Isoprene levels in the exhaled breath of 200 healthy pupils within the age range 7–18 years studied using SIFT-MS

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Isoprene levels in the exhaled breath of 200 healthy pupils within the age range 7 to 18 years studied using SIFT-MS.

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Abstract.

The published results of breath isoprene studies, to date largely involving adults, are briefly reviewed with special attention given to the work done on this topic during the last ten years using selected ion flow tube mass spectrometry, SIFT-MS. Then the new data recently obtained on isoprene levels in the exhaled breath of some 200 healthy children and young adults (pupils) with ages ranging from 7 to 18 years measured using SIFT-MS are presented in detail. A concentration distribution has been constructed from the data obtained and compared to those for healthy adults also obtained from SIFT-MS data. Although there is overlap between the two distributions, which are close to log normal in both cases, the median level for the young cohort is much lower at 37 parts-per-billion, pbb, geometric standard deviation, GSD 2.5, compared to that for the adult cohort of 106 ppb with a GSD of 1.65. Further to this, there is a clear increase in the mean breath isoprene concentration with age for the young cohort with a doubling of the level about every five to six years until it reaches the age-invariant mean level of that for adult cohort. Should this trend be extrapolated downwards in age it would indicate a near-zero breath isoprene in the newborn that was indicated by a previous study. Indeed, in this study isoprene was not detected on the breath of two young children. The results reveal mean breath isoprene levels (± SD) for pupils within the given age ranges as: 7 to 10 year (28±24 ppb) and 10 to 13 years (40±21 ppb), 13 to 16 years (60±41 ppb) and 16 to 19 years (54±31 ppb). The more rapid increase that occurs between the second and third age ranges is statistically highly significant ($p=0.001$) and we attribute this phenomenon to the onset of puberty and the spurt in growth that occurs during this phase of development. There is no significant difference in mean breath isoprene between males and females for both the adult cohort and the younger cohort.

1. Introduction.

The hydrocarbon isoprene, 2-methyl-1,3-butadiene, has been known to be present in exhaled breath since the inception of breath analysis [1, 2]. This compound is also produced by plants and in several industrial processes and is widely used in industry. Hence, it is distributed throughout the troposphere at a mean level of about 0.3 parts-per-billion, ppb. As such it is constantly being inhaled by all animals and human beings. Nevertheless, the major fraction, if not all of the isoprene present in exhaled breath, is genuinely endogenous. There have been several studies that have shown it to be the most abundant hydrocarbon in exhaled breath in the healthy adult population, being present at concentrations ranging from a few ppb to several hundred ppb with a mean level of about 100 ppb, [1, 3, 4, 5]. At the onset of this discourse on breath isoprene it is important to note that in all the measurements carried out (prior to our very recent SIFT-MS studies described later) it is **mouth-exhaled** breath that has been analysed and unless stated otherwise this is implicit in quoting breath isoprene levels. Fortunately, as we stress later, isoprene is not generated in the oral cavity and is a genuine systemic compound and so the considerable amount of work on mouth-exhaled breath is not compromised by oral generation.

The biochemical origin of isoprene in the body is via the melavonic acid pathway of cholesterol biosynthesis [6] leading to the production of the isoprene based molecule, isopentenyl pyrophosphate (IPP). The rate controlling enzyme in this pathway is 3-hydroxyl-3-methyl glutaryl-CoA reductase or HMGR, and it is this enzyme that is the target of the group of drugs called statins [7]. Thus, it seems plausible that taking statins to lower cholesterol will reduce breath isoprene and there have been some studies to investigate this [2, 4, 8], but further research work is required before definite conclusions can be drawn. One study designed to explore any correlation between breath isoprene and blood cholesterol level failed to find a correlation [9]. However, it has been shown that the level of exhaled breath isoprene apparently peaks during the night when the rate of cholesterol synthesis is at its greatest [10].

A study has been carried out to investigate whether there is any difference between breath isoprene in diabetics and healthy children [11]. The motivation for this was presumably because HMGR, which is the rate limiting enzyme in isoprene production, is itself regulated by another enzyme, AMP-activated protein kinase (AMPK). Once activated, AMPK inhibits HMGR and hence cholesterol (and isoprene) synthesis, but it also affects insulin secretion by pancreatic beta cells [12] so a link between isoprene levels and diabetes seems plausible. In addition, AMPK stimulates muscle glucose uptake. During periods of exercise, AMPK activity is induced in skeletal muscle and this in turn will lead to inhibition of HMGR. This may be one reason why it has been reported that breath isoprene concentration increase after beginning exercise followed by a slow decrease (typically below pre-exercise levels) [2, 13, 14]; a very recent, more detailed study of exhaled isoprene during controlled exercise has been carried out using proton transfer reaction mass spectrometry, PTR-MS [15]. It has also been demonstrated that breath isoprene concentrations are increased following exposure to ozone which led to the proposal that breath isoprene could be a non-invasive marker of a physiological response to oxidant-induced injury to epithelial membranes and to the fluid linings of the lower respiratory tract [13].

Breath isoprene studies have largely involved the healthy adult population (> 20 years of age) and these studies have mostly indicated that there is no significant variation of breath isoprene level with age and gender [2, 14]. However, a recent study [16] of exhaled isoprene carried out using PTR-MS has indicated that 19-29 year old subjects exhale significantly less isoprene than do older adults and that breath isoprene levels were significantly higher in male subjects, but a very recent paper has indicated a small but statistically significant lowering of breath isoprene level with increasing age in adult men but not in adult women [17].

During our detailed and comprehensive studies of the trace compounds in exhaled breath during the last few years using selected ion flow tube mass spectrometry, SIFT-MS, (more about which is given below) we have opportunistically been able to sample the exhaled breath of some very young healthy children and seen indications that breath isoprene levels are clearly lower than in adults. This is consistent with a very thorough study of exhaled isoprene in newborn infants and children with diabetes mellitus [11], which showed that the newborn had undetectable isoprene in their exhaled breath. The isoprene levels apparently increased with age, healthy school children having higher levels than healthy pre-school children. No significant difference in breath isoprene was found between healthy and diabetic children.

During the last two-to-three years we have placed some accent on the study of the trace compounds in the exhaled breath of significant cohorts of young adults and observed that some trace compounds are indeed lower in the exhaled breath of the young compared to adults, notably ammonia and acetone [18, 19]. One valuable feature of SIFT-MS is that analyses of single breath exhalations can be achieved in real time simply by asking the donor to exhale normally at the sample inlet port of the instrument. This is not an uncomfortable or difficult procedure so the exhaled breath of both symptomatic patients and, significantly, young children can readily be obtained. We have carried out a detailed study of the exhaled breath of some 200 children and young adults in the age range 7 years to 18 years using SIFT-MS, measuring the concentrations of some ten metabolites, including isoprene. These experiments and the general results obtained are presented in a recent paper [20]. They have revealed that breath isoprene levels for children are much lower than the breath levels for the adult population, which is consistent with the earlier study [11]. In this paper we present for the first time the detailed results of these studies of breath isoprene in children and young adults and compare them with the data obtained for the adult population using the SIFT-MS analytical method, which we now briefly review.

1.1.Brief summary of breath isoprene measurements previously obtained by SIFT-MS.

The first systematic SIFT-MS measurements of breath isoprene were made in the context of a short study of the influence of a protein calorie meal on the levels of several breath metabolites in just six healthy volunteers carried out during a single day. This study showed that whilst obvious changes in breath ammonia (increase) and acetone (decrease) occurred during the six hours following the ingestion of the protein meal, there was no obvious change in breath isoprene levels, which were seen to be sensibly invariant for each individual, with all values lying within the range 40 to 150 ppb [21]. Subsequently, in a similar study, the same six healthy volunteers ingested a carbohydrate meal and again their breath isoprene levels were not modified (unpublished observations). A near contemporaneous study involved the acquisition of 119 breath samples from 29 healthy volunteers obtained over a period of a few weeks, which allowed a crude concentration distribution of breath isoprene levels to be obtained [22]. This study revealed a range of breath isoprene levels from 20 ppb to 240 ppb with a mean level of 83 ppb (standard deviation 45 ppb).

Following the above, we embarked on a study of several metabolites in the exhaled breath of patients suffering from end-stage renal failure by locating the SIFT-MS instrument in a busy haemodialysis unit. The breath of some 19 patients was analysed immediately prior to a haemodialysis session and then immediately after the session [23]. Prior to dialysis, the mean breath isoprene level (+standard deviation, SD) was 138 (63) ppb for this patient cohort, which is significantly higher than the mean values for healthy individuals. After dialysis, the mean level was even higher at 183 (95) ppb. These observations are supported by independent observations using techniques other than SIFT-MS that breath isoprene increases following haemodialysis [24, 25]. It has been suggested that isoprene levels are connected to mental and

oxidative stress [2], but these suggestions have not been confirmed. It is also likely that the increased isoprene following dialysis is due to bio-incompatibility of the dialysis membranes (which are known to disrupt circulating blood cells), haemodynamic stress and even inflammation, as discussed in [23].

The next phase of this continuing breath isoprene saga involved a longitudinal study of the breath isoprene of only five healthy volunteers over a 30 day period, sampling the breath of each volunteer in the early morning of each working day [26]. The results of this study showed the lowest individual mean breath isoprene level was 50 ppb to the highest individual mean level of 120 ppb with a mean coefficient of variation for all five subjects of 0.4. The latter is somewhat greater than those for the more abundant compounds ammonia and acetone of 0.3 as measured in the same study [26].

More recently, a longitudinal study involving 30 healthy volunteers (19 males, 11 females) carried out over a six-month period in which each volunteer gave breath samples each week, provided 481 mean values (from three sequential exhalations/inhalations) of breath isoprene [27]. From this study the level distribution for this cohort was obtained (illustrated later in Figure 1) that showed a wider variation of levels of breath isoprene ranging from 0 to 474 ppb with a mean level of 118 ppb and a standard deviation of 68 ppb with no obvious correlation with gender or body mass index. The breath isoprene levels increased immediately after moderate exercise, but returned to normal within 2-3 minutes after exercise ceased, consistent with similar previous studies referred to above. A possible reason for this exercise effect may be the extreme volatility of isoprene at the alveolar interface and its relatively weak endogenous production; so a measurable time is required to replace the isoprene in alveolar blood (see also [15]. However, as described earlier, it may be that the exercise induces AMPK activity, which regulates the enzyme HMGR, reducing isoprene production. Blood cholesterol levels were measured for only three of the volunteers and this strictly limited amount of data did not reveal any correlation of blood cholesterol with breath isoprene, a conclusion reached by the recent, more thorough study mentioned above [17].

A very recent SIFT-MS study has investigated the origin of several trace gases in exhaled breath [28]. Thus, analyses have been performed of the breath of three healthy adult volunteers as exhaled via the **mouth** and the **nose,** and also of the static air in the oral **cavity** during breath hold. Nine trace compounds have been quantified and concentration distributions have been constructed from longitudinal data obtained over each morning of each working day for four weeks. Of these compounds, the levels of acetone, methanol and isoprene are the same in the mouth-exhaled and the nose-exhaled breath; hence, we deduce that these breath compounds are totally systemic in origin. The levels of ammonia, ethanol and hydrogen cyanide are much lower in the nose-exhaled breath than in the mouth-exhaled breath and highest in the oral cavity, indicating that these compounds are largely generated in the mouth with little being released at the alveolar interface. The mean mouth/nose breath level of isoprene for these three volunteers is about 100 ppb and thus falls within the expected range, as indicated in the above summaries. Thus, it is fortuitous that all the previous measurements of isoprene in mouth exhaled breath were not compromised by oral production of this compound, in common with the important new results relating to children and young adults described below.

2. Methods.

2.1. Brief description of SIFT-MS and the analysis of exhaled breath.

Detailed descriptions of the SIFT-MS analytical technique for trace gas analysis of air and exhaled breath have been given previously [29, 30]. Thus, it is sufficient to say here that it involves the ionisation of the trace gases in an air/breath sample (to the exclusion of the major gases N_2 and O_2 inevitably present in the sample) in their reactions with pre-selected precursor ions H_3O^+, NO^+ and O_2^+ in a helium-buffered flow tube. The signal levels of the precursor ions and the product ions of their reactions with each trace gas compound are recorded by a downstream mass spectrometer /ion detection system. The essential point is that in this way, analyses of several trace compounds can be obtained in single breath exhalations in real time avoiding the collection of breath samples into bags or trapping trace metabolites from the samples onto surfaces for later release and analysis, techniques that may lead to inaccurate quantification. The precursor ion species of choice in these SIFT-MS studies to quantify isoprene was NO^+, which avoids complications that can arise when using H_3O^+ precursor ions [27]. As has been discussed in previous papers [29, 30], the proper set up of the instrument was checked using the simultaneously measured level of exhaled water vapour, which is close to 6% at normal body temperature. Thus, in these studies only data was accepted that showed alveolar water vapour levels >5%; lower levels were indicative of a poor exhalation and a need for repeat sampling.

2.2. Subjects and ethical issues.

The measurements of the exhaled breath of 200 children were made over a period of a few weeks by locating a SIFT-MS *Profile 3* instrument (*Instrument Science Limited, Crewe, UK*) in a school near to the Keele laboratories and the University Hospital of North Staffordshire, Stoke-on-Trent. All healthy children aged between 7 and 18 years of age attending this school were eligible for inclusion in the study. It was established by correspondence with the UK National Research Ethics Service – NRES (http://www.nres.npsa.nhs.uk/) that this study can be carried out in a non-NHS (National Health Service) establishment as long as informed consent from the parents of each child and the approval of the head teacher of the school were obtained. Hence, we obtained approval from the head teacher of the school involved as well as the appropriate class teachers and also obtained signed consent from the parents. The demographics of the volunteer cohort are exactly as reported in the previous paper [20]: 105 male and 95 female, the age of the youngest volunteer was 7.0 years and of the oldest was 18.5 years.

2.3. Protocol.

For simplicity, these measurements involving a large cohort of children were performed on mouth-exhaled breath only. Prior to providing breath samples, the volunteers were asked to complete a questionnaire concerning their dietary intake during the previous 12 hours and of any prescribed medication. No dietary or time constraints were placed on these measurements, which were taken at convenient times during the normal school attendance. No unusual patterns in the questionnaire answers were recognised by the attending paediatrician and so these data were not considered in the assessment of these isoprene data. No effort was made to obtain physiological data such as heart rate and breathing frequency in the already constrained situation in a school. Each child was simply asked to rest for a few minutes before the breath analysis because, as mentioned above, it is known that exercise temporarily changes the level of exhaled breath isoprene [4, 14]. As is our common approach to on-line breath analysis, the volunteers were encouraged to take three long, slow inhalations

and exhalations through a disposable viral filter placed at the sampling inlet port of the instrument. To ensure maximal exhalation via their mouths, they wore a soft plastic nose-clip during the sampling period. The variation of the isoprene level between the three exhalations was typically 20%; hence, the mean value of the three isoprene concentrations provided values that were reliable to better than this percentage.

3. Results.

A concentration distribution constructed from the mean levels of three sequential exhalations from the 200 children and young adults is shown in Figure 1. As can be seen, the distribution is a reasonable approximation to log normal, as indicated by the continuous line, although there are just a few values that are at very low, even zero values. Zero values were consistently seen for one healthy volunteer (17 measurements over a six-month period) in our earlier longitudinal data [27] and such was seen in another study of the breath of the newborn [11]. The zero breath isoprene levels are both very interesting and perplexing and must be clues to the true origin of endogenous isoprene. The mean level, standard deviation, SD, median level and geometric standard deviation, GSD, for breath isoprene are given in Figure 1 for this cohort. The median value 37 ppb has been reported before in [20] for the same measurements together with geometrical mean of 33 ppb. The arithmetic mean (44 ppb) is larger than the median and the geometric mean due to the skewed nature of the log-normal distribution of the isoprene breath levels.

The most significant feature of these data is that the median value of 37 ppb is much lower than that obtained for adults of about 90 to 110 ppb from the several studies summarised earlier. Included in Figure 1 is the distribution obtained from the longitudinal study of adult breath isoprene [27], which clearly shows this difference. Note the larger GSD (2.5) for the children and young adult distribution compared to that for the adult cohort (1.6). Presumably, this is largely a reflection of the age variation of the breath isoprene that is clearly indicated in Figure 2, where the individual mean breath isoprene levels are plotted as a function of age on a semi logarithmic scale to show the wide variation. These data strongly support the findings of the previous study by Nelson et al [11] that there is a variation of breath isoprene with age amongst younger healthy volunteers. Notice how the values for the older children approach those for the sensibly age-independent mean values for the adults.

The coefficient of determination of the linear regression through the logarithmically transformed data represented by the exponential function, $R^2 = 0.18$, as indicated in Figure 2. The statistical significance that the slope of this line is non-zero is remote, since according to the regression statistics, $p < 10^{-7}$. The exponential function corresponds to a doubling of the isoprene concentrations each 5 to 6 years. Linear regression of these data without logarithmic transformation provides the following results: $R^2 = 0.14$, i.e. lower than for the exponential function, with a confidence level of 95%; the slope of the linear plot is greater than 2.8 and less then 5.8 ppb/year, the most probable value being 4.3 ppb per year (or 43 ppb per 10 years). Should this trend continue downwards to even younger children and babies it certainly would indicate near-zero breath isoprene levels in the newborn, as seen by Nelson et al. [11]. Similar plots of the isoprene levels against weight and height indicate correlations with both parameters (for both, $R^2 = 0.12$), but not as good as that with age.

What is so interesting is that discernible within these SIFT-MS data given in Figure 2 is a more rapid increase in breath isoprene level when moving into the age range 13 to 16 years. This increase is seen in the vertical bar charts in Figure 3 that presents the mean breath isoprene levels and the associated standard deviations for five age ranges. Thus, the mean values and standard deviations (given as mean ± S.D) in the following age groups of n volunteers were found to be: 7 to 10 years (n=57) 28±24 ppb; 10 to 13 years (n=63) 40±21 ppb; 13 to 16 years (n=69) 60±41 ppb; 16 to 19 years (n=11) 54±31 ppb. The S.D values are

represented by the vertical bars in Figure 3. The more rapid increase of the breath isoprene between the age ranges 10-13 and 13-16 years is very significant, analysis of variance, ANOVA, giving $p < 0.001$, whereas the decrease between ages ranges 13-16 and 16-19 years is not significant ($p = 0.66$). The most likely explanation for this faster-than-average increase in breath isoprene around age 13 years is the onset of puberty and the spurt in growth that occurs at this time. Clearly, beyond puberty, growth slows down and eventually stops as adulthood is reached and this is reflected in the steady mean breath isoprene level. There is some evidence [16] that breath isoprene begins to reduce beyond age 65, which might be associated with the reduction of lean body mass that occurs, but much more experimental work is needed if this is to be substantiated.

4. Discussion.

The mean projected level for breath isoprene at age 20 years is close to 70 ppb and if this is projected to age 60 years it would exceed 200 ppb. However, the study by Lechner et al. [16] indicates that breath isoprene continues to increase with age up to only about 29 years and then ceases, but it should be said that only a small fraction of the Lechner et al. cohort (11 individuals) fell into the age bracket 19 to 29 years. Clearly, the above projection to a mean value of about 200 ppb at 60 years old is obviously unwise and the mean value of the distribution for the older healthy volunteers, as measured by SIFT-MS [27] and shown by the dotted/shaded distribution in Figure 1, is more appropriate, noting also the insignificant variation of the mean value with age for this adult cohort as shown by the open circles in Figure 2. The absolute levels of isoprene amongst the Lechner et al. cohort are significantly lower than would be expected based on our several SIFT-MS studies and these lower values are most probably due to the assumption that the only identifying product ion for isoprene in those PTR-MS experiments is that at a mass-to-charge ratio of 69 only, which is now known to represent only about half of the total product ions [17].

Within the cohort of children and young adults involved in this SIFT-MS study, the mean level of breath isoprene is 44 ppb for the females and 45.0 ppb for the males with a common standard deviation of 30 ppb, the median values being 36 and 37 ppb respectively. Statistically, there is no significant difference between the breath isoprene for the males and females ($p = 0.77$) in accordance with the majority of the adult data. The conclusion to be drawn from all these studies must be that the increase in breath isoprene in the young is related to growth development and essentially ceases when growth stops.

5. Concluding remarks.

Breath isoprene has received considerable attention during the last 10 years, not least using the SIFT-MS analytical method for its quantification. It is now positively established as an endogenous trace compound [28] even though definite clinical significance has not been established for this metabolite. Its concentration in exhaled breath and its variations over days and weeks has been defined for healthy individuals by studies involving small cohorts and large cohorts of healthy adults. The results from the various studies are very consistent in that the mean level for healthy adults is typically 100 ppb, but ranging from 20 to 200 ppb, with rare cases of near-zero breath isoprene. These studies mostly indicate no difference in mean values for males and females and little or no age variation for adults. However, our recent studies on a large cohort of children and young adults have shown that the mean level of breath isoprene are much lower at about 40 ppb, and that there is a slow but definite increase with age in the range 7 to 19 years. This trend had been seen in an earlier study, which also showed that isoprene is not present in the exhaled breath of the newborn. The indications from the collected data shown in Figure 2 are that the greatest rate of increase in exhaled

breath isoprene occurs during the first few years of life. There is no significant difference in breath isoprene between the genders in the cohort of 200 children and young people, but there is a clear indication of a more rapid increase in breath isoprene around the expected age of puberty (see Figure 3), although this interesting and potentially important observation merits further measurements that must be subject to careful statistical scrutiny.

Concerted studies of breath isoprene levels in the diseased state have not been carried out, although opportunistic evidence has been obtained that this trace compound is significantly higher in the breath of patients with end-stage renal failure and is further increased following haemodialysis treatment [23, 24, 25]. That breath isoprene levels are lower in the very young and increase with age towards adulthood is perhaps a clue as to the real origin of this metabolite, since it appears to be related to growth and development. The collective evidence shows that breath isoprene levels for particular healthy adults remain sensibly constant for years, but the inter-person differences are wide. It remains to be seen if breath isoprene levels are indicators of particular diseases, but breath isoprene has not been found to be different between healthy and diabetic children. Perhaps long term measurements of breath isoprene and other breath metabolites might be indicators of developing disease as individuals acts as their own control.

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Figure 1.
Histogram of isoprene levels in parts-per-billion, ppb, obtained in the exhaled breath of 200 children and young adults in the age range 7 to 18 years in this case shown as a percentage, %, within the chosen ppb window. Both the mean level and the standard deviation (SD) and the median level and the geometric standard deviation (GSD) are given. Note the few outliers at low and zero isoprene levels. For comparison, the distribution for adults obtained by Turner et al [27] is shown as a dotted/shaded distribution.

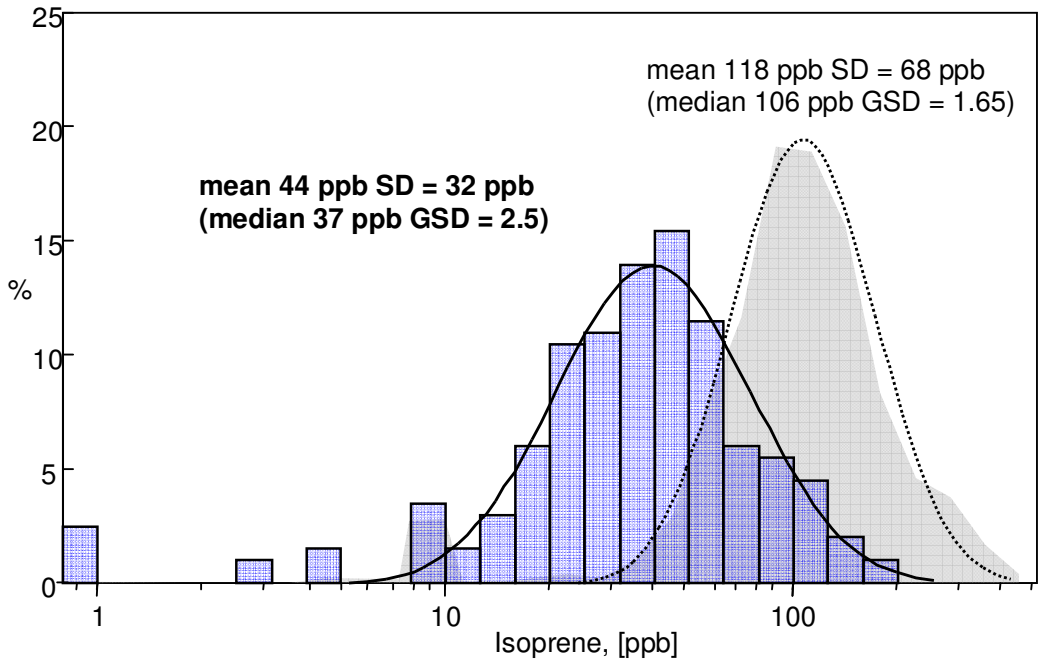
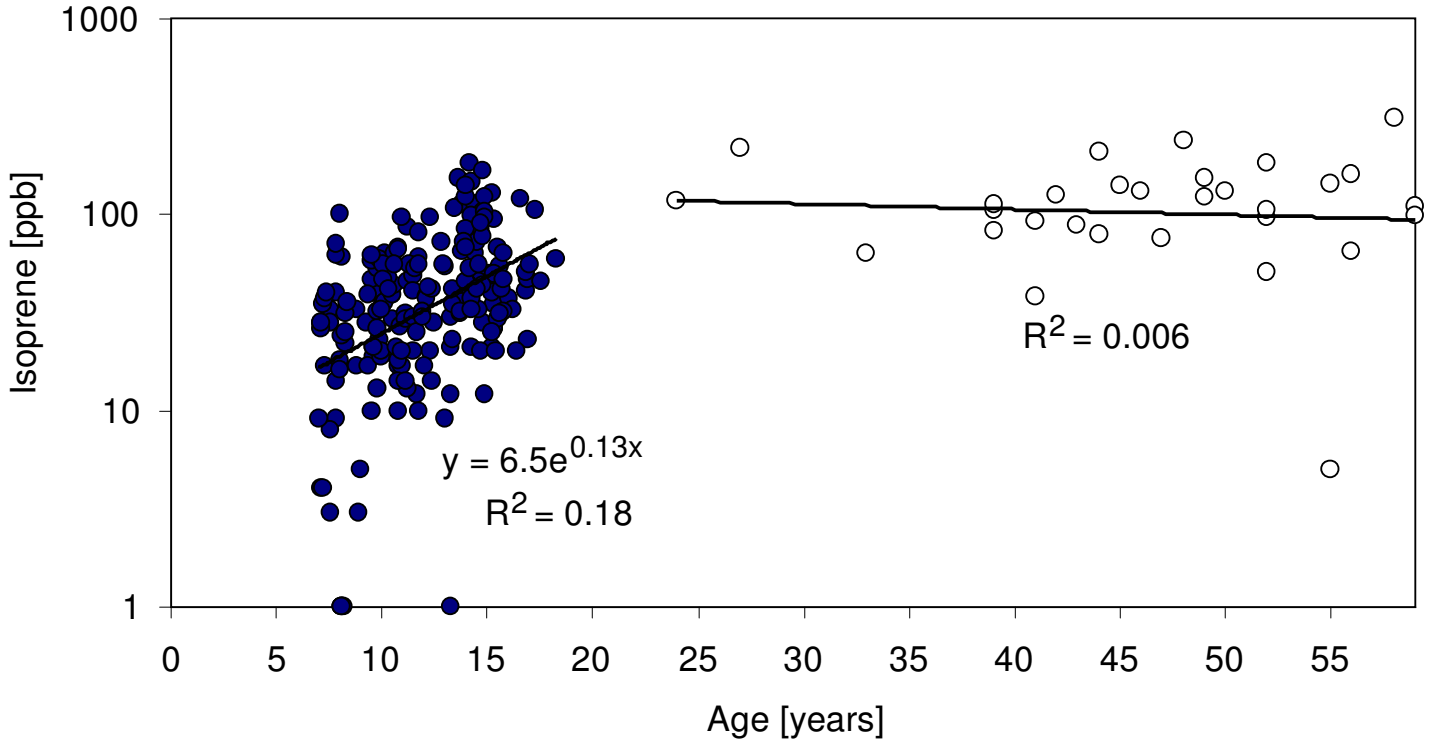


Figure 2.
A plot of the isoprene concentration in parts-per-billion, ppb, against age (years) in the exhaled breath of two cohorts of healthy individuals as measured using SIFT-MS. Filled circles: children and young adults in the age range from 7 to 18 years. Open circles: adults in the age range 24 to 59 years [27]. Note the six outlier low values amongst the children cohort and the single outlier for the adult cohort. The coefficient of determination, R^2, indicates a significant variation if breath isoprene level with age amongst the younger volunteers but not so for the adults. Excluding the outlier point from the adult values leads to a slight positive correlation with age but which is not so significant with $R^2 = 0.012$.



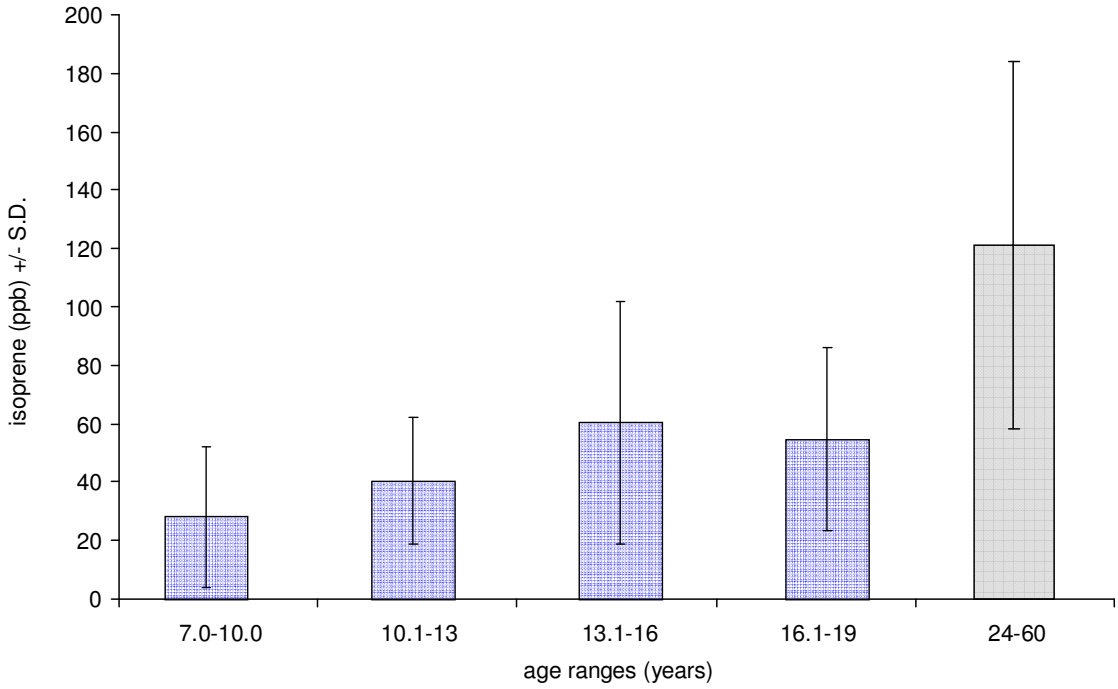


Figure 3.
The mean levels of breath isoprene in parts-per-billion, ppb, together with the corresponding standard deviations, SD (vertical bars) for healthy persons within the age ranges indicated. The more rapid increase in the mean levels seen in moving from the 10 to 13 age range to the 13 to 16 age range is statistically highly significant ($p < 0.001$) and is probably due to the onset of puberty. There is no statistically significant difference in isoprene levels between the male (M) and female (F) volunteers, because the difference is much smaller than the SD. For the 13-16 year old (pubescent) group, the mean levels are M=62 ppb, F=57 ppb ($p = 0.51$).