present in the drift tube, although in much lower concentrations. For example, at an operational reduced electric field of 130 Td, two additional reagent ions are present, namely NO\(^+\) and \( \text{O}_3^+ \). However, these are at low concentrations, having relative to the \( \text{H}_2\text{O}^+ \) reagent ion intensity 0.8%–1.6% and 2%–5% levels for \( \text{NO}^+ \) and \( \text{O}_3^+ \), respectively, and hence they can be safely ignored when determining the product ions.

An issue with using PTR-MS for the analysis of breath in real time is breath’s high humidity, which changes the humidity in the reaction chamber leading to higher concentrations of protonated water clusters for a given reduced electric field. Hence, the effects of humidity on PTR-MS behaviour have been investigated in this study for the volatiles of interest.

The data presented in this paper have been taken using an Ionicon Analytik GmbH (Innsbruck, Austria) PTR-TOF 8000. For the measurements, the settings on the ion source used in this study were as follows: ion source current of 3.5 mA, source voltage 160 V, source-out voltage 140 V, and source valve opening 40%. The PTR-TOF 8000’s drift tube was maintained at a pressure of 2.3 mbar and a temperature of 60 °C. The voltage drop across the drift tube was varied from 365 up to 965 V, resulting in a range of reduced electric field from approximately 80 Td up to 210 Td.

To ensure that the product ions identified were coming from the volatile compound of interest, and not from an impurity in the sample, a multicapillary column (MCC) for gas chromatographic pre-separation was connected to the front of the PTR-TOF 8000 for product ion determinations only (i.e. the MCC was not present for the reduced electric field measurements or for real breath sampling). Product ions were identified at three reduced electric field values of 80 Td, 140 Td and 180 Td. For these MCC measurements there was a flow of 50 ml min\(^{-1}\) of \( \text{N}_2 \) through the column, which was isothermally heated to 40 °C. The set-up for the MCC-PTR-TOF 8000 combination is described in detail by Ruzsanyi et al [24].

The mass spectral scans converted from the drift times of the ions in the ToF-MS analyser ranged from approximately \( m/z \) 3 to \( m/z \) 200, and were acquired in a time of 1 s by co-adding 25 000 single 40 μs extraction period recorded at a sampling frequency of 10 GHz. The mass resolution in the present experiment obtained from the detected peaks was \( \approx 2400 \) at \( m/z \) 100. The total duration of a single measurement was 14 min, which corresponds to 60 mass spectra acquired per single \( E/N \) value. The averages of the ion signal levels at each \( m/z \) value from these 60 spectra were used to calculate the percentages of the product ions resulting from each of the compounds. Data were analysed using PTR-MS Viewer 3.2.8, performing mass calibration and peak identification. The areas under the individual product ion peaks were added together and peaks whose intensities were found to be less than 3% of this total area for all reduced electric fields were excluded from further analysis. The product ion distributions were then investigated for fourteen distinct reduced electric field values (from about 80 Td up to about 210 Td) in incremental changes of approximately 10 Td by an appropriate adjustment of the drift tube voltage.

The PTR-MS Viewer automatically adjusts the raw peak data by applying a \( m/z \) transmission curve. Therefore, often the data that are shown in the literature have had a transmission correction. This predominantly makes an allowance for the higher efficiency of transmission of ions with increasing \( m/z \) through a ToF-MS. However, this transmission has been provided by Ionicon Analytik GmbH for just one \( E/N \) (130 Td). Hence, it is questionable whether this manipulation of the raw data is appropriate at any other reduced electric field. Therefore, in the following presentation of results only the raw data have been used - no attempt of allowing for \( m/z \) transmission dependences has been made.

The signal intensity of \( \text{H}_3\text{O}^{18}\text{O}^+ \) is too large to be measured directly. Therefore, the signal intensity for the spectral line peaking at \( m/z = 21.02 \), corresponding to \( \text{H}_3^{18}\text{O}^+ \), was recorded. The \( m/z = 19.02 \) intensity, corresponding to \( \text{H}_3^{16}\text{O}^+ \), was determined in the normal manner by multiplying the \( m/z = 21.02 \) signal by 487. Similarly, the \( m/z = 37.03 \) signal intensity, corresponding to \( \text{H}_4\text{O}^+ \cdot \text{H}_2\text{O} \), was not measured directly. Instead the signal intensity at \( m/z = 39.03 (\text{H}_4\text{O}^+ \cdot \text{H}_2\text{O} \text{ or } \text{H}_3\text{O}^+ \cdot \text{H}_3\text{O}) \) was recorded and multiplied by 243.

The ion mass (\( m/z \) ) calibration was regularly checked using the presence of the ions of known \( m/z \) values, namely: \( \text{H}_3^{18}\text{O}^+ (21.022) \) and \( \text{NO}_2^+ (45.9924) \).

Normalized counts per second (ncps) referred to in a number of figures later in the results section refer to normalizing the product ion signal intensities to \( 10^6 \) reagent ions per second, using the sum of the reagent ions \( \text{H}_3\text{O}^+ \) and \( \text{H}_2\text{O}^- \cdot (\text{H}_2\text{O}) \).

### 2.3. Sampling procedures

For reduced electric field measurements, various volumes (0.1–5 ml) of headspace above a given volatile were taken depending on the compound being analysed and injected into a Tedlar\(^\circ\) bag previously filled with 2 l of either dry or humid \( \text{N}_2 \). Humid \( \text{N}_2 \) was generated by passing high purity \( \text{N}_2 \) through water into the Tedlar\(^\circ\) bag, which was kept in an oven at 50 °C to avoid condensation on the bags surface. The outlet of the heated bags was connected to the inlet PTR-TOF 8000 via a heated (45 °C) PEEK (polyetheretherketone)
transfer line of approximately 1.5 m in length and 1 mm internal diameter.

Owing to potential impurities from the Tedlar® bag, a blank measurement using a bag filled with high purity N₂ was made before each volatile measurement. The resulting concentration levels were subtracted and the inlet tube temperature was set at 2.3 mbar and 60 °C.

The inlet tube temperature (Ionicon Analytik GmbH buffered end-tidal individual breaths could be obtained through a device connected to the inlet of the drift tube, so that each breath had gone through the BET. This resulted in a total time of measurements of several minutes for each time point. To set base levels, breath samples were taken 15 min before the peppermint oil capsule was swallowed and immediately (t = 0) after it was ingested. Breath samples were then taken every 15 min after taking the capsule starting from 30 min up to 90 min and then at start time points of 120, 150, 165, 210, 240, 285 and 360 min. Five breath samples were taken for each time point, with a pause between each breath to ensure that each breath had gone through the BET. This resulted in a total time of measurements of several minutes for each time point. For the breath studies presented, the PTR-TOF 8000 was operated at 130 Td operating bag and inlet temperatures of 70 °C.

2.4. DFT calculations
DFT calculations have been undertaken to determine the PA and GB of the water monomer, dimer and trimer, and for the key volatiles of interest contained in the peppermint oil in order to support the experimental work. These calculations were conducted using the Gaussian09W program with the GaussianView05 for Windows interface and the B3LYP functional with 6-31 + G(d,p) basis set [26].

2.5. Chemicals
With the exception of D-limonene, all chemicals were purchased from Sigma Aldrich (Merck). D-limonene was purchased from Fluka (Honeywell Research Chemicals). These chemicals were used directly without further purification for the 7890A GC-MS, MCC-PTR-TOF 8000, and PTR-TOF 8000 headspace analyses reported in this paper. The stated purities for the chemicals were given as: menthol (95%), menthone (96%), 1,8-cineole (99%), α-pinene (97.5%), β-pinene (99%), mentholum (99%) and limonene (98%).

3. Results and discussion
3.1. GC-MS determination of the volatiles present in peppermint oil
A large number of volatile compounds were identified from the GC-MS analysis of the headspace of the peppermint oil contained in the specific capsules (Boots Pharmaceuticals, UK) that are being used by the peppermint consortium. The following volatile compounds (CAS number) have been identified; 3-methylbutanal (590-86-3), 2-methyl-butanal (96-17-3), 2-ethyl-furan (3208-16-0), 3-methyl-1-butanol (123-51-3), 2-methyl-1-butanol (137-32-6), 2,5-diethyltetrahydrofuran (41239-48-9), α-thujene (2867-05-2), α-pinene (80-36-8), camphene (79-92-5), β-phellandrene (555-10-2), β-pinene (127-91-3), β-myrcene (123-35-3), α-phellandrene (99-83-2), α-terpine (99-86-5), limonene (138-86-3), m-cymene/o-cymene/p-cymene (535-77-3/527-84-4/99-87-6), 1,8-cineole (470-82-6), γ-terpine (99-85-4), terpinolene (586-62-9), 2,6-dimethyl-2,4-hexatriene (7216-56-0), menthofuran (494-90-6), menthone (10458-14-7), menthol (1490-04-6), pulegone (89-82-7), menthyl acetate (89-48-5), and Caryophyllene (87-44-5). However, many of these are present in too low concentrations to be relevant for measurements at 70 °C). The use of PEEK with a narrow internal diameter (1.0 mm) reduces surface effects, but surface effects will still be present and hence may affect breath sampling. In order to investigate inlet effects, we added the headspace of peppermint oil into a 2 l Tedlar® bag containing dry nitrogen at a level such that the concentrations of the volatiles are comparable to those found in breath. Temporal profiles of product ion intensities were obtained at the operating bag and inlet temperatures of 70 °C.
peppermint oil breath studies. We therefore selected volatile compounds in the capsules which contribute more than 3% to the total intensity. This selection is illustrated in figure 1, which provides a single chromatogram of the headspace of the peppermint oil contained in one of the peppermint capsules. The volatiles highlighted in bold were then selected for further investigation using PTR-ToF-MS. The key volatiles that were selected for further investigation using PTR-ToF-MS, both for the reduced electric field studies and for breath sampling measurements, are highlighted in bold, namely three monoterpenes (α-pinene, β-pinene and limonene), 1,8-cineole (eucalyptol), menthofuran, menthone and menthol. The other identified peaks in the chromatogram, namely 2,5-diethyltetrahydrofuran, α-thujene, camphene, β-phellandrene, β-myrcene, α-phellandrene, α-terpinene, p-cymene, γ-terpinene, terpinolene, and 2,6-dimethyl-2,4,6-octatriene each contribute less than about 3% to the total signal, and are therefore considered not to be of significant for breath studies.

3.2. DFT results
Table 2 presents the calculated PA and GB for the water monomer, the water dimer, and the key volatiles selected for study from the GC-MS investigation. These values are also given for the PA and GB of water for reactions with H3O+ and two waters for reactions also with H3O+·H2O. Important, the calculations show that the PA of all the volatiles of interest are sufficiently high so that they can all accept a proton from protonated water and the
Table 1. The major volatile compounds (bold) identified in the headspace of a peppermint oil capsule from GC-MS measurements in order of increasing retention time (minutes), providing percentage mean concentrations and standard deviations (SD) obtained from the measurements of nine separate peppermint capsules from the same bottle. The molecular formula and the molecular weight, for the lightest isotopomer, for each volatile are also provided. Details on some of the minor volatile constituents, some of whom are highlighted in figure 1, are also provided to illustrate that their individual contributions to the total percentage mean concentration are less than about or approximately 3%. The CAS numbers of the volatiles are provided.

| Retention time (minutes) | Volatile (CAS) | Molecular formula (monoisotopic mass (Da)) | Mean ± SD% |
|--------------------------|----------------|------------------------------------------|------------|
| 12.54                    | 2,5-diethyltetrahydrofuran (41239-48-9) | C_{14}H_{20}O (128.12) | 0.4 ± 0.1 |
| 13.30                    | α-thujene (2867-05-2) | C_{10}H_{14}O (136.12) | 0.7 ± 0.2 |
| 13.59                    | α-pinene (80-56-8) | C_{10}H_{18}O (136.12) | 7.0 ± 1.8 |
| 14.26                    | camphene (79-92-5) | C_{10}H_{18}O (136.12) | 0.4 ± 0.2 |
| 15.18                    | β-phellandrene (555-10-2) | C_{10}H_{16}O (136.12) | 1.8 ± 0.2 |
| 15.28                    | β-pinene (127-91-3) | C_{10}H_{16}O (136.12) | 6.2 ± 1.2 |
| 15.57                    | β-myrcene (123-35-3) | C_{10}H_{16}O (136.12) | 2.0 ± 0.3 |
| 16.13                    | α-phellandrene (99-83-2) | C_{10}H_{16}O (136.12) | 0.6 ± 0.1 |
| 16.62                    | α-terpinene (99-86-5) | C_{10}H_{16}O (136.12) | 1.9 ± 0.3 |
| 16.98                    | limonene (138-86-3) | C_{10}H_{16}O (136.12) | 13.7 ± 1.2 |
| 17.19                    | m-, α-, p-cymene (535-77-3/527-84-4/99-87-6) | C_{10}H_{14}O (134.11) | 3.4 ± 0.4 |
| 17.40                    | 1,8-cineole (470-82-6) | C_{10}H_{18}O (154.14) | 19.1 ± 1.0 |
| 17.98                    | γ-terpinene (99-85-4) | C_{10}H_{16}O (136.12) | 2.4 ± 0.3 |
| 18.92                    | terpinolene (586-62-9) | C_{10}H_{18}O (136.12) | 0.8 ± 0.1 |
| 20.40                    | 2,6-dimethyl-2,4,6-octatriene (7216-56-0) | C_{16}H_{20}O (204.19) | 0.5 ± 0.1 |
| 21.98                    | menthofuran (494-90-6) | C_{10}H_{18}O (150.10) | 6.8 ± 0.7 |
| 22.53                    | menthone (10458-14-7) | C_{10}H_{18}O (150.10) | 20.7 ± 1.6 |
| 23.02                    | menthol (1490-04-6) | C_{10}H_{18}O (150.10) | 8.7 ± 1.5 |
| 25.55                    | pulegole (89-82-7) | C_{10}H_{18}O (150.10) | 0.6 ± 0.1 |
| 26.28                    | methyl acetate (89-48-5) | C_{10}H_{18}O (150.10) | 1.8 ± 0.4 |
| 29.07                    | caryophyllene (87-44-5) | C_{10}H_{18}O (150.10) | 0.5 ± 0.2 |

Table 2. Proton affinities (PA) and gas-phase basicities (GB) for the water monomer, dimer and trimer and for the volatiles, α-pinene, β-pinene, D-limonene, 1,8-cineole (eucalyptol), γ-terpinene, menthone, and menthol. Calculations were performed using the B3LYP Functional and the 6-31+G(d,p) basis set at 298 K.

| Volatile | PA (kJ mol\(^{-1}\)) | GB (kJ mol\(^{-1}\)) |
|----------|----------------------|----------------------|
| Water    | 684                  | 653                  |
| Water dimer | 842                | 777                  |
| Water trimer | 937                | 841                  |
| α-pinene   | 873                  | 845                  |
| β-pinene   | 885                  | 857                  |
| Limonene   | 887                  | 807                  |
| 1,8-cineole | 885                 | 853                  |
| γ-terpinene | 861                 | 829                  |
| Menthofuran | 930                 | 901                  |
| Menthone   | 896                  | 866                  |
| Menthol    | 865                  | 833                  |

protonated water dimer, and for menthofuran also from the protonated water trimer.

For limonene and γ-terpinene two sets of values are given, owing to the two possible sites for protonation. For limonene the first set of calculations refer to protonation on the carbon double bond of the ring and the second to protonation on the carbon double bond on the side chain. The values obtained from our DFT calculations for limonene are in good agreement with both experimental and theoretical (DFT) values obtained by Fernandez et al who report an experimental PA and GB of 842 ± 5 kJ mol\(^{-1}\) and 875 ± 5 kJ mol\(^{-1}\), respectively, and theoretical PA calculations of 869.6 kJ mol\(^{-1}\) (B3PW91/6-31G*) to 873.9 (BLYP/6-31G*) kJ mol\(^{-1}\) [32]. For the γ-terpinene, the first set of results refer to protonation on the C=C sites either adjacent to isopropyl or to the methyl group. Menthofuran has three protonation sites, the furan ring C=C, cyclohex C=C and oxygen, with associated calculated PA of 930, 896, and 760 kJ mol\(^{-1}\), respectively.

For α-pinene, β-pinene, limonene, 1,8-cineole, menthofuran and menthol, no obvious fragmentation pathways were found in the calculations, although ring opening of the 1,8-cineole could lead to some fragility. For menthol, the calculations show that upon protonation the C=O bond becomes lengthened and that the loss of H_2O from the protonated parent is barrierless. Hence, for menthol it is expected that dissociative proton transfer will dominate, and no protonated parent, at a nominal value of m/z 157, should be observed in the PTR-MS measurements.

3.3. PTR-ToF-MS results

3.3.1. H_2O***(H_2O)***(n = 0, 1 and 2) reagent ions

The relative intensities of the hydronium ions (H_3O\(^{+}\)) to the protonated water clusters at any given E/N value
depend on the humidity in the drift tube and the value of the reduced electric field being used (see figures 2(a) and (b)). It needs to be appreciated that even if a dry buffer/carrier gas is used in the drift tube, diffusion of water from the ion source always results in some humidity in the drift tube. This is referred to as ‘normal’ operating conditions. When a water saturated buffer gas is used, then this is referred to as ‘humid’ operating conditions [33]. Above about 130 Td, the protonated water clusters are present in low concentrations under both ‘normal’ and ‘humid’ conditions. For example, the H$_3$O$^+$·H$_2$O reagent ions have an intensity of approximately 2%–3% to that of the protonated water monomer. However, at lower reduced electric fields (<100 Td), H$_3$O$^+$·H$_2$O, which is formed from a third body association reaction of H$_3$O$^+$ with H$_2$O, becomes an important reagent ion (figure 1(a)) the intensity of which is very much dependent on the humidity present in the drift tube (figure 1(b)). Only at very low $E/N$ (about 80 Td for ‘normal’ operating conditions and below 110 Td for the saturated operating conditions) is the protonated water trimer observed, and then only with a much smaller intensity compared to that of the protonated water dimer. This is the reason why $n = 0$ and 1 are only considered important in reaction (1).

### 3.3.2. MCC-PTR-ToF-MS results

Following the identity of the key volatiles contained within the peppermint oil being ascertained through GC-MS, a MCC PTR-TOF 8000 was used to categorically identify the product ions resulting from reactions of the reagent ions in the drift tube specifically to individual compounds at three $E/N$ values (low, medium and high values); 80 Td (low)—at which value the protonated water dimer becomes a significant reagent ion (see figure 1(a)), 140 Td (medium, and a commonly used reduced electric field for analytical purposes), and at 180 Td (high, for which any fragment product ions will be more easily identified owing to their increase in intensity). The $m/z$ values (lightest isotopomer), the molecular ion formula (aided by the accurate measurement of the mass spectral peak) and distributions of the product

![Figure 2. Ion intensities in counts per second (cps) of the water reagent ions (H$_3$O$^+$·(H$_2$O)$_n$, $n$ = 0, 1 and 2) recorded at the detector of the PTR-TOF 8000 under (a) ‘normal’ and (b) ‘humid’ operating conditions as a function of reduced electric field (approximately 80–200 Td).](image-url)
ions (percentages) at the three E/N values selected are provided in Table 3. This section only presents the product ions that have been unambiguously identified to derive from a given volatile and which result in a branching percentage of 3% or above at any given reduced electric field value, i.e. product ions have been included whose branching percentages are 3% or greater at a higher E/N than the maximum value used in Table 3 (180 Td) (see next section). A discussion of the reaction pathways follows in the next section, which deals with the results from a more detailed PTR-ToF-MS E/N investigation for both normal and humid drift tube conditions, as defined previously.

3.3.3. Reduced electric field (E/N) investigations of the individual key volatiles contained in the peppermint oil
A key objective of this study is to not only determine which product ions should be monitored for breath sampling with PTR-MS, following the ingestion of a peppermint capsule, but also to investigate how the product ion distributions vary as a function of reduced electric field under ‘normal’ and ‘humid’ drift tube conditions. This has been undertaken to investigate whether changing the reduced electric field could be used to enhance the selectivity of the isomeric compounds. Thus, following the unambiguous identification of the m/z values for product ions coming from a specific volatile using the MCC PTR-TOF 8000, a more detailed E/N study of the product ion percentages was undertaken for each volatile, the results for which are summarized in Figure 3 for (a) α-pinene, (b) β-pinene, (c) limonene, (d) 1,8-cineole, (e) menthofuran, (f) menthone and (g) menthol under ‘normal’ drift tube conditions. Although there were some differences in the product ion branching percentages for the humid conditions, especially at low reduced electric fields, the differences are not significant enough to warrant individual figures, and hence the humid results are not graphically presented, other than for 1,8-cineole, which had the largest humidity affect observed. For the other volatiles the effects of humidity are described in the following text.

For the determination of the product ion branching percentages, only raw data have been used in the calculations, i.e. no attempt to make adjustments for dependence of ion transmission has been made owing to the large E/N range used. (Ion transmission characteristics cannot be easily obtained for each E/N value. This is another reason why calibration of each PTR-MS is needed using a gas standard containing the key volatiles to be monitored for the breath studies.) Furthermore, although in Table 3 only the lightest isotopomer is presented, when calculating the product ion percentage distributions all isotopes (mainly 13C) were taken into account.

It should be appreciated that for comparisons with other PTR-MS measurements that the product ion percentage distributions reported here are specific

| Volatile       | Product ions, m/z (lightest isotopomer) | E/N (Td) 80 |
|----------------|----------------------------------------|------------|
| α-pinene       | C₆H₁₀⁺, 137.13                         | 64 48 30   |
|                | C₆H₁₁⁺, 95.09                          | 1 2 7     |
|                | C₆H₁₄⁺, 93.07                          | 1 3 4     |
|                | C₆H₁₅⁺, 91.05                          | — 1 2     |
|                | C₆H₁₈⁺, 81.07                          | 34 46 50  |
|                | C₆H₁₉⁺, 79.05                          | — 4      |
|                | C₆H₂₀⁺, 77.04                          | — 1      |
|                | C₆H₂₁⁺, 39.02                          | — 2      |
| β-pinene       | C₆H₁₀⁺, 137.13                         | 62 48 27  |
|                | C₆H₁₁⁺, 95.09                          | 1 3 9     |
|                | C₆H₁₄⁺, 91.05                          | — 1      |
|                | C₆H₁₅⁺, 81.07                          | 37 48 54  |
|                | C₆H₁₉⁺, 79.05                          | — 1      |
|                | C₆H₂₀⁺, 77.04                          | — 1      |
|                | C₆H₂₁⁺, 39.02                          | — 3      |
| Limonene       | C₈H₁₂⁺, 137.13                         | 74 39 21  |
|                | C₈H₁₃⁺, 95.09                          | 2 7 12    |
|                | C₈H₁₆⁺, 81.07                          | 24 54 59  |
|                | C₈H₁₇⁺, 79.05                          | — 5      |
|                | C₈H₁₈⁺, 39.02                          | — 3      |
| 1,8-cineole    | C₉H₁₇O⁺, 155.14                        | 7         |
|                | C₉H₁₈O⁺, 137.13                        | 90 52 31  |
|                | C₉H₂₀⁺, 95.09                          | — 2      |
|                | C₉H₂₁⁺, 81.07                          | 3 46 55   |
|                | C₉H₂₂⁺, 79.05                          | — 4      |
|                | C₉H₂₃⁺, 39.02                          | — 3      |
| Menthofuran    | C₉H₁₇O⁺, 151.11                        | 100 98 91 |
|                | C₉H₁₈O⁺, 149.10                        | — 2 4    |
|                | C₉H₂₀⁺, 133.10                         | — 1      |
|                | C₉H₂₁⁺, 93.07                          | — 1      |
|                | C₉H₂₂⁺, 91.05                          | — 1      |
|                | C₉H₂₃⁺, 79.05                          | — 1      |
|                | C₉H₂₄⁺, 77.04                          | — 1      |
|                | C₉H₂₅⁺, 39.02                          | — 1      |
| Menthone       | C₁₀H₁₈O⁺, 155.14                        | 96 47 1   |
|                | C₁₀H₁₉O⁺, 137.13                       | 3 21 20   |
|                | C₁₀H₂₁⁺, 95.09                         | — 6 18   |
|                | C₁₀H₂₂⁺, 81.07                         | 1 26 44   |
|                | C₁₀H₂₃⁺, 79.05                         | — 6      |
|                | C₁₀H₂₄⁺, 39.02                         | — 11     |
| Menthol        | C₁₀H₁₇O⁺, 139.15                       | 88 1 1    |
|                | C₁₀H₁₈⁺, 95.09                         | — 1 1    |
|                | C₁₀H₂₀⁺, 83.09                         | 7 38 3    |
|                | C₁₀H₂₁⁺, 81.07                         | 2 9 27    |
|                | C₁₀H₂₂⁺, 69.07                         | — 4 1    |
|                | C₁₀H₂₃⁺, 57.07                         | 3 12 1    |
|                | C₁₀H₂₄⁺, 55.05                         | — 26 6   |
|                | C₁₀H₂₅⁺, 41.04                         | — 7 6    |
|                | C₁₀H₂₆⁺, 39.02                         | — 2 48   |
to our instrument under the specified operating conditions. Hence the results presented only serve as a guide for other investigations using different PTR-MS instruments. This results from differences in reagent ion concentrations of the protonated water monomer and protonated water clusters in the drift tubes for any given reduced electric field, possibly differences in the internal energies of the reagent ions as a result of differences in the way they are formed in the hollow cathode [34], differences in the inlets, different operating conditions for a given E/N (temperature and pressure of the drift tube), differences in extraction voltages of the ions from the drift tube to the analyser (resulting in fragmentation outside of the drift tube) and differences in ion transmissions on m/z through the analyser. Therefore, for any breath standardization investigations, each PTR-MS must be suitably calibrated if the data from it are to be of use in comparison studies. A calibration gas containing the appropriate volatile compounds in known trace concentrations will therefore be needed for any pharmacokinetic studies if useful comparisons between different analytical techniques are to be made.

PTR-TOF 8000 reduced electric field studies of the monoterpenes, α-pinene, β-pinene and limonene have been previously reported in detail under the ‘normal’ conditions and over a slightly larger E/N range than we explored (60–240 Td) by Materic et al [35]. Although the same PTR-ToF-MS model has been used in the two studies, differences in product ion branching percentages between this study and ours are apparent. There are a number of possible reasons for this. In part this is associated with our decision that any product ion that has a branching percentage less than 3% over the entire reduced electric field investigated will not be taken into account, because it would have too low intensity to be of any use in any breath studies. Furthermore, we used a MCC pre-separation to clearly identify the product ions that are coming from a given volatile compound, and not from an impurity in the sample. However, in addition to this is the fact that we did not allow for any m/z transmission dependence. The study by Materic et al used the supplied Ionicon Analytik GmbH m/z transmission dependence calibration determined at one reduced electric field only, 130 Td. As mentioned earlier, we decided against this, because there is no justification to use the supplied ion transmission curve for all values of the reduced electric field being investigated. However, by applying Ionicon’s ion transmission curve to our data, we have found that these cannot account for the observed differences in product ion intensities. For example, for both α-pinene and β-pinene, Materic et al report that the product ion at m/z 39.02 (C6H13+) dominates for reduced electric fields above about 180 Td, whereas we find it to have only a small branching percentage at such high reduced electric field values, even when allowing for a m/z transmission dependence.

For all E/N values we find in our measurements that the protonated parent at m/z 137.13 (C10H17+) and the fragment ion at m/z 81.07 (C6H9+) are the dominant product ions from the reactions involving the monoterpenes. In this regard, both Materic et al and our results are in good agreement with those obtained in an earlier PTR-Quadrupole-MS study reported by Tani et al for a smaller reduced electric field range of 80–120 Td [36]. The branching product ion percentages we observe for limonene are qualitatively in better agreement with the results presented by Materic et al but there are still significant differences, e.g. again for the intensity of the m/z 39.02 product ion and for the value of reduced electric field value at which the product ion m/z 81.07 becomes dominant. The cross over from the protonated parent to a fragment ion being the dominant reagent ion is much lower in Materic et al measurements (approximately 90 Td) than in ours, and as reported by Tani et al (approximately 130 Td). The significant differences between our results and those obtained by Materic et al are unexpected given that they were taken on the same PTR-ToF-MS model (both studies using different PTR-TOF 8000 instruments). This highlights that even when using the same PTR-MS model the results from one instrument may not be reproduced in another, and hence any published data on product ion distributions just give a guide of what to expect in terms of the intensities of the product ions at a given reduced electric fields, and what should be monitored for an analytical application. Thus, for comparisons of measurements between instruments, every PTR-MS instrument, even when using the same model, needs to be suitably calibrated. Gas standards are therefore required for calibrating any PTR-MS, particularly if results from one PTR-MS study are to be compared with another, as they are meant to be in the peppermint standardization programme.

For all of the monoterpenes, the product ions m/z 137.13 (protonated parent) and m/z 81.07 (fragment ion) dominate over the entire range of reduced electric fields investigated, having approximately equal intensities in our measurements at E/N 140 Td. Although the cross over E/N value for which the fragment ion dominates is found to be relatively insensitive to the humidity in the drift tube, the actual intensity of the ion signals at low reduced electric field has some dependency on humidity, with more protonated parent being observed at the lower reduced electric fields in the higher humid drift tube conditions. This can be explained from the fact that at the lower reduced electric fields the dominant reagent ion is no longer H2O+, but the protonated water dimer. Proton transfer from the protonated water dimer to a monoterpene will result in a lower energy change than occurs in a reaction involving H2O+, and hence the amount of
dissociative proton transfer will be consequentially reduced.

For similar reasons, for the monoterpenoid, 1,8-cineole, the protonated parent \((m/z 155.14, C_{10}H_{19}O^+)\) is found to have a greater intensity at low \(E/N\) under the more humid drift tube operating conditions. But even at these low reduced electric field values, the dissociative proton transfer is found to dominate through a loss of water from the protonated parent resulting in a product ion at \(m/z 137.13\), which is of course indistinguishable from the product ion (the protonated parent) produced by the reactions with the monoterpenes. With increasing reduced electric field the product ion at \(m/z 81.07\) (\(C_6H_{10}^+\)) becomes more and more intense, until at about 140 Td it becomes the dominant product ion, which again is a non-specific product ion.

A detailed PTR-MS study of 1,8-cineole has also been previously reported by Beauchamp et al.\(^{[37]}\), but only at one low reduced electric field in order to suppress fragmentation. The main objective of the study by Beauchamp et al., which used a Ionicon Analytik GmbH High Sensitivity PTR-Quadrupole-MS, was to provide a real-time gas analysis of breath after the ingestion of an eucalyptol-containing capsule, i.e. similar to what is being proposed and undertaken for

![Product ion distributions (branching percentages) as a function of \(E/N\) resulting from reactions \(H_3O^+\cdot(H_2O)_n\) \((n = 0\) and 1) (actual intensities of the reagent ions are dependent on the reduced electric field value (see figure 2)) with the individual volatiles contained in peppermint oil using dry air samples as the buffer gas in the drift (reaction) tube of a PTR-TOF 8000 instrument for reduced electric field values ranging from about 80 Td up to about 210 Td in steps of 10 Td for (a) \(\alpha\)-pinene, (b) \(\beta\)-pinene, (c) limonene, (d) 1,8-cineole (eucalyptol), (e) menthofuran, (f) menthone and (g) menthol. In the case of 1,8-cineole measurements for both 'normal' and 'humid' drift tube operating conditions are illustrated to provide one illustration of the effects of humidity on the product ion distributions. The \(m/z\) values given for the product ions are for the lightest isotopomer, but the product ion distribution percentages have taken into account the contributions from the \(^{13}\text{C}\) containing product ions.

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the research programme of the peppermint consortium. The study by Beauchamp et al was the first to apply PTR-MS to investigate the pharmacokinetics of an ingested volatile, thereby illustrating the on-line capabilities of PTR-MS to record temporal changes in breath concentrations of a swallowed exogenous compound. Of relevance to this study, Beauchamp et al presented a PTR-Quadrupole-MS headspace analysis of pure (98%) 1,8-cineole. Although the reduced electric field is not directly specified, from the provided details of the drift tube’s voltage, temperature and total operating pressure, a reduced electric field of approximately 100 Td was being used. The fragmentation pattern they observed at this reduced electric field is in good agreement with what we have observed in terms of the product ion at m/z 137 (approximately 70%) being the most dominant, followed by m/z 81 (approximately 20%) and then m/z 155 (approximately 10%), which is in reasonable agreement with our product ion branching percentages of approximately 75%, 20% and 5%, respectively.

To our knowledge, we report here the first PTR-MS study of menthofuran. Of the volatiles investigated in this study, menthofuran is found to be the most stable to proton transfer, with the protonated parent being the dominant product ion for the full range of reduced electric fields investigated. Even at the highest reduced electric field investigated the total intensity of the product ions resulting from fragmentation of the protonated parent is only about 30%.

Menthone shows no fragmentation until about 120 Td, when the branching percentage of the protonated parent at m/z 155.14 (C₁₀H₁₉O⁺) begins to drop owing to dissociation to m/z 137.13 (resulting from the loss of water from the protonated parent) and m/z 81.07 (C₆H₁₁⁺). With increasing E/N other product ions are observed, particularly m/z 95.09 (C₇H₁₁⁺). Using a PTR-Quadrupole-MS at 140 Td, Tietz et al [38] observed the same product ions as we have found in our study, but as expected with considerably different intensities, with m/z 81 being found to be the most dominant (60%), followed by m/z 95 (18%), m/z 137 (15%) and m/z 155 (7%). The differences in intensities illustrate once again the problems of comparing non-calibrated results from different PTR-MS instruments and the highlights again the need for a calibration standard for any benchmarking activities.

As expected from the DFT calculations, the loss of water from the protonated menthol readily occurs, and hence the protonated parent is never observed for any value of the reduced electric field. The product ion observed at m/z 139.15, resulting from the loss of water, is the dominant species up to about 100 Td. With increasing reduced electric field, the intensity of the product ion C₆H₁₁⁺ at m/z 83.09, increases and becomes dominant above approximately 110 Td. With increasing reduced electric field other product ions are observed at m/z 81.07 (C₆H₁₉⁺), m/z 69.07 (C₅H₁₀⁺), m/z 55.05 (C₄H₇⁺), m/z 41.04 (C₃H₅⁺) and m/z 39.02 (C₂H₅⁺). The product ion at m/z 39.02 becomes the most dominant above about 170 Td.

An earlier PTR-Quadrupole-MS E/N study of menthol, recorded over a more limited reduced electric field range than in our study, namely 90-133 Td, was reported by Gordon et al [39]. They identified four product ions at m/z values 55, 81, 83 and 139, but with considerably different product ion branching percentages than we obtained from our measurements. According to Gordon et al the most abundant product ion at 133 Td is m/z 83 (with a branching percentage of approximately 43%). This is followed by m/z 55 (approximately at 33%), m/z 81 (approximately at 16%) and m/z 139 (approximately at 8%). In comparison, from our PTR-ToF-MS measurements, we do find that the most dominant product ion at 130 Td is that associated with m/z 83, but with a higher branching percentage of approximately 60%.
3.4. Inlet effects affecting standardization and PTR-MS breath profiles following ingestion of a peppermint oil

3.4.1. Sampling from a heated Tedlar® bag

Figure 4 provides the temporal profiles of product ion intensities for $m/z$ 81.07 and 137.13 (with contributions from the monoterpenes and 1,8-cineole), $m/z$ 83.09 and 139.15 (menthol), $m/z$ 151.11 (menthofuran), and $m/z$ 155.14 (menthone) resulting from continuous sampling over a period of 10 min of trace quantities of peppermint headspace placed in a Tedlar® bag heated to 70 °C which is attached to the PEEK inlet, also maintained at 70 °C.

Figure 4. Temporal profile of the product ion intensities of $m/z$ 81.07 + 137.13 (monoterpenes and 1,8-cineole), $m/z$ 83.09 + 139.15 (menthol), $m/z$ 151.11 (menthofuran), and $m/z$ 155.14 (menthone) resulting from continuous sampling of trace quantities of peppermint headspace placed in a Tedlar® bag heated to 70 °C which is attached to the PEEK inlet, also maintained at 70 °C.

3.4.2. Effects on breath profiles

The sorption effects we have observed have significant implications for breath sampling of these volatiles, because even when using a BET system, which increases the sampling time, the measured volatiles in the individual breaths never reached a steady state in our measurements and hence the true volatile concentrations in the breath were not accurately determined under the operating conditions we used. This is illustrated in figure 5(a), which shows a real-time measurement of the key volatiles in a single exhaled breath from a volunteer 30 min after the ingestion of a peppermint oil capsule. For this exhaled breath the volunteer inhaled to completely fill the lungs and then immediately exhaled slowly into the BET. The initial start of the breath profiles shows a similar, but not identical behaviour to that found for the continuous sampling measurements. The observed differences are most probably associated with the transitory phase during which the breath concentrations will be increasing with duration of exhalation. Independent of the cause, this figure demonstrates that the intensities of the volatiles in the breath profile cannot reach a steady state value, which would provide a true representation of the true concentrations of the volatiles in the exhaled breath. Furthermore, there will be issues with the duration of expiration. We have found that a plateau is not reached even after 20 s of expiration. This is similar to the results reported by O’Hara et al for isoprene and acetone, for which positive slopes in time-dependent expirograms were observed [40], with the slope of the isoprene expirogram being persistently linear. This study by O’Hara et al raised concerns with regards to the use of uncontrolled single on-line exhalations for providing reliable measurements.

No surface effects are expected for CO$_2$, as is confirmed by monitoring CO$_2$H$^+$ (figure 5(a)). Despite the complication of surface effects with the volatiles, the reproducibility of the five breath samples taken at each measurement start time point, the duration of which took about 2–3 min to record using the BET for each time point, is good, as illustrated in figure 5(b). Hence, when determining washout characteristics, we can conclude that inlet issues are not a problem providing breath samples are collected in a consistent way.

Averages of areas under five exhalations, starting after the dead-space transitional phase as defined by the CO$_2$ level so that the end-tidal breath phase is being
analysed, were obtained for each time point. In this way the loss of the volatile compounds from the body can be determined. For illustrative purposes, figure 6 provides a result from a series of PTR-ToF-MS breath measurements for the same volunteer. This looks promising. However, from the small number of volunteers we have...
so far investigated, we have found inter- and intra-individual differences with regards to the washout characteristics, but that will be the subject of another paper dealing with results from pharmacokinetic investigations involving the research groups within the peppermint consortium who are using PTR-MS. Nevertheless, for this example, the figure shows that by approximately 120 min both menthol and menthone breath concentrations are down to background levels, whereas the signals associated with the monoterpenes, 1,8-cineole and menthofuran reach background levels after about 210 min. Our findings are somewhat different from a peppermint oil capsule study recently reported by Gaude et al.[31]. In their study they continuously collected end-tidal breath samples for 4 min onto Tenax TA/Carbo grind STD sorbent tubes (Markes International) using a ReCIVA breath sampler (Owlstone Medical). Thermal-desorption gas chromatography-mass spectrometry (TD-GC-MS) was then used to analyse the breath samples. Hence, they can monitor the individual compounds and therefore observe the different washout characteristics of the different monoterpenes. Although they also report a marked rise in breath concentrations of α-pinene, β-pinene, limonene, eucalyptol and menthone, within 30 min of ingestion compared to baseline levels, breath concentrations above the baseline are still observed even after 8 h from ingesting the peppermint oil capsule.

4. Concluding remarks

Our results suggest that a suitable reduced electric field to operate a PTR-MS for breath analysis of the key volatiles contained in a peppermint oil capsule is at 130 Td. At this reduced electric field value, the ion signals at m/z 137 and m/z 81 monitor a combination of the monoterpenes and 1,8-cineole, and hence a pharmacokinetic study would produce details relating to an average washout of these compounds. m/z 155 predominantly monitors the washout of menthone (because any contribution from 1,8-cineole to m/z 155 at 130 Td is negligible). The product ions at m/z 83 + 139 and m/z 151 are unique to menthol and menthofuran, respectively, and hence the temporal profiles of those product ions provide specific details on the washout characteristics of those volatiles.

Although 130 Td is useful for the proposed breath analysis, it does not provide the best sensitivity, which is usually provided at lower reduced electric field values. Whilst operating at 80 Td would result in more sensitivity, there would be issues of distinguishing menthone from 1,8 cineole, as both would contribute significantly to the ion signal intensity at m/z 155. However, this problem could be overcome if selective rapid electric field switching were to be adopted[42]. Although demonstrated for enhancing PTR-MS compound specificity with explosives, this electric field compound selectivity can be applied to any analytical area where there are rapid changes (e.g. seconds) in volatile concentrations, such as happens when sampling a real-time breath exhalation.

From the above discussion, it is clear that, without any pre-separation, the use of PTR-MS for pharmacokinetic studies in the peppermint oil consortium programme has some limitations in terms of individually monitoring the washout characteristics of some of the key breath volatiles owing to the production of non-selective product ions; namely m/z 81 and 137 coming from the monoterpenes and 1,8-cineole. Use of a pre-separation of the breath samples, e.g. by using GC or fast-GC column, is needed to determine the individual washout characteristics for the monoterpenes and 1,8-cineole.

With regards to standardization between different PTR-MS instruments, inlet system issues and ion transmission characteristics need to be taken into account. Therefore, direct comparisons of relative intensities between different PTR-MS instruments (even if using the same model) is not possible without the use of a calibration gas standard, owing to differences in the inlet conditions, operating conditions for fixed E/N (e.g. temperature and humidity) and m/z transmission dependencies of ions from the drift tube to the transfer optics and then through the mass spectrometer, followed by any m/z detection efficiencies of the detector. Hence, although the results presented in this paper inform other PTR-MS users as to what product ions should be monitored under specific operating conditions, the product ion distributions only give a broad indication of which ones dominate at any given reduced electric field. Thus, comparisons between different breath studies will only be useful if each PTR-MS (including inlet systems) is individually calibrated using a gas standard containing known concentrations of menthol, menthone, menthofuran, limonene, α-pine ne, and β-pinene. And given the difficulties associated with identical m/z values for several of these compounds, only certain volatiles contained within the peppermint oil capsules can monitored to provide individual pharmacokinetic profiles, namely menthol, menthone, and menthofuran.

We have demonstrated that although when using a BET system and heated PEEK inlet lines there are issues relating to real-time breath sampling in terms of surface effects, the system nevertheless provides an adequate quantitative method for pharmacokinetic measurements. Therefore, we propose that this method should be adopted to provide a standardization for PTR-MS breath analysis rather than using direct on-line tidal breath sampling, which would result in less accurate measurements[43]. We have found that to achieve the best results, during exhalation a volunteer should breath out normally at a constant rate. Directly monitoring protonated CO2 aids in determining the best conditions for exhalation. It is
also best to have a volunteer practice on the BET systems a number of times before taking any measurements. Alternatively, to overcome these inlet conditions, PTR-MS measurements could be done off-line by using capnography controlled sampling to collect only the end-tidal phase of about three to four breaths into 100 ml glass syringes as discussed by Fernández del Río et al [3].

With regards to the use of peppermint oil capsules, a simpler procedure that could be adopted for a possible second-phase of measurements for PTR-MS investigations in the peppermint oil consortium would be to have specially prepared medical capsules containing just menthol, menthone, and menthofuran. This possible second phase of measurements for the consortium, especially for the PTR-MS community, would be adopting a method used by Winkler et al [44], who used medical gelatine capsules in their PTR-MS pharmacokinetic study of ingested isotope-labelled ethanol. These capsules were filled by the researchers so that the concentrations are accurately known. Furthermore, by adopting this procedure of using medical grade capsules, just selected compounds could be added, which would provide unambiguous product ions in the PTR-MS. An alternative, and perhaps a simpler method, is to administer chemical compounds to be monitored by dissolving them in water, which are then drunk. This protocol was used effectively by Ruzsanyi et al to monitor the conversion of 2-propanol-1,1,1-d3 to d3-acetone by alcohol dehydrogenase [2]. However, moving away from using health supplement capsules would require a new and more detailed ethical approval.

Compounded to the above difficulties for the proposed peppermint oil breath standardization programme, are issues relating to inter-individual and intra-individual variations and differences in the ways the volatiles are removed in the body. For examples, 1,8-cineole is not metabolized efficiently in the body, being predominantly removed from the blood via gas exchange in the lungs, whereas menthol is metabolized into menthol glucuronide in the liver and urinated out of the body. This explains the differences in the observed washout for these two volatiles. More detailed studies are required to investigate the influence of differences in metabolism between individuals, and these will be obtained from the research programmes being adopted by the peppermint consortium. However, from the preliminary results presented in this paper, the goal of the peppermint consortium of having a standardized breath sampling and analysis, which will bring a robustness and valid inter-laboratory comparisons using the same and different analytical tools, will be extremely hard to achieve. Nevertheless, the peppermint consortium will generate a considerable amount of useful benchmark data, which should provide the first step in a way forward for the standardization of breath sampling and analysis, and also shed light on the variability of breath analysis.

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