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*Original Article*

**Akira Tiele <sup>1</sup>, Anne Daly <sup>2</sup>, John Hattersley <sup>3</sup>, Alex Pinto <sup>2</sup>, Sharon Evans <sup>2</sup>, Catherine Ashmore <sup>2</sup>, Anita MacDonald <sup>2</sup> and James A. Covington <sup>1,\*</sup>**

<sup>1</sup> School of Engineering, University of Warwick, Coventry CV4 7AL, UK

<sup>2</sup> Dietetic Department, Birmingham Children's Hospital, Birmingham B4 6NH, UK

<sup>3</sup> Human Metabolism Research Unit, University Hospitals Coventry and Warwickshire, Coventry CV2 2DX, UK

\* Corresponding author: [J.A.Covington@warwick.ac.uk](mailto:J.A.Covington@warwick.ac.uk)

## Abstract

In clinical practice, caregivers of children with phenylketonuria (PKU) report that their children have breath malodour. This might be linked to the regular consumption of low phenylalanine (Phe)/Phe-free protein substitutes (PS), which are an essential component of a low-Phe diet. Oral malodour can negatively affect interpersonal communication, lead to bullying, low self-esteem and social isolation. In this longitudinal cross-over study, exhaled volatile organic compounds (VOCs) were measured using gas chromatography – ion mobility spectrometry (GC-IMS). 40 children (20 PKU, 20 controls) were recruited. Subjects with PKU took either L-Amino Acid (L-AA) or Casein Glycomacropeptide (CGMP-AA) exclusively for 1 week, in a randomised order. On the 7<sup>th</sup> day, 7 exhaled breath samples were collected over a 10-hr period. Subjects then transferred to the other PS for a week and on day 7, provided 7 further breath samples. All subjects had a standardised menu using low-Phe food alternatives and all food intake was measured and recorded. In the PKU group, the aim was to collect samples 30-min after consuming PS. In 3 subjects, breath was collected 5-min post-PS consumption. Fasted L-AA and CGMP-AA breath samples contained a similar number of VOC peaks (10-12) as controls. Longitudinal breath testing results demonstrate that there was no significant difference in the number of exhaled VOCs, comparing L-AA or CGMP-AA with controls, or between PS (12-18 VOC peaks). Breath analysed immediately after consumption of PS (n=3) showed an immediate increase in the number of VOC peaks (25-30), but these were no longer detectable at 30-min post-consumption. This

suggests PS have a transient effect on exhaled breath. Measurements taken 30-min after consuming L-AA or CGMP-AA were not significantly different to controls. This indicates that timing food and drinks with PS consumption may be a potential solution for carers to reduce or eliminate unpleasant PS-related breath odours.

**Keywords:** Phenylketonuria (PKU); glycomacropeptide; PKU supplements; breath analysis; breath malodour; GC-IMS; volatile organic compounds (VOCs)

## 1 Introduction

Phenylketonuria (PKU) is an inherited metabolic disorder, caused by a deficiency in the enzyme phenylalanine hydroxylase (PAH), which is responsible for converting the amino acid phenylalanine (Phe) into tyrosine. This metabolic disruption causes Phe levels to accumulate in blood, which if untreated, causes neurotoxicity [1] leading to severe and irreversible neurological impairments [2]. PKU has been reported in all ethnic groups, affecting males and females in similar numbers. Incidence rates are around 1 in every 10,000 births but with a high prevalence in Turkey, Poland and Ireland [3]. In the UK, infants with PKU are detected by new-born screening [4]. While there is no cure for PKU, effective management of the condition is achieved through dietary restriction of Phe (in the form of a strict low-protein diet) and supplementation with Phe-free protein substitutes (PS) [5]. PS are essential, supplying adequate nitrogen for growth, suppressing blood Phe

concentrations, and most will provide comprehensive vitamin and mineral supplementation meeting age appropriate requirements.

Most children with PKU tolerate <10g/day natural protein. This means that high protein foods are avoided with a controlled intake of some vegetables and cereals and children must eat different foods from their friends, compromising normal socialisation. They are usually recommended to take PS 3 times daily with meals. While there have been significant improvements in the taste, appearance and presentation of PS [6], overall PS tastes unpleasant and patients often associate intake with bad breath. This is a concern of parents and caregivers, since oral malodour can result in psychological distress, interfere with social interactions, lower self-esteem and body-image [7]. Breath malodour in PKU has received little clinical or research attention, and it is unknown if this is an issue more common in PKU than a healthy population.

This study focuses on 2 types of PS; Phe-free L-amino acids (L-AA) and low-Phe casein glycomacropeptide (CGMP-AA). Conventional L-AA consist of single amino acids and have a characteristic odour associated with their pure amino acid composition. Casein glycomacropeptide is a peptide, derived from the whey fraction during the cheese manufacturing process. It is naturally low in phenylalanine and after the addition of some essential amino acids, has been adapted for use as a PS in PKU. CGMP-AA has a different composition from L-AA PS, being made of 60% peptide and 40% L-AA. In PKU, no studies have compared L-AA with CGMP-AA and reported impact on breath malodour. It is suggested that CGMP-AA is associated with less oral malodour.

Since there is limited research, the authors propose 3 possible causes for PKU-associated breath malodour: (1) residue from the PS in the oral cavity

leads to a temporary change in breath composition (i.e. breath smells like the PS); (2) residue and regular replenishment of sulphur-containing amino acids from the PS (such as cystine and methionine) serve as a substrate for bacterial degradation in the oral cavity [8] resulting in the release of volatile sulphur compounds (VSCs) such as hydrogen sulphide, methyl mercaptan, and dimethyl sulphide (DMS), which are typically associated with halitosis [9]; and (3) modifications to the exhaled breath composition of subjects with PKU is caused by changes in metabolism or the gut microbiota due to their low-protein diet [10], resulting in oral malodour. These factors are not necessarily mutually exclusive and could be produced by a combination of these effects.

Currently, 3 methods for measuring oral malodour are available, including: organoleptic measurement, sulphide monitors and gas chromatography – flame photometric detector (GC-FPD) [11]. The organoleptic method is generally considered the gold standard halitosis test, and involves assessing oral malodour, as perceived by the human nose of a trained investigator [12]. However, this method is expensive (requiring a highly trained professional), subjective (cannot accurately quantify the oral malodour level) and is unsuitable for testing large study groups. Portable commercial instruments such as the OralChroma (Nissha FIS, Osaka, Japan) and Halimeter (Interscan Cor., Simi Valley, CA, USA) provide a relatively inexpensive and easy-to-use alternative to quantify odour levels. The disadvantages of these devices are that they can only measure VSCs with relative concentration measurements and cannot provide chemical identification of other volatile organic compounds (VOCs) in exhaled breath [13]. GC-FPD is a reliable, objective and reproducible method of quantifying breath contents. However, these

instruments are bulky, expensive and require trained operators [14]. In recent years, alternative GC-based technologies, such as combining GC with ion-mobility spectrometry (collectively known as GC-IMS), have produced portable analysers for exhaled breath, which can measure VOCs in the low parts-per-billion (ppb) range and are suitable for sampling vulnerable subjects, including children.

Therefore, in a collaborative research effort, undertaken by Birmingham Children's Hospital, the University of Warwick and University Hospitals Coventry and Warwickshire, this study aims to investigate the phenomenon of paediatric PKU breath malodour by analysing exhaled breath using GC-IMS technology.

## **2 Materials and methods**

### *2.1 Subjects*

Forty children (20 PKU, 20 healthy controls) were recruited for this longitudinal cross-over pilot study. This study received ethical approval (IRAS ref: 211894) and was conducted in accordance with the Declaration of Helsinki. Participants were recruited to create a balanced cohort (gender, age, and ethnically matched). The median age was 11 years (range 7-15.5 years), including 18 males and 22 females. The Phe concentrations of subjects with PKU were measured by weekly blood spots 12 months prior to starting the study. Median Phe concentrations for children  $\leq 12$  years ( $n=17$ ) was 270  $\mu\text{mol/L}$  (range 150-360  $\mu\text{mol/L}$ ), and children  $>12$  years ( $n=3$ ) was 330  $\mu\text{mol/L}$  (range 315-350  $\mu\text{mol/L}$ ). Both these groups show excellent Phe control within

the European target recommendations [15]. An overview of the demographic data for the study is shown in Table 1.

**Table 1.** PKU breath study demographic data of subjects.

Parameter	Subjects with PKU (n=20)	Controls (n=20)
Age (SD)	10.5 (2.5)	10.6 (2.5)
Gender ratio M:F	9:11	9:11
Siblings (Twins)	3 (1)	4 (0)
Regular medication (No. of subjects)	Ritalin (1) Neurotransmitter medication (1)	N/A

Eight sets of siblings were involved in this study; 4 sets in both groups and 1 set provided a control and a child with PKU. Recruiting siblings can reduce the possible effects of confounding factors, such as lifestyle and environment [16]. Confounding factors can be accounted for in the design-stage by recruiting a balanced cohort and in the analysis stage by separately investigating factors, such as age and gender. For these purposes, age and gender groups were defined as [ $\leq 10$  vs  $> 10$  years] and [male vs female], respectively. The confounding factor groups are shown in Table 2.

**Table 2.** Confounding factor groups.

Factor	Groups	PKU	Controls	Total
Age	$\leq 10$ years	10	10	20
	$> 10$ years	10	10	20
Gender	Male	9	9	18
	Female	11	11	22

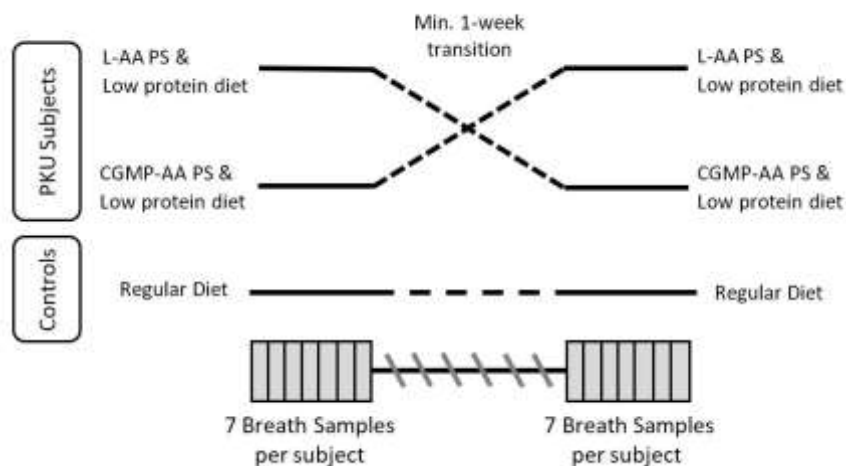


## 2.2 Study Design

To better understand the effects of paediatric PKU breath malodour, the recruited subjects were asked to complete a questionnaire relating to how they perceive their breath. Subjects with PKU took all their PS requirement from either L-AA or CGMP-AA exclusively for 7 days, in a randomised order. On the 7th day exhaled breath samples were collected over a 10 hour period. The subjects arrived fasted and samples were collected first thing, post-morning meal, pre- and post-midday and evening meals, and an afternoon sample taken at a standard time. Subjects with PKU then transferred to the other PS for 7 days and on day 7, provided 7 further breath samples as before. When randomised to L-AA, 17 children were on liquid and 3 on powdered products. The liquid L-AA PS were: PKU Lophlex LQ juicy berry n=3, (Nutricia Ltd), PKU Cooler red n=12, PKU Cooler Air red n=2, (Vitaflo, Liverpool, UK). The flavours of the L-AA powder PS were: PKU express raspberry n=1, PKU gel raspberry n=1, (Vitaflo, Liverpool, UK), PKU First Spoon unflavoured n=1 (Nutricia Ltd). They were all reconstituted with water. CGMP-AA was also a powdered PS, available in vanilla n=2 and berry n=18 flavours (Vitaflo Ltd, Liverpool, UK) and was reconstituted with water.

For the first breath sample (before morning meal) all children attended the research unit without brushing their teeth. Breath samples were collected in a staggered timetable to maintain standard interval times between samples for all subjects. A maximum of 4 subjects were sampled each day, including control, L-AA and CGMP-AA subjects on most days. All subjects consumed a standardised menu on the days of breath sampling using low-Phe alternatives, e.g. low-protein pasta for subjects with PKU and regular pasta for controls. All

food intake was measured and recorded. The cross-over study design and staggered timetable are shown in Figure 1 and Appendix A, respectively.



**Figure 1.** Longitudinal cross-over study design: subjects with PKU consumed L-AA or CGMP-AA exclusively, by random order for 7 days, and on day 7, provided 7 breath samples. Subjects with PKU then transferred to the other PS for 7 days and provided 7 further breath samples on day 7.

In the PKU group, the aim was to collect samples 30 minutes after consuming the PS to allow subjects to digest and metabolise their meals and PS. This enabled measurements in potential changes in exhaled breath composition related to the metabolism of PS (i.e. pharmacokinetic changes), instead of residue from the PS.

### 2.3 Gas Chromatography – Ion Mobility Spectrometry

This study utilised a commercially-available, gas chromatography – ion mobility spectrometry (GC-IMS) instrument (G.A.S. BreathSpec, Dortmund, Germany). This technology uses GC as a pre-separator, followed by a drift-

tube IMS detector. The BreathSpec GC-IMS was equipped with a MXT-200 midpolarity column (Thames Restek, Saunderton, UK) and fitted with a circulator gas flow unit (G.A.S. CGFU, Dortmund, Germany). This unit recirculated and filtered the air and allowed the unit to operate without the need of an external gas supply. When the breath sample was introduced to the GC, chemical interactions with the column cause individual compounds to elute at different times – known as the retention time. This stage was followed by the IMS detector, where the pre-separated analytes were ionised and injected into a drift tube. The ions drifted against a buffer gas, under influence of a uniform electric field (400 V/cm). This enabled ions to achieve different velocities, inversely proportional to their size, mass and charge [17]. They were then collected on a Faraday plate, to provide a time-dependent signal corresponding with ion mobility. The buffer gas flow rate was 150 ml/min and carrier flow rate through the instrument was 15 ml/min, while the sample was being introduced. The carrier flow rate was then ramped to 50ml/min over the 11-minute measurement time. The temperatures of the GC column and IMS were set to 45°C. The device can measure substances down to the low ppb range, depending on the proton affinity of the compound. The instrument was suitable for a clinical setting and was placed on a work surface (dimensions: 45 x 50 x 30 cm; mass: 20 kg).

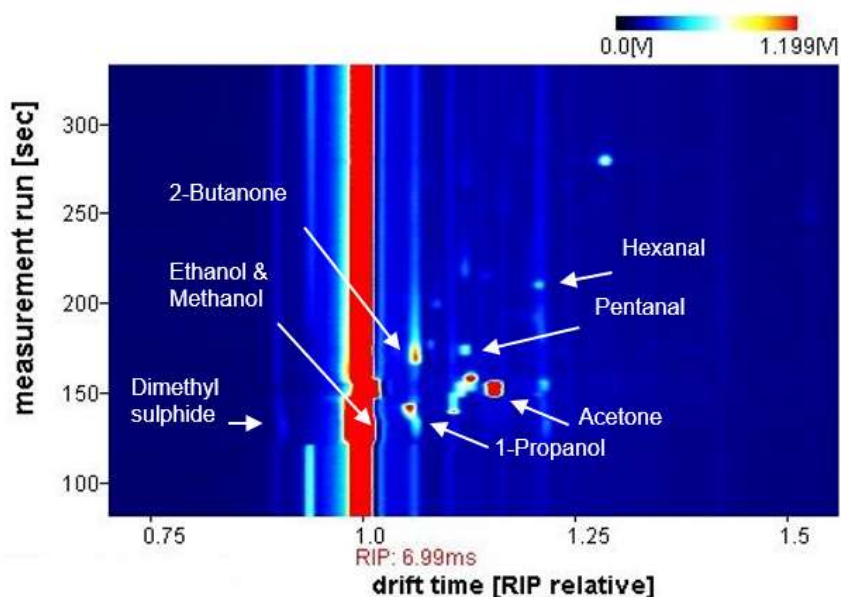
#### *2.4 Breath Sampling*

The BreathSpec instrument collects end-tidal breath using an integrated sampling system. Subjects were provided with a disposable polypropylene mouthpiece (G.A.S., Dortmund, Germany), which attached to a holder

(connected to the front-panel of the instrument). A new mouthpiece was provided for each sample. The mouthpiece is a tapered and open-ended tube, with a one-way valve. As the subject exhales through the tube, air is displaced, which separates dead-space from end-tidal breath. This portion of exhalate is pulled into the 10mL sample loop, prior to being introduced to the GC column. The transfer tubing between the mouthpiece holder and instrument/sample loop was heated, with a temperature set to 45°C. The sampling procedure only required around 4 seconds of exhaled breath. Subjects were not required to exhale until their lungs were as empty as possible; instead, sampling only required a single normal breath. This procedure improved reproducibility and was appropriate for paediatric subjects.

### *2.5 Data Analysis*

A typical output response (chromatogram) from the GC-IMS is shown in Figure 2; specifically, a fasted sample from a control subject. The chromatogram is represented as a topographic map, whereby each data point was characterised by the retention time in the chromatographic column (in seconds), the drift time (in milliseconds) and the intensity of the ion current signal (in volts), as indicated by colour. The reactant ion peak (RIP) refers to the constant peak in the spectrum that results from the carrier gas being always present in the measurement process. This peak can be used to align samples for data analysis. As a result, the drift time is represented as a RIP relative value. Laboratory Analytical Viewer (LAV) software (v2.2.1, G.A.S., Germany) was used to analyse chromatograms.



**Figure 2.** Typical GC-IMS BreathSpec output chromatogram (control subject).

For data analysis, a custom program was written in MATLAB (R2018b, MathWorks, Natick, MA, USA) to detect, count and extract VOC information. This data analysis approach attempts to represent breath samples as VOC ‘fingerprints’ by quantifying the number and types of endogenous VOCs (compounds that originate from within the body). This method has been previously used to demonstrate that patients with Alzheimer’s disease have different exhaled breath VOC fingerprints than healthy controls [18]. The first step involves a pre-processing stage. The aim of which was to remove the background (that contains no useful information). A typical GC-IMS dataset contains 11 million data points. The individual regions of high intensity, which correspond to individual VOC peaks, are thus made up of tens of single data points. Since we were only interested in these areas, we could remove the background by setting a static threshold. The remaining clusters of non-zero

data points can then be considered as individual VOC peaks and totalled. In addition, their locations and maximum intensities can be extracted. Maximum peak intensities were selected, as we are able to correspond this with the abundance of the compound and it is independent of the applied background threshold. Using the locations of the VOC peaks, GC-IMS Library Search software (v1.0.1, G.A.S., Dortmund, Germany) was used to identify the compounds, based on gas chromatographic retention times and ion mobility drift times. These were linked to a NIST database, which included 400,000 annotated retention indices and an estimated 83,000 compounds entries [19]. Some common breath VOCs have been labelled in Figure 2.

### *2.6 Quality Assurance and Control*

For quality assurance, the position and quality of the RIP on the GC-IMS was regularly checked for signs of contamination. Samples were collected in the same setting, by the same operator, throughout the study. This ensured consistent sampling procedures since the collection process was manually triggered by the operator, while the subject exhaled through the mouthpiece. Additional quality control procedures were implemented. This involved collecting 4 room air samples at suitable intervals between breath samples (8:00, 11:00, 15:15 and 18:45) to monitor changes in ambient air and identify exogenous VOCs (compounds that do not originate from within the body).

To match the GC-IMS Library Search software with the equipped column, the GC-IMS instrument was normalised using a standard ketone mix (2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone). The locations of common breath VOCs, such as acetone, 2-butanone, pentanal,

hexanal and 1-propanol were also verified using chemical standards. The locations of other compounds, such as dimethyl sulphide and limonene were identified using the GCxIMS Library Search software. In addition to normalising the column for compound identification, it is possible to develop quantification curves for some VOCs. This was achieved using a dilution series of the target compound to generate a calibration function, which allows the voltage signal from the GC-IMS output to be converted into ppb concentrations.

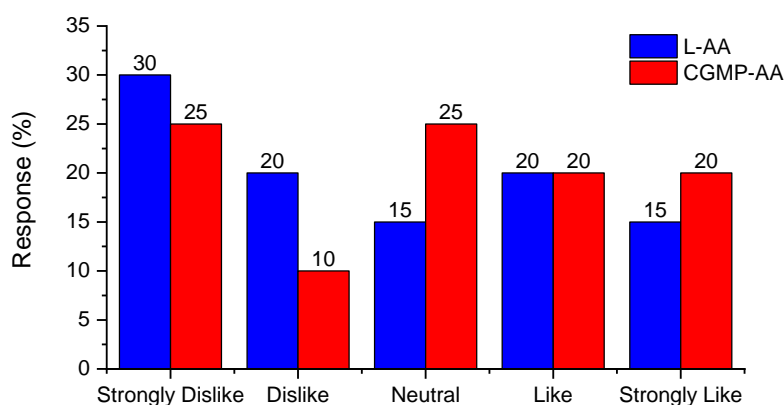
### **3 Results**

#### *3.1. Questionnaire Responses*

The questionnaire responses are shown in Figures 3 to 8. The results indicate that 50% of subjects with PKU dislike or strongly dislike the taste of L-AA, compared to 35% with CGMP-AA (Figure 3). Subjects with PKU worry more about their breath smelling when consuming L-AA, over CGMP-AA (Figure 4 and Figure 8). Controls stated that they worried little or not at all about their breath and that others do not comment on their breath (Figure 4 and Figure 5). This suggests that the problem is associated with PS consumption. While most subjects (controls and subjects with PKU) noticed their breath smelling when fasting in the morning, subjects with PKU also observed their breath odour in the afternoon and after consuming PS (Figure 6). A limited number of subjects from both PS groups worried their breath smell might affect friendships (Figure 7). The questionnaire results indicate that subjects with PKU experience breath malodour in greater numbers than

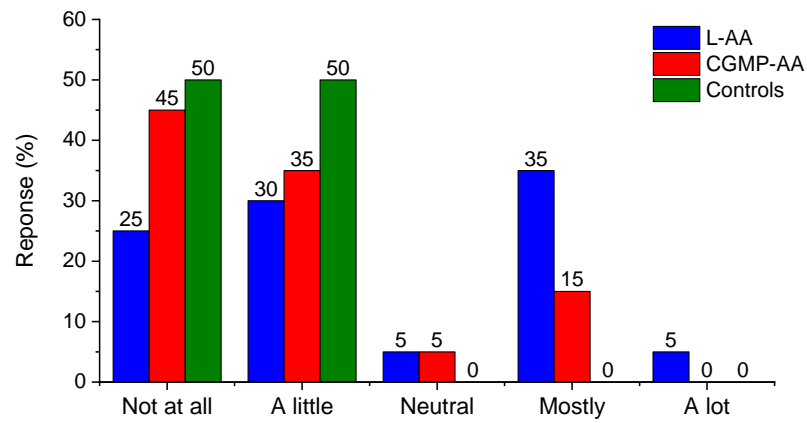
controls and that these subjects notice their breath smelling more when consuming L-AA, compared to CGMP-AA.

Descriptive statistics were applied to the questionnaire results, using one-way ANOVA analysis and Wilcoxon matched t-test. A significant difference between the L-AA compared to the CGMP-AA and control groups was evident when children were asked 'Q2: Do you worry about your breath smelling?' ( $p = 0.02$ ), 'Q3: Do others notice/comment your breath smells?' ( $p = 0.03$ ), 'Q5: Does your breath affect your friendships?' ( $p = 0.04$ ), 'Q6+7: Do you notice your breath smells more with L-AA than CGMP-AA, or CGMP-AA than L-AA?' ( $p < 0.0001$ ).



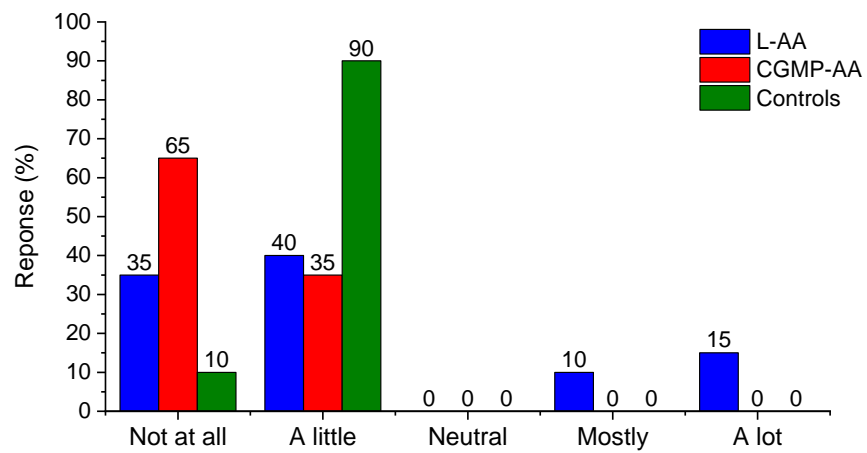
**Figure 3.** Questionnaire - Responses to Question 1 (Q1): Do you like/dislike the taste of your L-AA or CGMP-AA supplements?





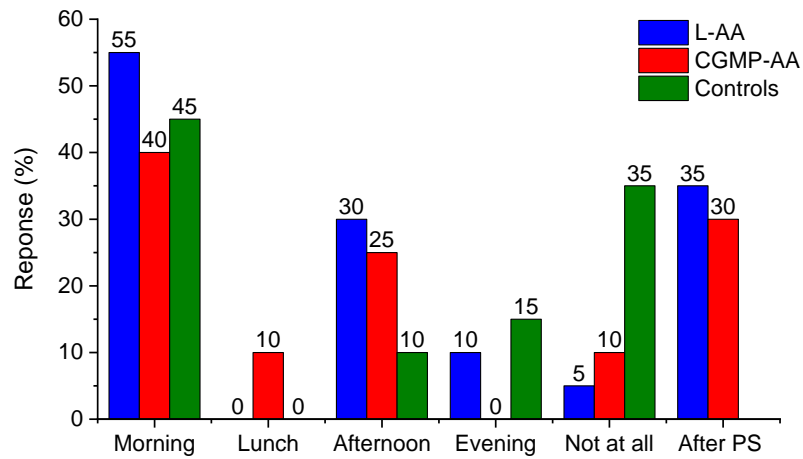
**Figure 4.** Questionnaire - Responses to Question 2 (Q2): Do you worry about your

breath smelling?

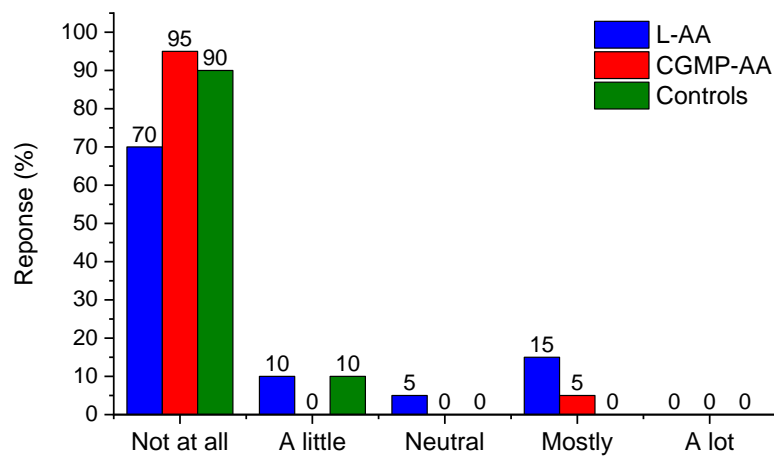


**Figure 5.** Questionnaire - Responses to Question 3 (Q3): Do others

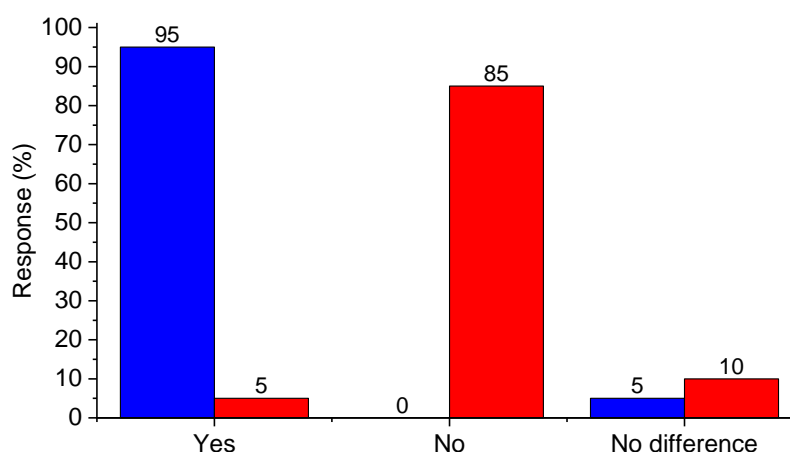
notice/comment your breath smells?



**Figure 6.** Questionnaire - Responses to Question 4 (Q4): When does your breath smell more?



**Figure 7.** Questionnaire - Responses to Question 5 (Q5): Does your breath affect your friendships?

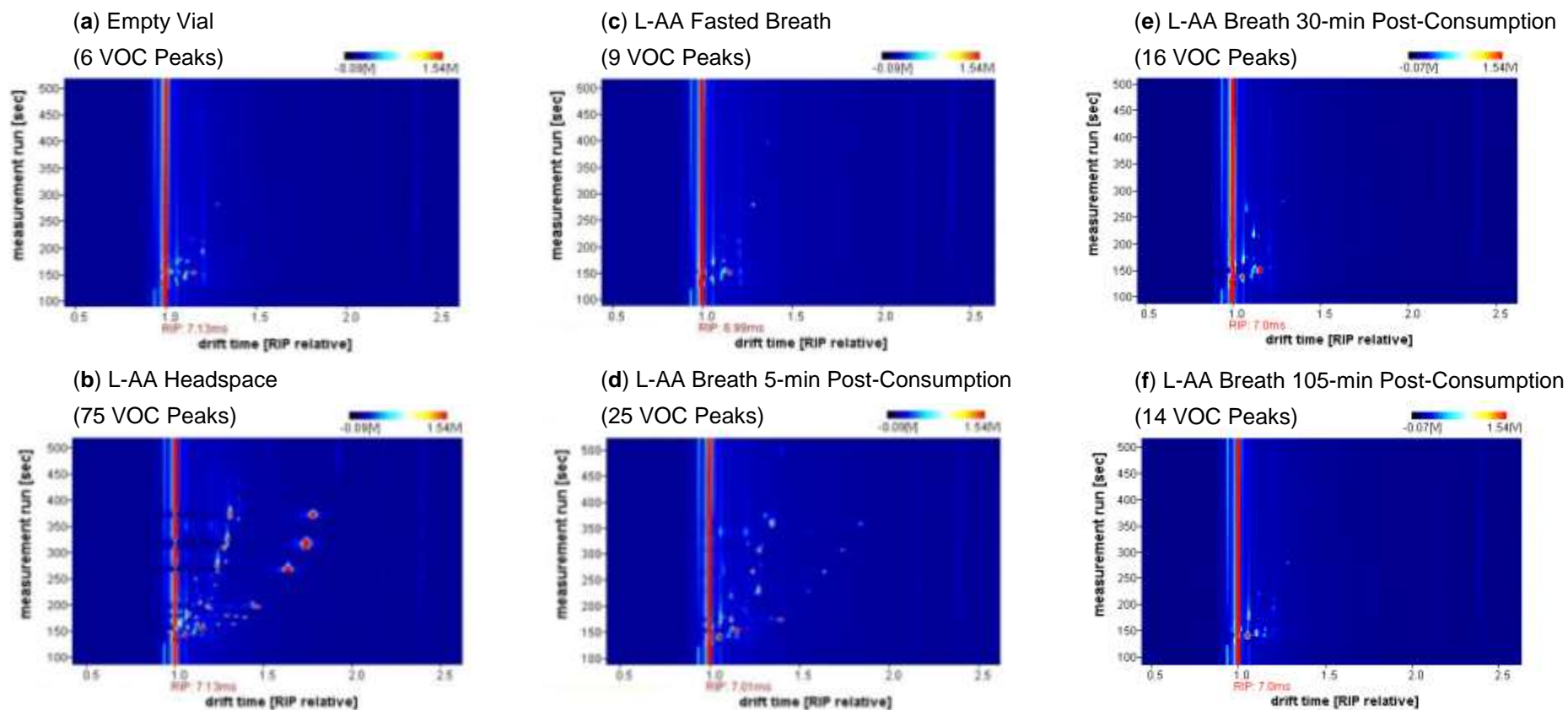


**Figure 8.** Questionnaire - Responses to Questions 6 and 7 (Q6+7): Do you notice your breath smells more with L-AA than CGMP-AA (blue) or CGMP-AA than L-AA (red)?

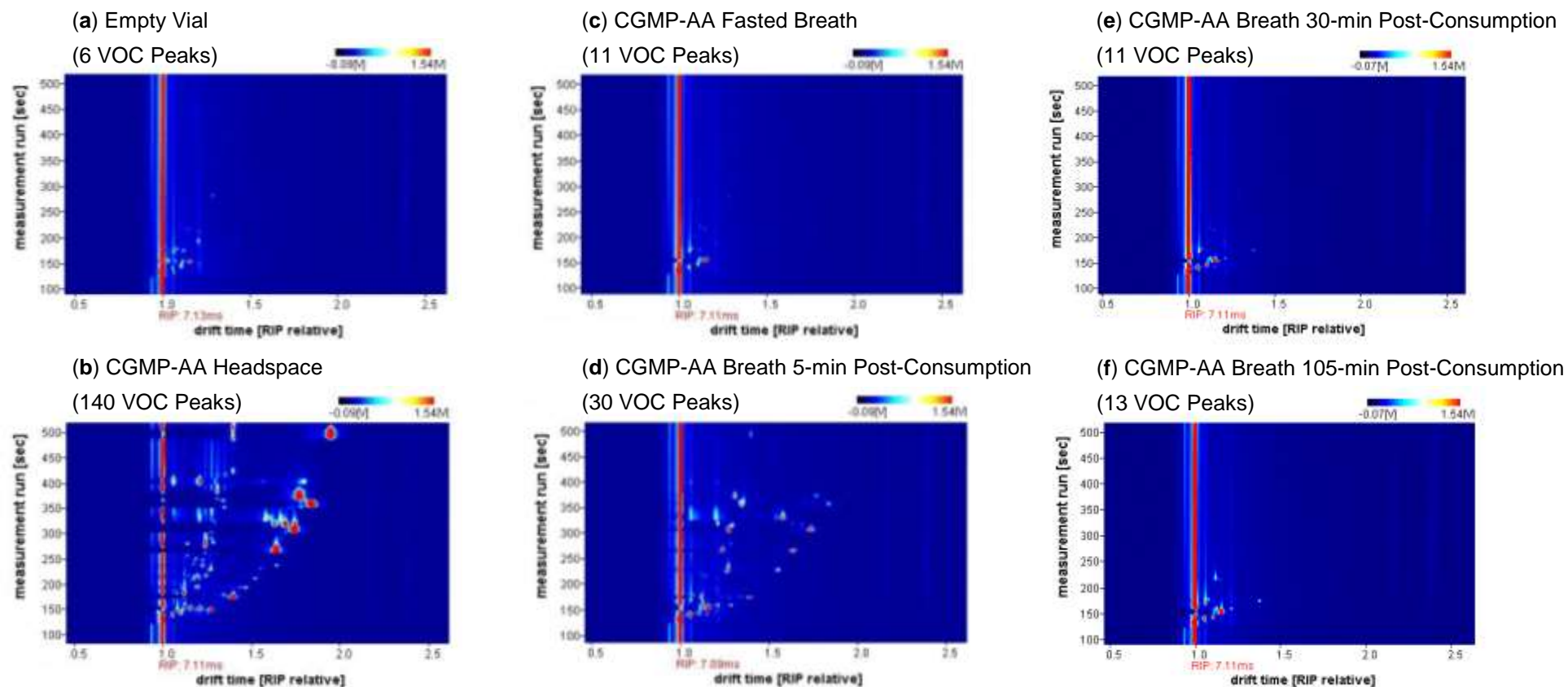
### 3.2. Headspace Gas and Exhaled Breath Outputs

To demonstrate that the BreathSpec GC-IMS could measure VOCs from the PS, analysis was conducted of the headspace gas generated from heating up a small portion (5mL) to 40°C (DB-2D Dri-Block, Techne, Stone, UK) in a 20mL glass vial, for 10 minutes. To illustrate the change in the GC-IMS output, an empty vial was also analysed.

For comparison, the exhaled breath of 1 subject with PKU was analysed, 5 minutes after consuming the PS. To demonstrate the change in the GC-IMS output, the exhaled breath of the same subject was analysed in a fasted state, 30 minutes after consuming the PS, and 105 minutes after consuming the PS. The results for L-AA and CGMP-AA are shown in Figures 9 and 10, respectively.



**Figure 9.** L-AA: (a) Empty vial; (b) L-AA headspace gas; (c) Subject with PKU fasted exhaled breath; (d) Subject with PKU exhaled breath 5-min post-consumption of L-AA; (e) Subject with PKU exhaled breath 30-min post-consumption of L-AA; (f) Subject with PKU exhaled breath 105-min post-consumption of L-AA.

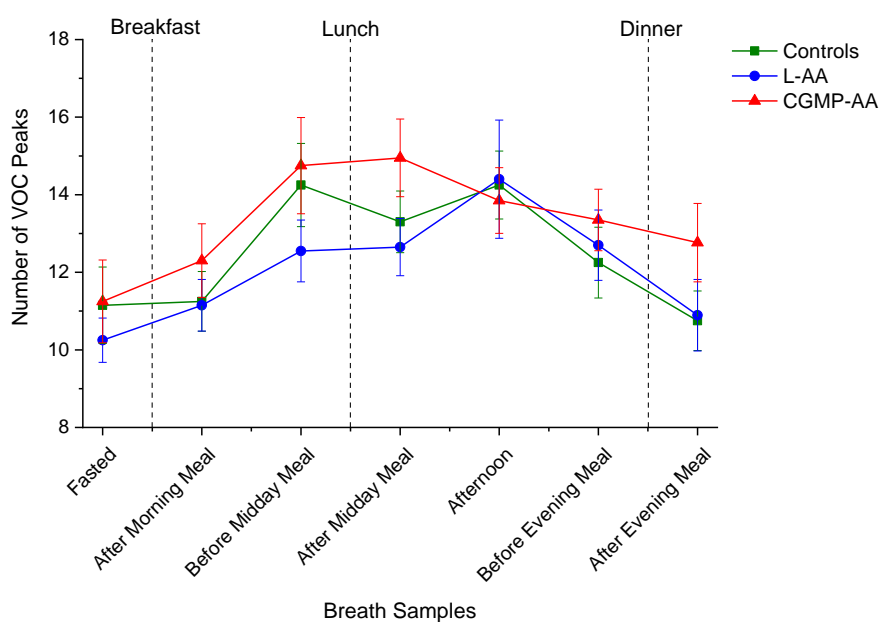


**Figure 10.** CGMP-AA: (a) Empty vial; (b) CGMP-AA headspace gas; (c) Subject with PKU fasted exhaled breath; (d) Subject with PKU exhaled breath post-consumption of CGMP-AA; (e) Subject with PKU exhaled breath 30-min post-consumption of CGMP-AA; (f) Subject with PKU exhaled breath 105-min post-consumption of CGMP-AA.

The VOC analysis program detected >75 VOC peaks in the headspace gas of supplement L-AA, compared to 25 in exhaled breath from a subject with PKU, 5 minutes post-consumption of PS. Supplement CGMP-AA had >140 VOC peaks in headspace gas and 30 VOC peaks 5 minutes post-consumption of PS. By comparison, there were 9 and 11 VOC peaks in the fasted breath samples of the same subject, respectively. The number of VOC peaks in samples collected 30- and 105-minutes post-consumption of either PS (11-16 VOC peaks) are significantly lower than in samples collected 5 minutes post-consumption. The extracted VOC peak locations from the PS established a strong alignment between VOCs found in the headspace gas and exhaled breath, shortly after consumption.

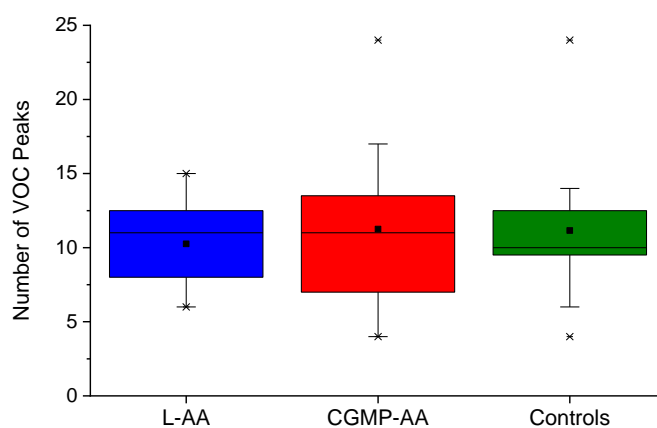
### *3.3. Longitudinal Breath Samples*

Figure 11 shows the longitudinal trends of the number of VOC peaks in the exhaled breath samples of controls, and subjects with PKU consuming L-AA and CGMP-AA, across all 7 sampling intervals. The plot represents mean values, with standard error whiskers.



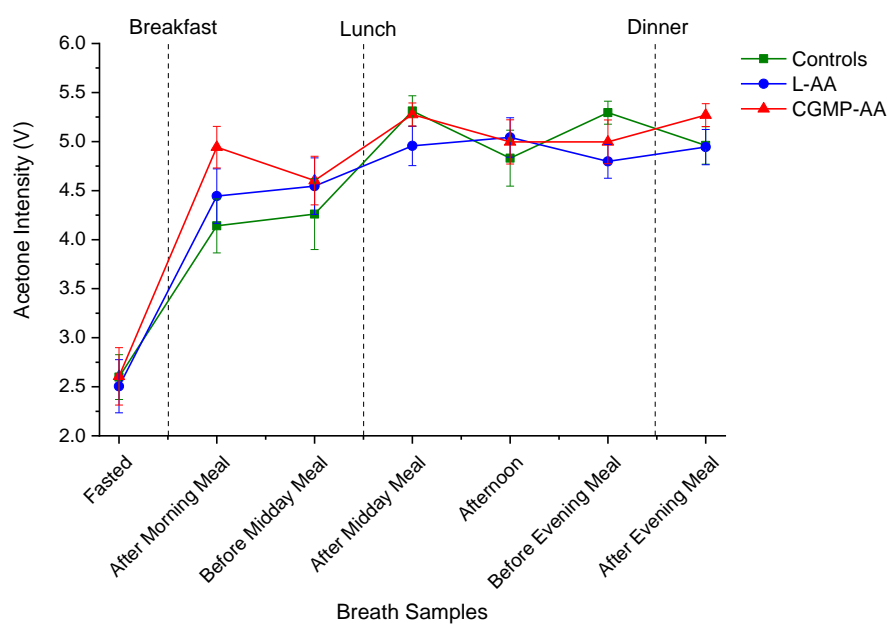
**Figure 11.** Number of VOC peaks by intervention groups.

Closer examination of the fasted sampling interval demonstrated that the number of VOC peaks for all groups start at similar levels. A box plot of the number of VOC peaks for the fasted samples is given in Figure 12. This demonstrated that the average number of VOC peaks in controls was slightly lower than L-AA and CGMP-AA, but not significantly different. CGMP-AA was associated with the greatest variance.



**Figure 12.** Box plot for number of VOC peaks from fasted sampling interval.

In addition to monitoring the number of VOCs peaks, it was possible to track the intensity of specific VOCs, such as acetone, dimethyl sulphide and 2-butanone (Figures 13-15, respectively). Dimethyl sulphide was of particular interest, since it is a compound typically associated with halitosis.



**Figure 13.** Acetone intensity changes by intervention groups.



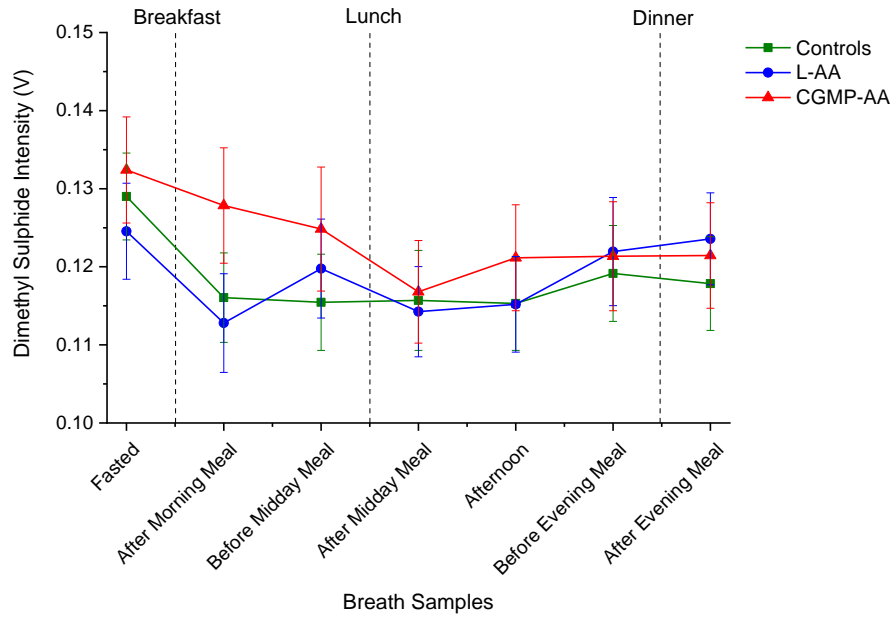


Figure 14. Dimethyl sulphide intensity changes by intervention groups.

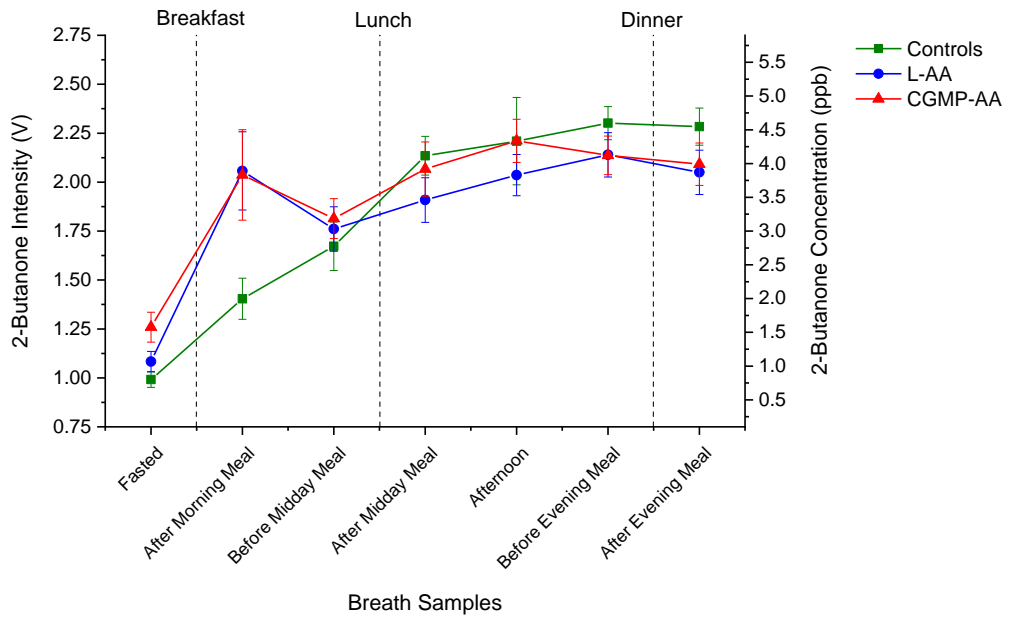
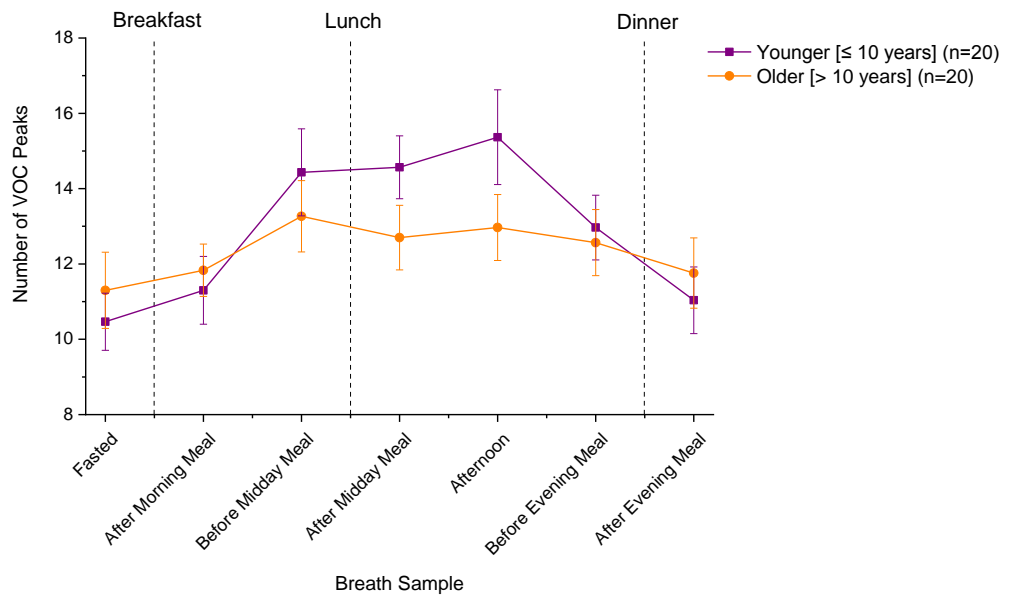


Figure 15. 2-butanone intensity/concentration changes by intervention groups.

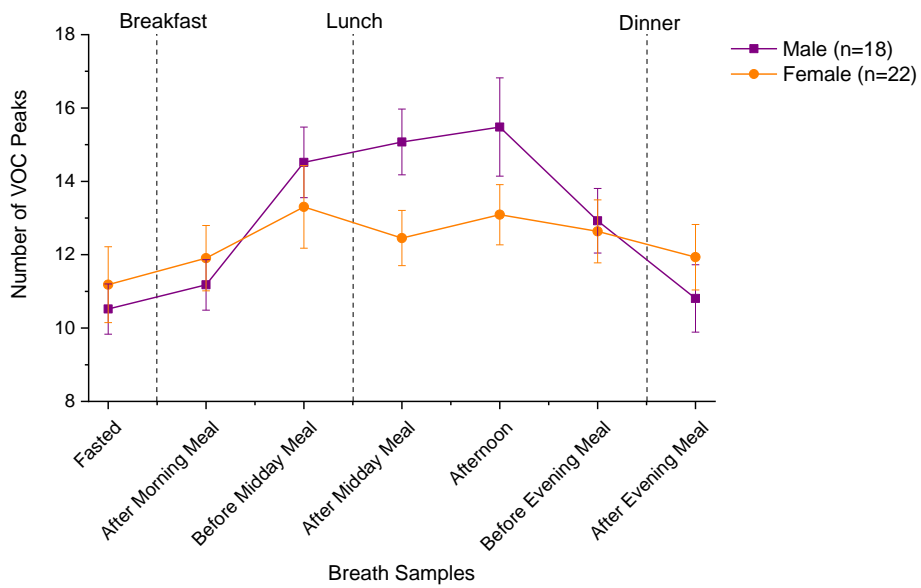
The intensity trends shown in Figures 13 and 14 indicate that acetone and dimethyl sulphide exhibit similar trends across all groups. The intensity trends of 2-butanone (Figure 15) show some differences between subjects with PKU and controls. The post-morning meal (post-fasted) sample is associated with a sharp increase in 2-butanone for subjects with PKU (both L-AA and CGMP-AA). To better characterise this behaviour, the quantification procedure described in Section 2.6 was completed for 2-butanone to convert voltage signals from the GC-IMS output into ppb concentrations. The quantification model indicates that the concentration of 2-butanone in the post-fasted samples for subjects with PKU was around 3.9 ppb, compared to 2.0 ppb for controls. Subsequent samples are comparable in intensity (around 3.0 ppb) for all groups and follow similar increasing trends for the rest of the day.

#### *3.4. Confounding Factors*

The analysis in Figure 11 was repeated after categorising the subjects based on confounding factor groups, instead of intervention groups, e.g. younger vs older subjects, instead of controls vs L-AA vs CGMP-AA. The longitudinal trends of the number of VOC peaks in exhaled breath samples, based on age and gender, are shown in Figures 16 and 17, respectively.



**Figure 16.** Number of VOC peaks: younger vs older (controls and subjects with PKU).



**Figure 17.** Number of VOC peaks: male vs female (controls and subjects with PKU).

## 4 Discussion

The results of this study provide an important insight into how subjects with PKU perceive their breath after consuming PS. The questionnaire results suggest that control subjects worried little or not at all about their breath, but subjects with PKU were concerned about breath odour and associated this with PS. However, the 2 types of PS were not perceived in the same way, since 95% of subjects with PKU stated that they notice their breath more with L-AA than CGMP-AA. Unlike CGMP-AA, L-AA was also associated with others noticing breath malodours. Study results from the questionnaire suggests that the effects of L-AA and CGMP-AA on exhaled breath are likely to be different, immediately post-consumption. The effects of PS consumption were analysed by locating and extracting VOC information from the headspace gas and breath samples for both PS.

Analysis of headspace gas from L-AA and CGMP-AA was associated with over 75 and 140 VOC peaks, respectively; while only 25-30 VOC peaks were detected in the exhaled breath from subjects with PKU, 5 minutes after PS consumption. VOCs in PS thus appear in reduced numbers and lower concentrations in exhaled breath, shortly post-consumption. CGMP-AA is likely associated with greater numbers of VOC peaks, due to additional flavourings and sweeteners, compared with L-AA. This is reflected in the questionnaire results (Figure 3) regarding PS taste, as 50% of subjects with PKU stated they dislike or strongly dislike the taste of L-AA, compared to 35% with CGMP-AA. Subjectively, CGMP-AA is described to have a sweet smell (like ice cream) and taste less like traditional L-AA. Experiments measuring breath odour post-PS administration demonstrate that there is an immediate

and objective change to the breath content of subjects with PKU, 5 minutes after consuming supplements. The strong alignment of VOC peaks in both the headspace gas and breath suggest that for a short duration, the subject's breath is perceived similarly to the PS. However, the immediacy of this intense taste in the mouth is likely to lead to subjective differences, since perceived taste and measurable difference in exhaled breath are not the same.

Longitudinal breath testing results show that the mean number of VOC peaks in exhaled breath increase after the fasted breath sample, in all 3 groups. Likewise, the initial number of VOC peaks in fasted breath are similar. This indicates that there is no fundamental difference in breath composition between controls and subjects on a low-protein diet, supplemented with PS – detectable using our analytical platform. Common breath compounds, such as acetone, 2-propanol, hexanal, 2-butanone and 1-propanol were observed in fasted samples, including the control group.

The longitudinal trends of control and L-AA groups are characterised by 2 increases in the number of measured VOC peaks; specifically, during the 'before midday meal' and 'afternoon' samples. In the CGMP-AA group, the number of VOC peaks were observed to increase until the 'after midday meal' sample and decrease thereafter. The increase in exhaled VOCs following meals, and throughout the day generally, is partially associated with the intake of food and drinks. For example, limonene was observed in postprandial samples from both control and subjects with PKU, especially those who drank orange cordial. It is well known that some dietary products can leave a residual taste and cause transient breath odours (e.g. onions, garlic and spicy foods) [9]. The increase in the number of exhaled VOCs throughout the day is also

linked to day-time metabolic processes and the microbiome in the gastrointestinal tract, which generates additional VOCs during the metabolism of nutrients [20,21]. Subjects with PKU are not observed to have significantly more, or different types of VOCs in exhaled breath, when sampled 30 minutes after consumption of either PS.

The headspace gas analysis enabled identification of the locations of VOC peaks relating to the PS. The locations of these VOCs were tracked across the longitudinal samples in Figure 11. However, none of the VOC peaks directly related to those in the headspace gas were detected in any breath samples from subjects with PKU, for either PS. These findings suggest that the effects of the PS disappear in less than 30 minutes after consumption. This strongly indicates that breath malodour from the supplements is reduced, or entirely removed, by the intervention of food and drinks. Comparing samples from subjects with PKU to those from controls, we did not observe any additional VOC peaks, which could be linked to by-products of pharmacokinetic effects and metabolic breakdown of the supplement.

Analysis of possible confounding factors indicate that differences in the number and trends of VOCs throughout the day are more likely related to factors of age and gender, rather than PKU-associated effects, since the differences in the results from confounding factor groups are greater than in the intervention groups. This provides further evidence to suggest that the composition of breath between subject with PKU and controls is not fundamentally different. The trends in Figure 16 and Figure 17 are quite similar, which could be related to more females being recruited (22F: 18M) and the mean age of female subjects being marginally higher than males; 11.0

years (standard deviation: 2.6) and 9.8 years (standard deviation 2.2), respectively.

Intensity monitoring of specific compounds, such as acetone and dimethyl sulphide, demonstrate similar trends across all groups. There is, however, an interesting difference in the concentration of 2-butanone between subjects with PKU and controls in the post-morning meal sample. While controls are associated with a gradual increase throughout the day, both L-AA and CGMP-AA are associated with a sharp rise after their morning meal (around 3.9 ppb for subjects with PKU, for both PS, compared to 2.0 ppb for controls). These levels drop subsequently and maintain similar trends to controls for the rest of the day. This could be a metabolic response to the PS and requires further investigation. Similar peaks are not observed following the midday and evening meal PS, which suggests that this only occurs post-fasting. The quantification results for 2-butanone can be used for comparison with other studies or serve as a reference for future studies. Regarding dimethyl sulphide, the intensity trend is shown to decrease throughout the day, after the fasted sample. It is possible that this compound is associated with malodorous breath upon awakening, known as 'morning breath'. These unpleasant odours are VSCs, which are produced by bacteria during sleep, as a result of decreased salivation (i.e. dry mouth) [22,23]. This effect usually lasts a few hours and can be reduced or relieved through oral hygiene (brushing of teeth) and/or consumption of breakfast. Findings from this study suggests that dimethyl sulphide concentrations in exhaled breath are similar in subjects with or without PKU, for both PS.

There are some limitations associated with this study. For example, the scope of this study focuses only on the PS and not on blood Phe levels. In future studies, blood Phe levels could be monitored in conjunction with breath measurements, to determine any links between exhaled breath composition and Phe levels. It has also been suggested that Phe could be converted to phenyl ketones, which may contribute to malodour [24]. This is also worth investigating.

A further limitation of this study is that targeted monitoring of sulphur-based compounds using dedicated VSC-monitors may provide insight beyond the generally screening approach taken here using GC-IMS technology. Another drawback is that only a small number of subjects (n=3) provided breath samples, 5 minutes after consuming the PS. While we were able to demonstrate that it was possible to measure the immediate effect of consuming L-AA and CGMP-AA PS, longitudinal breath testing using GC-IMS could not establish any significant and measurable changes between controls and subjects with PKU, for either PS.

These findings strongly suggest that the reported effects of breath malodour are transient in nature and can be reduced, or completely removed, by the intervention of food and drinks. Further work should include an investigation of intervention methods to determine which are most effective at reducing or completely removing temporary breath malodour, following the consumption of L-AA or CGMP-AA. For example, a simple intervention study comparing the composition of exhaled breath of subjects with PKU who rinse their mouth with water post-consumption of PS, to those who do not.



## 5 Conclusions

In this longitudinal cross-over pilot study, exhaled VOCs were measured using GC-IMS technology when subjects took both L-AA and CGMP-AA protein substitutes. Headspace gas from L-AA and CGMP-AA were associated with over 75 and 140 VOC peaks, respectively; while only 25-30 VOC peaks were detected in the exhaled breath from subjects with PKU, 5 minutes after consuming the PS. The strong alignment of VOC peaks in both headspace gas and breath suggest that consuming PS has an immediate impact on breath composition. Fasted L-AA and CGMP-AA breath samples contained a similar number of VOC peaks (10-12) as controls. Monitoring of specific VOCs indicate that acetone and dimethyl sulphide demonstrate similar trends across groups. Post-fasted samples from subjects with PKU, for both PS, were associated with a sharp increase in 2-butanone concentration, which was not observed in the control group. This should be further investigated, as it could be a metabolic response relating to the post-fasted consumption of PS. Intensity tracking of dimethyl sulphide suggests a subsiding trend of 'morning breath' across all groups. Longitudinal breath testing showed that there were no significant differences in the number and types of exhaled VOCs compared to controls or between PS (12-18 VOC peaks). PS are thus shown to have a transient effect on exhaled breath. This indicates that any PS-related unpleasant breath odours are eliminated by food or drinks.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## 6 Appendix A – Staggered Breath Sampling Time

Time	Subject 1	Subject 2	Subject 3	Subject 4
08:00	Room Air Sample 1			
08:15	Arrive	Arrive	Arrive	Arrive
08:30	Fasted Sample	Break	Break	Break
08:45	Morning Meal	Fasted Sample	Break	Break
09:00		Morning Meal	Fasted Sample	Fasted Sample
09:15			Morning Meal	
09:30	After Morning Meal	Morning Meal	Morning Meal	Morning Meal
09:45	Break		After Morning Meal	After Morning Meal
10:00		Break	Break	
10:15		Break	Break	Break
10:30 – 10:45				Break
11:00	Room Air Sample 2			
11:15 – 11:30	Break	Break	Break	Break
11:45	Before Midday Meal	Break	Break	Break
12:00	Midday Meal	Before Midday Meal	Break	Break
12:15		Midday Meal	Before Midday Meal	
12:30			Midday Meal	Before Midday Meal
12:45	After Midday Meal	Midday Meal	Midday Meal	Before Midday Meal
13:00	Break		After Midday Meal	Midday Meal
13:15		Break	After Midday Meal	After Midday Meal
13:30		Break	Break	
13:45	Afternoon Sample	Break	Break	Break
14:00			Afternoon Sample	
14:15		Break	Afternoon Sample	Break
14:30	Break		Afternoon Sample	
14:45	Break		Break	Break
15:00				Break
15:15	Room Air Sample 3			
15:30 – 16:15	Break	Break	Break	Break
16:30	Before Evening Meal	Break	Break	Break
16:45	Evening Meal	Before Evening Meal	Break	Break
17:00		Evening Meal	Before Evening Meal	
17:15			Evening Meal	Before Evening Meal
17:30	After Evening Meal	Evening Meal	Evening Meal	Evening Meal
17:45	Finished		After Evening Meal	After Evening Meal
18:00		Finished	Finished	
18:15		Finished	Finished	Finished
18:30				Finished
18:45	Room Air Sample 4			

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