Joint use of retention index and mass spectrum in post-mortem tests for volatile organics by headspace capillary gas chromatography with ion-trap detection

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Abstract

A method for an unbiased search for volatile organics is described. It is based on direct headspace extraction, capillary GC separation on an apolar stationary phase and ion-trap detection. By automatic reconstruction of the chromatogram with each ion in the scanned mass range (29–199 u), peaks that did not appear on the total ion chromatogram could be spotted. The concentration of some organics at the limit of detection was in the range 0.03–8 μmol/l in blood. Owing to the high sensitivity, the mass spectrum of a peak was not reliable as the sole proof of a substance present at a low concentration. The identification was therefore made on the basis of joint data from mass spectra, searched on-line in a library, and retention indices, retrieved from the literature. To show the value of the method, examples are given of some scarce intoxicants found in post-mortem samples.

1. Introduction

To reveal a toxin in post-mortem samples is a challenge. A reason for this is the common lack of information as to possible agents involved, and in these instances a search for the "general unknown" has to be made. It is true that a number of efficient methods are now available for screening body fluids or tissues for alien organics. However, these tests are mainly applicable to spotting substances derived from drugs or narcotics. Methods for an unbiased search for volatile organics are more sparse, and to some extent also more demanding. The broad range of polarity and the complex nature of the organic volatile fraction with components often present at low concentrations are circumstances that contribute to detection and analysis problems.

Most of the current methods for screening biological materials for volatile organics are based on headspace (HS) extraction and gas chromatographic (GC) separation. Even though the huge number of volatile substances necessitate separation by high-resolution capillary GC [1], most laboratories involved in toxicological analysis still prefer packed columns for screening purposes [2–7]. The choice of extraction method, notably direct HS or purge and trap of the HS fraction, depends on the detection method being used. With flame ionization (FID) or electron-capture detection (ECD), both of which yield high sensitivity, direct HS has been used [3–9]. When the substance search was done by mass spectrometry (MS) with a quadrupole [2] or magnetic [10–12] instrument run in the full-scan mode, on the other hand, the more efficient purge and trap extraction had to be employed.

Unlike these "conventional" MS methods, ion-trap detection (ITD) offers nearly the same sensitivity with the mass scanning set over a wide

range as tuned on a single selected ion. Along with direct HS extraction of blood before GC separation on a polar capillary (DB-WAX), this detection mode has also been found useful for spotting endogenous polar volatile organics in human samples [13]. However, low-molecular-mass volatile substances often give unspecific mass spectra and also occur in a biological sample only at low concentrations. It can be difficult, therefore, to judge whether an ion comes from a target substance or from some interfering ion that by chance has the same mass. Also, compounds in the same series, e.g., the hydrocarbons, may generate very similar mass spectra. To single out a volatile "general unknown" based only on its mass spectrum is, hence, often uncertain, and the search for a match may result in a number of candidates.

The retention time in a GC capillary may also be a fair, but not perfect, marker for a substance. One advantage is that this parameter is easily proved in a reconstituted mass chromatogram, even if the substance concentration is low. The versatile use of the retention time as an identification tool in an unbiased search for the "general unknown" requires, however, access to literature data on retention indices, and that these be transferable between GC systems. From the works of Kováts seven rules arose. Two of these stated that the retention indices of an apolar compound (alkane) assayed on various types of stationary phases, or the retention indices of any substance assayed on various apolar stationary phases, should be close to each other [14]. This means that the most versatile use of retention indices reported in the literature may be gained by a screening test with GC separation on an apolar stationary phase.

Based on mass spectra obtained with ITD, and on retention indices determined by capillary GC with an apolar stationary phase, a comprehensive, coupled HS–GC–ITD method for an unbiased search of post-mortem samples for the volatile "general unknown" is described here. Some examples of its use with post-mortem samples for spotting, identifying and determining solvents or gases rarely seen in intoxications are also given.

2. Experimental

2.1. Chemicals

Propane and n-butane were purchased from Aga Gas (Sundbyberg, Sweden). The other n-alkanes (C_5–C_{12}), used for indexing the retention times, and all reference substances, used for the final proof of a detected substance, were obtained from Aldrich-Chemie (Steinheim, Germany) or Merck (Darmstadt, Germany).

2.2. Instrumentation and software

HS extraction was carried out with a Hewlett-Packard Model 19395A autosampler together with an 18906B accessory kit for constant heating time. The gas chromatograph was a Hewlett-Packard Model 5890 with a DB-1 capillary from J&W Scientific (Folsom, CA, USA). It was inserted without a flow restrictor directly into the ion source of a Finnigan MAT ITD800 ion-trap detector. The tuning of the latter was done manually to resolve the m/z 69 and 70 peaks and the m/z 131 and 132 peaks. Evaluation of the raw data was carried out with a Datamaster II (program version 1.3; Finnigan MAT).

2.3. Specimens and sample preparation

The samples to be searched for volatile organics were post-mortem samples sent to our laboratory for routine toxicological analysis. To determine the limit of detection or to construct the calibration graphs, blood from blood donors was used. The body fluids were collected in a 25-ml polystyrene container with a polyethylene screw stopper (3-64211 universal container; Nunc, Denmark). After potassium fluoride, at a final concentration of about 1%, had been added to the body fluids as a preservative, the samples were sent by mail to the laboratory. The analyses were carried out on aliquots of 1.5 ml of fluids added to a 20-ml HS vial (No. 092357; Apodan, Copenhagen, Denmark) which contained 1.8 g of sodium chloride.

2.4. Analytical procedures

The analytical process, generating the raw data, was fully automated with a capacity of 21 samples per batch and a turnover time for each sample of 33 min. The parameters used for the chemical analysis and the instrumental operations are given in Table 1.

For detecting a "general unknown", the total ion current of the mass chromatogram was reconstituted with each single ion in the recorded mass range (29–199 u). This first step of the search was carried out automatically by a data program, written in the "procedure language" (version 2.01) of Datamaster II. The chromatograms, formed and frozen in a spooler file (171 chromatograms on top of one another on 22 screen pages), could then easily be surveyed for random peaks. To be able to judge whether a peak arose from the background noise or from a "general unknown", the limit of detection, *i.e.*, a signal response equal to three times the standard deviation of the gross blank signal, was measured according to the method of Knoll [15]. This was done on control samples at the proper mass number or combination of added mass numbers.

To identify a spotted "general unknown", the library retrieval program of Datamaster II was used. This is a forward library search, in which the spectrum of the "general unknown" is compared with a number of library entries [National Bureau of Standards/National Institute of Health/Environmental Protection Agency (NBS)], and the ten best matches along with their Chemical Abstracts Service (CAS) Registry Numbers are reported. Normally, the retrieval was done with the molecular mass set at 0–220. To obtain a "purity" index also of a compound that was suspected but not among the ten candidates, the molecular mass range was narrowed until the substance was listed. Before searching for "purity" matches of a low-intensity mass spectrum, which might be partly obscured by background ions, it was edited based on the peak height as measured in the reconstituted mass chromatograms.

The retention index was measured and calculated according to the equation [16]

$$I_{calc} = 100(t_{R(unknown)} - t_{R(z)})/(t_{R(z+1)} - t_{R(z)})$$
$$+ 100z$$

where I_{calc} is the retention index of the "general unknown", $t_{R(unknown)}$ is the total retention time for the "general unknown", $t_{R(z)}$ and $t_{R(z+1)}$ are the total retention times for the *n*-alkanes that bracket the "general unknown" and *z* is the number of carbon atoms in the *n*-alkane standard that elutes just before the "general unknown".

To be able to compare I_{calc} with literature data, references that show the retention index (I_{lit}) of a "general unknown" was searched in the ESA–IRS database "Chemabs" using the CAS number along with the search word "retention index". The I_{lit} values referred to in this paper were drawn from the work of Streete *et al.* [9] or from different papers quoted in the review by Evans and Haken [14].

If the I_{lit} was not found, an estimate on a substance's retention index was obtained by using the equation [17]

$$I_{bp} = 10^{0.00134052T(bp)+2.558916} - 440.5$$

where I_{bp} is the boiling point index in retention index units and $T(bp)$ is the boiling point of the compound in kelvin at atmospheric pressure.

As a final proof of the identity of an organic compound, its chromatographic and mass spectrometric traits were compared with those of the matching reference substance. If the signal response of an identified substance exceeded ten standard deviations of the background noise [15] and if relevant for the toxicological survey, a quantitative assay according to the external standard method was carried out on the blood. Calibration graphs were constructed using normal blood spiked with suitable concentrations of the analyte.

3. Results

The above-described method for screening post-mortem samples for low-molecular-mass

Table 1
Experimental conditions

Headspace extraction		Gas chromatography		Ion-trap detection	
Equilibration temperature	50°C	Capillary dimensions	30 m × 0.25 mm I.D.	Sample introduction	Direct coupling
Equilibration time	33 min	Coating	1 μm of methylsiloxane	Transfer line temperature	220°C
Valve/loop temperature	54°C	Carrier gas (He) flow-rate	18 ml/min	Ionization mode	Electron impact (50–80 eV)
Auxiliary gas pressure	130 kPa	Column head pressure	75 kPa	Multiplier voltage	1900 V
Vial pressurization time	15 s	Injector temperature	60°C	Automatic gain control	On
Sample loop volume	1 ml	*Oven temperature programme*		Background mass ejected	<29 u
Sweep gas (He) flow-rate	70 ml/min	Initial value	40°C	Analytical scan rate	0.5 s per scan
Injection mode	Split	Initial hold	4 min	Microscans/analytical scan	4
Vent/loop fill time	1 s	Ramp to 200°C	10°C/min	Acquisition time	1.45–20 min
Injection time	2 s	Ramp to 250°C	50°C/min	Mass range examined	29–199 u

volatile organics has been in routine use for about 8 months. During this period 43 different substances were detected and identified, and in some instances also determined. They constituted different types of compounds, e.g., hydrocarbons, alcohols, aldehydes, ketones, esters and ethers. The I_{calc} values ranged from less than 350 to over 1100, and the I_{calc}/I_{lit} ratio was 0.997 ± 0.010 (mean ± S.D.; $n = 40$). The I_{calc} values for the hydrocarbons were also related to the boiling points, with $I_{calc}/I_{bp} = 1.008 ± 0.016$ (mean ± S.D.; $n = 19$). For the non-hydrocarbons, on the other hand, there seemed to be no simple relationship between I_{calc} and the boiling points, as indicated by the I_{calc}/I_{bp} ratio of 0.874 ± 0.121 (mean ± S.D.; $n = 24$).

Fig. 1 shows the chromatograms of volatile organics found in samples from two subjects who had died following the intake of solvents. The upper chromatogram reveals the findings in the blood of a 35-year-old diabetic and alcoholic. The pathologist found no direct cause of the death, but as the victim was also a solvent thinner sniffer, a screen for solvents was asked for. The total ion current of the mass chromatogram showed two main peaks, which held methanol and nitromethane. The concentration of methanol in the blood was 28.1 mM (measured by the routine method of the laboratory) and that of nitromethane was 3.4 mM (determined from a calibration graph generated at four concentrations in the range 2.5–25 mM, $r = 0.999$). The lower chromatogram in Fig. 1 shows the data from the study of the gastric contents in a 78-year-old man who died suddenly without a previous history of illness. The pathologist found no direct cause of the death, but he noticed a sweet smell when opening the victim's stomach, and, therefore, asked for a screen of its contents for volatile compounds.

Fig. 2 shows the chromatograms of volatile organics found in the blood from two subjects following death caused by inhalation of gases. The upper mass chromatogram is from a 34-year-old man who had inhaled town gas and the lower chromatogram is from a 23-year-old woman who had inhaled car exhaust gas. In both instances over 70% of carbon monoxide–haemoglobin was
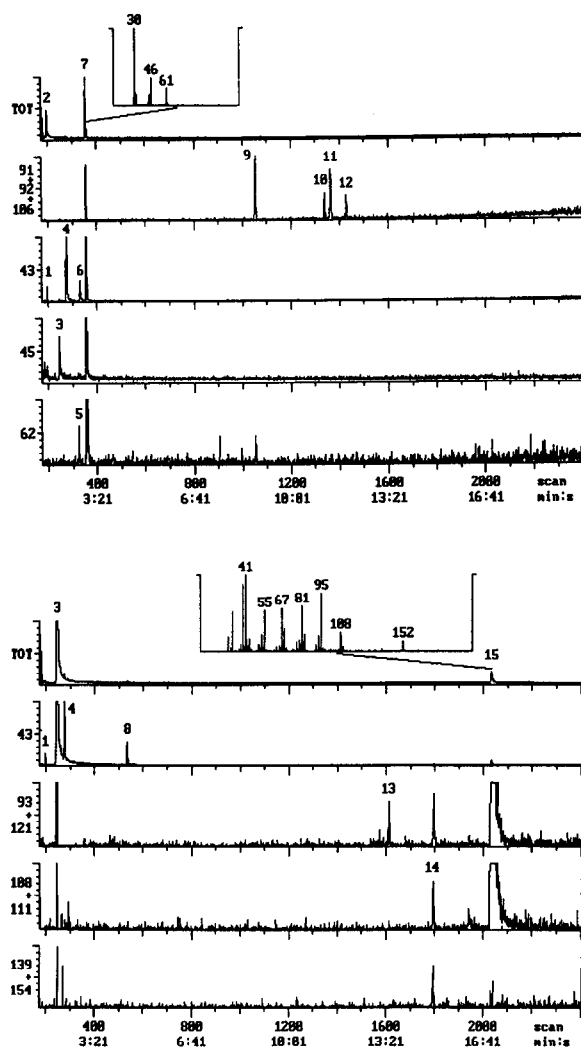

Fig. 1. Total ion current and reconstituted mass chromatogram of volatile organics in post-mortem samples from two deaths caused by intake of solvents. Peaks: 1 = acetaldehyde; 2 = methanol; 3 = ethanol; 4 = acetone; 5 = methyl sulphide; 6 = methyl acetate; 7 = nitromethane; 8 = ethyl acetate; 9 = methylbenzene; 10 = ethylbenzene; 11 = 1,3- and/or 1,4-dimethylbenzene; 12 = 1,2-dimethylbenzene; 13 = camphene; 14 = eucalyptol; 15 = camphor.

also measured along with 15–26 mM of ethanol (measured by the routine method of the laboratory).

As seen in both Figs. 1 and 2, most of the substances were present in such low concentrations that they showed up as peaks only on the chromatograms that had been reconstituted
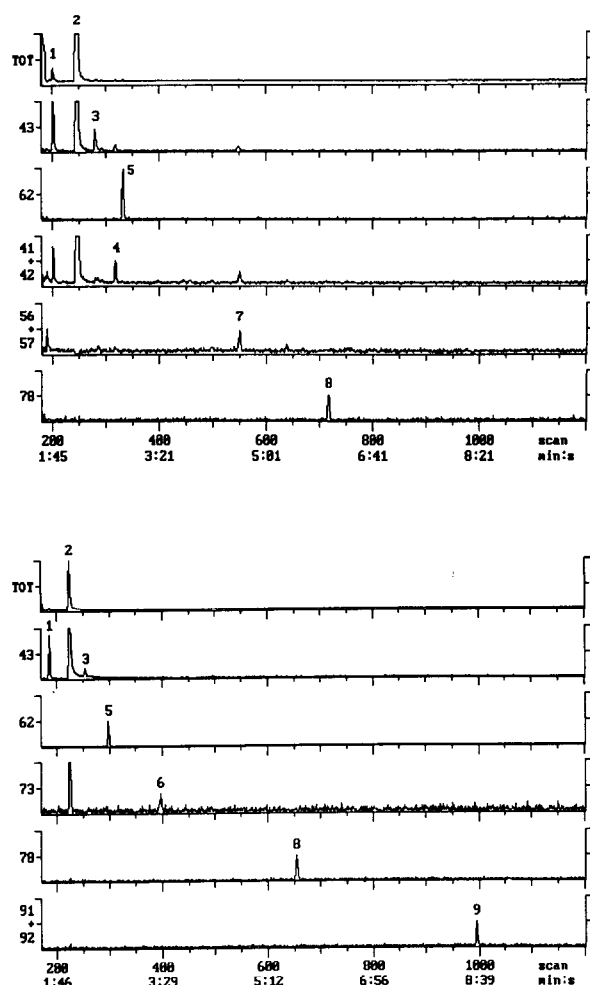
Fig. 2. Total ion current and reconstituted mass chromatogram of volatile organics in post-mortem samples from two deaths caused by inhalation of gases. Peaks: 1 = acetaldehyde; 2 = ethanol; 3 = acetone; 4 = pentane; 5 = methyl sulphide; 6 = methyl tert.-butyl ether (MTBE); 7 = hexane; 8 = benzene; 9 = methylbenzene.

high enough to be used as the sole proof of identification. Except for acetone, MTBE and hexane, the analyte was listed among the ten most probable alternatives. The I_{calc} values, used as a paired identification proof, agreed reasonably well with the I_{lit} values for all substances, and also with the I_{bp} values for the hydrocarbons.

4. Discussion

Exposure to volatile substances causes numerous deaths each year [9]. The variety of this type of compound in trade products used in daily life is also abundant [18], and a laboratory carrying out toxicological analyses should therefore have the means for the efficient search and assay of such organics.

Packed columns are today the most commonly used separation tool in the search for volatile organics [2–7]. To widen the range of detectable substances, two columns with different polar stationary phases have been used [6], and also recently recommended for routine toxicological work [7]. The single capillary with an apolar stationary phase used in the work presented here seemed to serve well to separate organics with a broad range of boiling points. This is in accord with a newly reported method, which allows the screening of blood or urine for 244 substances with a wide-bore apolar capillary [9]. However, methanol and acetaldehyde, two organics often found in post-mortem samples, were not separated on the apolar phase. With the present method they could be easily separated based on their different fragmentation patterns.

Another means of widening a GC screening for volatile organics is to use two detectors, FID and ECD, coupled to a packed column [5] or to a wide-bore capillary [9]. In this context it is worth mentioning that ITD can be regarded as a multiple detection method, with each mass number combination as a single detector. At the same time ITD gives high sensitivity, as shown in Table 2 for some intoxicants and also reported previously for a number of alcohols, ketones and esters [13].

with the proper mass number, but not in the total ion current. A peak height exceeding three times the standard deviation of the gross blank signal was judged to indicate a spotted "general unknown". Table 2 gives the concentrations of some of the compounds at this limit of detection.

Table 3 shows the data from the process of identifying the volatile organics in Figs. 1 and 2. As can be seen, the "purity" of a target substance's mass spectrum was in most instances not

As can be seen in Figs. 1 and 2, a number of

Table 2
Limits of detection (LOD) of volatile organics in blood

Substance	LOD (μmol/l)	Substance	LOD (μmol/l)
Pentane	0.10	*m-/p*-Xylene	0.05
Methyl sulphide	0.19	Styrene	0.13
Nitromethane	1.44	*o*-Xylene	0.08
Methyl *tert.*-butyl ether (MTBE)	0.03	Propylbenzene	0.74
Hexane	0.11	Camphene	1.24
Benzene	0.07	Eucalyptol	3.11
Methylbenzene	0.04	Camphor	8.33
Ethylbenzene	0.06		

The LOD, equal to three times the standard deviation of the background noise, was calculated according to Knoll [15].

Table 3
Summary of identification criteria of substances shown in Figs. 1 and 2

Substance	Mass spectrum library search: purity[a] (rank number)[b]	Retention index I_{calc}[c]	Retention index I_{lit}[d]	Retention index I_{bp}[e]
Acetaldehyde	903(1), 659(1), 845(1)	342	352	457
Methanol	821(2)	348	353	588
Ethanol	578(1), 547(1), 813(1), 798(1)	428	427	633
Acetone	661(>10), 360(>10), 499(>10), 488(2)	465	460	562
Pentane	592(2)	501	500[f]	500
Methyl sulphide	451(1), 761(1), 740(1)	506	508	499
Methyl acetate	451(1)	507	512	562
Nitromethane	803(1)	521	526	511
MTBE	156(>10)	556	560	556
Ethyl acetate	745(1)	600	598	626
Hexane	540(>10)	601	600[f]	600
Benzene	591(2), 556(4)	651	655	636
Methylbenzene	741(2), 558(6)	758	763	744
Ethylbenzene	552(1)	854	861	839
m-Xylene	421(4)	863	869	851
p-Xylene	421(3)	863	870	847
o-Xylene	596(2)	886	892	871
Camphene	418(8)	954	944	934
Eucalyptol	506(1)	1031	–	1001
Camphor	708(2)	1142	1160	1163

[a] Measure of the mass spectral resemblance rated from zero (no peaks in common) to 1000 (identical library and target substance mass spectra).
[b] The order of the target substance among the top ten matches based on the purity criterion.
[c] Measured data.
[d] Literature data.
[e] The boiling point index in retention index units.
[f] Value by definition.

volatile "general unknowns" were present only in trace amounts and, therefore, never showed up on the total ion chromatogram. A general problem, then, was to activate the right "detector" to make them visible. A useful help in the search for hidden peaks was the data program for automatic reconstitution of the chromatogram with each of the recorded 171 mass numbers. These data formed the basis for further manual processing, by which the sum of two or three of the mass numbers in any combinations could be made up and used to fine tune the reconstruction of the mass chromatogram.

To identify a volatile "general unknown" that had been spotted on the total ion chromatogram or in the reconstituted total ion current, a library search for mass spectrum matches was first done. However, as shown in Table 3, the spectrum resemblances were often far from ideal, resulting in a number of possible candidate target substances. One reason for this was difficulties in sorting out mass fragments of background compounds from fragments of an analyte present in trace amounts. Another was the, under some experimental conditions, inherited ITD problem with distortion of electron impact mass spectra, giving rise to enhanced $[M+1]^+$ peaks. As reported while this work was in progress, a decrease in the ITD manifold temperature or in the helium flow-rate through the capillary may, however, improve the same spectrum [19].

To narrow the number of possible target substances listed in the mass spectrum search, the I_{calc} value of the unknown peak was compared with the I_{lit} values for the different candidates. A similar approach, but with in-house retention indices, has been used by other workers to identify drugs in body fluids of comatose patients [20], polycyclic compounds of environmental interest [21] and hydrocarbons in aviation fuels [22]. As shown in this paper, the I_{calc} and I_{lit} values agreed well enough to be used as a complementary identification marker. Also, the I_{bp} values are a useful tool for the hydrocarbons, should no reference with the key information on I_{lit} be found.

The method described has served in routine toxicology, and to show its potential some findings of scarce intoxicants are presented. The detected compounds nitromethane, camphene, eucalyptol and camphor shown in Fig. 1, seem not to have been reported earlier in any post-mortem body fluid. Even though accidental ingestions of camphor are not rare, the compound has so far only been shown in the serum of two hospital patients [23].

Town gas, which caused the death represented in the upper trace in Fig. 2, contains over 90% of hydrogen, methane, carbon dioxide and carbon monoxide. None of these, though, can be seen by the present method. However, the gas also contains some unspecified hydrocarbons, which according to the producer amount to no more than 2%. By analysis of town gas, sampled in an evacuated HS vial, nine hydrocarbons, which eluted with I_{calc} values in the range 472–651, were identified. Of these, pentane, hexane and benzene were spotted in the blood of the deceased. The other subject represented in Fig. 2, killed by inhalation of car exhaust gas, had two hydrocarbons in the blood along with the octane booster MTBE, which is an additive in motor gasoline. These organics do not seem to have been reported previously in post-mortem samples from deaths caused by inhalation of car exhaust gas.

The method has been applied to blood samples from living persons, but owing to contaminants freed from the rubber septum of the vacutainer tubes used for specimen sampling, the results were difficult to assess. In the HS of one septum type, about fifteen substances were spotted at m/z 57 with I_{calc} values in the range 557–1189. Some of these organics were identified as 3-methylpentane, hexane, methylcyclopentane, cyclohexane, isooctane and toluene. From another brand of septa, tert.-butanol, MTBE, methylcyclopentane and cyclohexane were released into the HS. It is therefore urgent that the sampling tools be tested for contaminants that may appear as the "general unknown".

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