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PAPER

Impact of food intake on *in vivo* VOC concentrations in exhaled breath assessed in a caprine animal model

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Abstract

Physiological processes within the body may change emitted volatile organic compound (VOC) composition, and may therefore cause confounding biological background variability in breath gas analyses. To evaluate the effect of food intake on VOC concentration patterns in exhaled breath, this study assessed the variability of VOC concentrations due to food intake in a standardized caprine animal model. VOCs in (i) alveolar breath gas samples of nine clinically healthy goats and (ii) room air samples were collected and pre-concentrated before morning feeding and repeatedly after (+60 min, +150 min, +240 min) using needle trap microextraction (NTME). Analysis of VOCs was performed by gas chromatography and mass spectrometry (GC-MS). Only VOCs with significantly higher concentrations in breath gas samples compared to room air samples were taken into consideration. Six VOCs that belonged to the chemical classes of hydrocarbons and alcohols were identified presenting significantly different concentrations before and after feeding. Selected hydrocarbons showed a concentration pattern that was characterized by an initial increase 60 min after food intake, and a subsequent gradual decrease. Results emphasize consideration of physiological effects on exhaled VOC concentrations due to food intake with respect to standardized protocols of sample collection and critical evaluation of results.

1. Introduction

Volatile organic compounds (VOCs) emitted in exhaled breath reflect the exhaled part of the metabolome present in an organism. Analysis of VOCs in exhaled breath potentially enables diagnosis and monitoring of metabolic and pathophysiological processes in a non-invasive way when volatile breath biomarkers can be related to conditions of health or disease. VOC analysis is a promising tool for diagnosis of various diseases in veterinary medicine, too. Within the last decade, a number of *in vivo* studies were carried out in domestic animals and large animal models, particularly in ruminants, to identify volatile biomarkers attributed to different diseases (Spinhirne *et al* 2004, Fend *et al* 2005, Knobloch *et al* 2009, Purkhart *et al* 2011, Peled *et al* 2012, Bayn *et al* 2013, Ellis *et al* 2014, Bergmann *et al* 2015, Cho *et al* 2015). Independent on the mammalian species, the majority of studies report differences between 'healthy' and 'diseased' conditions of well-defined groups taking many aspects of methodological standardization into account.

Despite efforts to implement a high degree of standardization for collection and analysis of exhaled VOCs, a complementary knowledge about biological variability of VOC composition is necessary if unknown samples should be classified. This aspect, however, has been hardly addressed in literature so far. Although a high inter-individual variability has been reported for composition of VOCs in breath gas studies in healthy humans (Phillips *et al* 1999, Turner *et al* 2006, Ligor *et al* 2008, Kischkel *et al* 2010, Mochalski *et al* 2013),

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systematic studies evaluating sources of biological variability are rare and comparative studies including a variety of mammalian species are lacking.

Endogenous and exogenous factors can have an impact on exhaled VOCs composition and require consideration as confounding factors impeding reliable identification of breath biomarkers. Beside VOCs generated endogenously, VOCs with exogenous origin can also be excreted via the lungs after previous uptake from the environment, e.g. via inhalation or ingestion, and distribution within the body (Beauchamp 2011). Volatile metabolites are the result of diverse intra- and extracellular biochemical processes within the body. Little is known about the capacity of internal physiological processes such as digestion or metabolism to influence the outcome of breath gas assessments. Initial findings confirmed an impact of the factors age (Taucher *et al* 1997, Spanel *et al* 2007) and diet (Taucher *et al* 1996, Lindinger *et al* 1997, Smith *et al* 1999, Spanel *et al* 2011, Baranska *et al* 2013, Kistler *et al* 2014) in humans on resulting VOC signatures detected in exhaled breath.

A previous study in juvenile healthy goats indicated high variability in VOC profiles due to somatic growth and type of diet (Fischer *et al* 2015). The present study exploiting the same large animal model focused on evaluating physiological effects of intake of plant-based concentrates (morning feeding) on the variability of VOC concentrations assessed in exhaled breath. Thus, VOC concentration pattern were assessed in the alveolar portion of exhaled breath before and until 4 h after food intake. To exclude confounding influences of exogenous inhaled compounds on exhaled VOC concentrations, environmental VOCs present in room air were assessed in parallel to exhaled breath samples.

This large animal model was chosen for two reasons. First, it completes the current knowledge of volatile biomarkers assessed in a caprine model of *Mycobacteria* infection (Purkhart *et al* 2011, Bergmann *et al* 2015). Second, defined conditions with respect to breed, sex, age, nutrition regime, husbandry, and indoor environment ensured standardized experimental conditions that can never be reached in a heterogeneous population where a similar study would require a much higher sample size to reach statistically significance.

Results of the study emphasize that not only the methodological requirements of standardized and controlled collection and analysis of VOCs are important. Regardless of the mammalian species, methodological standardization needs to be completed by defining and standardizing biological conditions; i.e. at which time points and under which circumstances exhaled breath is collected for diagnostic purposes.

2. Animals, materials, and methods

2.1. Legislation and ethical approval

This study was carried out in strict accordance with the German Animal Welfare Act, and animals were housed in conformity with the guidelines for animal welfare set forth by the European Community. The study protocol was approved by the Committee for the Ethics of Animal Experiments and the Protection of Animals of the State of Thuringia, Germany (Registration No. 04-002/12). All experiments were conducted under supervision of the authorized institutional Agent for Animal Protection. Every effort was made to minimize discomfort and suffering throughout the duration of the study.

2.2. Animals, housing conditions, and health status

Nine male goats (breed: 'Thüringer Wald Ziege') were involved in this study. The animals originated from one farm, were colostrum-fed after birth, and were conventionally raised for the first few days. Aged nine to 17 d and weighing 4.6 to 8.0 kg (6.0 ± 1.0 kg; mean ± SD), they were purchased, and immediately transferred to the Federal Research Institute for Animal Health (Friedrich-Loeffler-Institut, Jena, Germany). Here, they were kept in-house, in one room, and under standardized ambient conditions (room climate: 20.9 ± 1.9 °C, 49 ± 6% relative humidity; mean ± SD) for about one year. They were bedded on straw that was completely renewed every two weeks.

To prevent the influence of sex hormones on the subsequent studies, each goat was castrated at the age of two months according to good veterinary practice. Routine microbiological screening and regular prophylactic treatment against endo- and ectoparasites ensured the absence of relevant infections. Confirmed by daily clinical examination (general behaviour, appetite, rectal temperature, etc.), all animals were apparently clinically healthy throughout the entire study. Because goats also served as healthy controls in another study, necropsies at the age of about 13 months were mandatory. Results of the pathological examination confirmed the physical health of all nine goats included.

2.3. Study design

This study was started when goats were eleven months of age (body weight: 38.3 ± 4.6 kg; mean ± SD). To assess reproducibility of results, each goat was examined twice with a 4-week interval between the two measurement campaigns. At the second measurement campaign, goats weighted 40.3 ± 4.9 kg (mean ± SD).

Nutrition regime was designed due to requirements of ruminant animals, i.e. water and roughage in the form of hay were available *ad libitum*. Daily morning feeding (food intake), based on a concentrated mixture of plants and plant products, i.e. barley, rape seed extraction meal, sunflower seed extraction meal, wheat bran, dried sugar beet pulp, and sugar beet molasses (Milchleistungsfutter, Landhandelsgesellschaft eG, Schmölln, Germany), was provided in portions of 200 g per animal at about 7:30 a.m. For supplementation of minerals, lickstones were freely available in form of mineral blocks (Blattimin, Höveler Spezialfutterwerke GmbH & Co. KG, Dormagen, Germany).

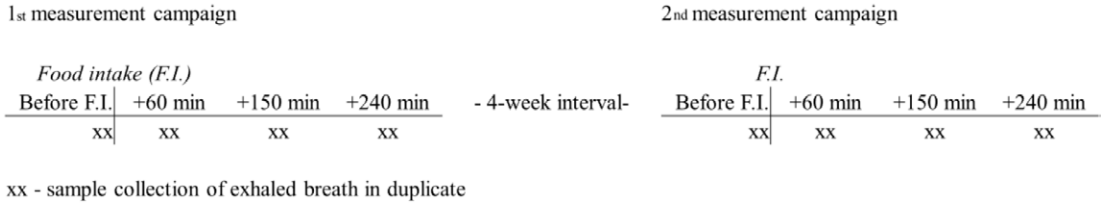

Figure 1. Schematic representation of the experimental design of the collection of VOCs from exhaled breath in each single animal.

Table 1. Schedule for collection of VOCs from exhaled breath of healthy goats (n = 9) and from room air at one measurement campaign.

	Day 1		Day 2		Day 3	
Schedule	room air	animals	room air	animals	room air	animals
Immediately before food intake[a]	n =1	n = 3	n = 1	n = 3	n = 1	n = 3
60 min after food intake	n = 1	n = 3	n = 1	n = 3	n = 1	n = 3
150 min after food intake	n = 1	n = 3	n = 1	n = 3	n = 1	n = 3
240 min after food intake	n = 1	n = 3	n = 1	n = 3	n = 1	n = 3

[a] Morning feeding of concentrated feed at about 7:30 a.m.

At the day of testing, VOCs were collected in three animals once immediately before and repeatedly after food intake (+60 min, +150 min, +240 min). One room air sample was collected per time point immediately before alveolar breath samples were taken. Afterwards, breath gas samples of each goat were collected in duplicate (details of sample collection see 2.4.), and respiratory rate and as well as any occurrence of eructation were recorded in parallel. Figure 1 illustrates the experimental design for one individual animal.

Three consecutive days of sample collections ensured the inclusion of all nine animals (table 1). The order of sampling of the individuals remained the same throughout the day. In a second measurement campaign 4 weeks later, the protocol was repeated in a randomized way, i.e. with a modified order of the goats. In total, 24 room air samples and 144 breath gas samples (16 samples per animal) were collected from the nine apparently clinically healthy goats.

2.4. Collection and processing of breath gas and room air samples

An automated alveolar sampling device (PAS Technology Deutschland GmbH, Magdala, Germany) combining needle-trap microextraction (NTME) and mainstream capnometry was used. This system was described previously for sample collection in humans (Trefz *et al* 2013b) and was recently adapted successfully for collection of breath gas samples in spontaneously breathing conscious goats by means of a tightly fitting face mask (Bergmann *et al* 2015, Fischer *et al* 2015).

Sampling of predominantly alveolar gas was realized by connecting the sampling device to a CO_2 sensor (Capnostat 5 CO_2 sensor, Hamilton Medical AG, Rhazüns, Switzerland) and a fast-responding, CO_2-triggered valve regulating the flow through the connected needle trap device (NTD). The flow rate during sampling was 21.5 ± 1.9 mL min^{-1} (mean ± SD) and total sample volume per goat per time point was set at 50 mL. Samples of the ambient air inside the animals' stable (room air samples; 50 mL each; flow rate 27.8 ± 2.69 mL min^{-1}; mean ± SD) were collected at head level of the animals using the automated NTME sampling device without CO_2-controlled opening of the valve.

The triple-bed NTDs were packed with divinylbenzene (DVB, 80/100 mesh, 1 cm), Carbopack X (60/80 mesh, 1 cm), and Carboxen 1000 (60/80 mesh, 1 cm). The NTDs were conditioned in a heating device (PAS Technology Deutschland GmbH, Magdala, Germany) at 250 °C for at least 12 h under permanent nitrogen flow (1.5 bar) before first use, and re-conditioned at 250 °C for 30 min before they were applied for the preconcentration of the samples. Each NTD was sealed by a Teflon cap (Shinwa LTD., Japan/PAS Technology Deutschland GmbH, Magdala, Germany) before and immediately after collecting a gaseous sample.

2.5. Identification and quantification of potential marker substances

VOC analyses were performed using gas chromatography–mass spectrometry (GC–MS). VOCs desorbed from the NTDs were separated by a gas chromatograph (Agilent 7890A) and detected by a mass selective detector (Agilent 5975C inert XL MSD), as previously described in Trefz *et al* (2013b).

Volatile compounds were tentatively identified by means of mass spectral library search (NIST 2005 Gatesburg, PA, USA). Then the analysis of pure reference substances verified the GC retention times and mass spectra of all selected marker substances for identification and quantification.

Humidified standards were prepared by means of a liquid calibration unit (LCU, Ionicon Analytik GmbH, Innsbruck, Austria). Eight concentration levels in a range from 1 ppbv to 1000 ppbv were analysed in

triplicate as previously described for NTME calibration (Trefz *et al* 2013a, Bergmann *et al* 2015). Additionally, 10 blank samples were measured and signal areas of baseline were integrated. The resulting mean values (m) and standard deviations (SD) were used for calculation of limits of detection (LOD = m + 3*SD) and limits of quantification (LOQ = m + 10*SD). Detected concentrations below the LOD were set to zero. Table 2 provides substance identities as confirmed through retention time and mass spectra as well as the limits of quantification (LOQs) of reference substances.

Pentane, hexane and, isoprene were purchased from Fluka/Sigma-Aldrich (Steinheim, Germany). 3-Methylpentane, cis-1, 3-pentadiene, and 2-ethyl-1-hexanol were bought from TCI Europe N.V. (Zwijndrecht, Belgium). Acetone was acquired from Ionicon Analytik GmbH (Innsbruck, Austria).

2.6. Selection of marker substances

Volatile substances in exhaled breath potentially affected by food intake were selected according to the following criteria:

1. Concentrations of VOCs detected in room air samples were significantly lower than in exhaled breath (Mann-Whitney *U*-Test).
2. There was a significant effect on concentrations of VOCs caused by different time points of collection of breath gas samples before and after the morning feeding (Multifactorial ANOVA).

In addition, acetone was included because it had been reported as an important marker for metabolic processes *in vivo* depending on nutrition and food intake (Smith *et al* 1999, Spanel *et al* 2011).

2.7. Statistical analysis

All 24 room air samples and 139 breath gas samples were included in statistical analyses of data (five breath gas samples were not evaluable due to methodological reasons and had to be considered as 'missing values'). SPSS (Version 19.0, IBM Corporation, NY, USA), R (version 2.15.2; 2012-10-26, R Development Core Team) with package agricolae (version 1.1–3; 2012-12-21), Excel (Microsoft Excel, Excel 2010, Microsoft Corporation, USA), and Statgraphics Centurion XVI (Version 16.1.18, Statistical Graphics Corporation) were used. Normally distributed data is provided as mean ± standard deviation (SD) while data without normal or with unknown distribution is provided as median and percentiles (25–75%). In box-and-whisker plots, outlier values (circles) were more than 1.5–3 times the length of a box away from the median. Extreme values (asterisks) are data beyond the three interquartile ranges.

The Mann–Whitney *U*-Test (exact test) was used to identify significant differences between two groups of unpaired data. A multifactorial analysis of variance (ANOVA) was performed for the global test of whether there is an effect caused by several factors on VOC concentrations detected in exhaled breath. An additional factor 'goat' was incorporated in the model to consider individual bindings of the data. Values of $P < 0.05$ were considered as statistically significant. For descriptive purposes, Tukey's HSD post hoc tests were applied for every feature to discern pairwise differences between specific time points. In Tukey's HSD tests, P-values were adjusted according to Bonferroni which guarantees the family-wise confidence level of 0.95. Significant differences are indicated using different letters starting with 'a' for the group with the highest responses.

Table 2. Analytical features of reference substances used for quantification of selected VOCs.

VOCs	Retention time (min)	R^2	LOQ (ppbV)
Pentane	6.18	0.991	10.34
Isoprene	6.78	0.999	3.92
cis-1, 3-Pentadiene	7.36	0.998	1.50
Acetone	7.49	0.991	10.92
3-Methylpentane	8.71	0.970	20.61
Hexane	9.26	0.998	6.42
2-Ethyl-1-Hexanol	23.83	0.989	0.16

VOC—volatile organic compound; R^2—coefficient of determination; LOQ—limit of quantification; ppbV—parts per billion by volume.

3. Results

Six substances that fulfilled the selection criteria (see 2.6.) could be identified and quantified by analysis and calibration of pure reference substances. These marker substances belonged to the classes of saturated hydrocarbons (pentane, 3-methylpentane, and hexane) and unsaturated hydrocarbons (isoprene and cis-1, 3-pentadiene), as well as alcohols (2-ethyl-1-hexanol). Acetone was added to the selected markers (see 2.6). Absolute concentrations of selected substances assessed in both exhaled breath and room air are given in table 3.

Before food intake in the morning, concentrations of 3-methylpentane, hexane, and cis-1, 3-pentadiene were not detectable in most of the breath gas samples (table 4). After feeding a mixture of plant materials, concentrations of both saturated and unsaturated hydrocarbons increased significantly. Peak concentrations were observed 60 min after food intake (figure 2(a)–(e)). In contrast, concentrations of 2-ethyl-1-hexanol decreased significantly after food intake (figure 2(f)). The concentration of acetone in exhaled breath expressed a higher variability after food intake, but did not differ significantly between different time points (figure 2(g)).

In addition to food intake, the effects of methodological (intra-individual measurements in duplicate, two measurement campaigns in a 4-week interval) and biological factors (respiratory rate, eructation) on

Table 3. Comparison of concentrations of selected VOCs in exhaled breath of healthy goats and in room air.

VOCs	VOC concentrations (ppbV) median (25–75% percentiles) Exhaled Breath $n = 139$	Room air $n = 24$	Mann-Whitney *U*-Test *P*-value
Pentane	19.20 (11.76–46.56)	0.00 (0.00–8.99)	<0.001
3-Methylpentane	21.75 (0.00–24.72)	10.47 (0.00–21.15)	<0.001
Hexane	9.80 (0.00–15.79)	0.00 (0.00–0.00)	<0.001
Isoprene	5.13 (4.30–7.09)	0.00 (0.00–3.98)	<0.001
cis-1, 3-Pentadiene	0.00 (0.00–1.52)	0.00 (0.00–0.00)	<0.001
2-Ethyl-1-Hexanol	0.39 (0.00–1.08)	0.00 (0.00–0.00)	<0.001
Acetone	139.60 (95.17–213.12)	10.43 (6.89–24.07)	<0.001

VOC—volatile organic compound; ppbV—parts per billion by volume; n—number of samples.

exhaled concentrations of the selected VOCs were tested in a multifactorial ANOVA (table 5). Respiratory rate and eructation (observed only eight times while collecting 139 samples of exhaled breath) did not present any significant impact on results. A potential relationship between the exhaled concentrations of 2-ethyl-1-hexanol and the respiratory rates, as suggested by ANOVA results in table 5, could not be confirmed by analyses of regression. As expected, substance concentrations depended significantly on the individual goat.

As also shown in table 5, VOC-results of two consecutive breath samples were highly reproducible. The only exception indicated by ANOVA for 1, 3-pentadiene (table 5) could not be attributed to biological relevance by graphical evaluation. Taking the two measurement campaigns (4-week interval) into account, absolute concentrations of VOCs differed significantly. Nevertheless, the time trend related to food intake was comparable at the two campaigns (figure 3(a)–(g)).

4. Discussion

4.1. Validity and biological relevance of the model

In large animal studies, particularly in ruminants, efforts were made to use breath VOC analysis for detection of infectious diseases that are zoonotic or cause high economic losses such as tuberculosis (Fend *et al* 2005, Peled *et al* 2012, Ellis *et al* 2014, Cho *et al* 2015), paratuberculosis (Knobloch *et al* 2009, Purkhart *et al* 2011, Bergmann *et al* 2015), or brucellosis (Knobloch *et al* 2009, Bayn *et al* 2013). Although preliminary results are promising for diagnosing infections by VOC analyses in future, aspects of biological variability have hardly been addressed so far. The controlled and standardized caprine animal model exploited for this study provided good prerequisites to evaluate specific effects of food intake on exhaled VOCs while retaining biologic diversity. The animals included in this study represented a uniform healthy cohort with defined characteristics regarding breed, sex, body weight and age, nutrition regime and environmental influences. Thus, many of the confounding factors present in human studies could be eliminated from this model. It is not meant to replace analogue studies in human medicine. From a translational point of view, results do complete our general understanding to what extend VOC concentrations may change due to food intake, and which compounds are significantly related to pure plant-based food.

The special feature of the digestion physiology of goats is the development of a symbiotic relationship with microbes in their forestomach system. Due to microbial fermentation cellulose and other complex carbohydrates from fibrous plant material that cannot be digested by mammalian enzyme systems, are degraded by the gastrointestinal microbiota into volatile fatty acids (VFAs, e.g. acetate, propionate and butyrate) that are used as the primary source of energy in strictly vegetarian ruminants (Weimer 1992, Russell *et al* 2009, Metzler-Zebeli *et al* 2013). For reasons of animal welfare, the nutrition regime was designed to meet natural conditions of ruminants. Consequently, the animals were not deprived of water and hay. The aim of this study was not to adjust the metabolism to complete fasting (which would be not physiological in either species). Accepting regular metabolism as baseline, the effects of first food intake in the morning were of particular interest. Morning feeding consisted of a mixture of concentrated plants and plant products. Thus, the degradation of plant products was evaluated while no effects of food products of animal origin, i.e. milk or meat, were assessed in this study.

Methodologically, the combination of needle trap microextraction (NTME) and an automated alveolar sampling device enabled standardized, automated CO_2-controlled collection and pre-concentration of VOCs in alveolar gas samples (Trefz *et al* 2013b). Recently, the application was adapted successfully to sample collection for breath gas analysis in goats (Bergmann *et al* 2015, Fischer *et al* 2015).

To minimize confounding effects from inhaled substance concentrations on results of breath gas analysis, significantly lower concentrations in room air samples compared to exhaled breath concentrations were a prerequisite for the selection of marker substances in our study.

Table 4. Concentrations of VOCs assessed in exhaled breath of healthy goats before and after food intake in the morning.

	before food intake			60 min after food intake			150 min after food intake			240 min after food intake		
	$n_{tot} = 35$			$n_{tot} = 34$			$n_{tot} = 36$			$n_{tot} = 34$		
VOCs	n_1	n_2	median (25–75%)	n_1	n_2	median (25–75%)	n_1	n_2	median (25–75%)	n_1	n_2	median (25–75%)
Pentane	8	27	13.37 (10.95–18.47)	3	31	47.49 (24.35–76.79)	2	34	30.22 (16.23–49.42)	4	30	19.54 (13.36–33.89)
3-Methylpentane	29	6	20.97 (20.94–21.02)	1	33	28.79 (25.59–31.39)	2	34	23.57 (21.87–24.75)	6	28	21.58 (21.27–22.20)
Hexane	35	—	—	1	33	18.70 (14.44–25.21)	2	34	12.42 (10.16–16.26)	8	26	9.94 (8.49–10.92)
Isoprene	4	31	4.51 (4.20–5.09)	—	34	7.05 (5.23–10.05)	3	33	6.20 (4.62–7.31)	5	29	5.16 (4.34–6.34)
cis-1, 3-Pentadiene	32	3	1.28 (1.25–1.45)[a]	11	23	1.67 (1.47–2.03)	18	18	1.62 (1.38–1.82)	16	18	1.47 (1.37–1.54)
2-Ethyl-1-Hexanol	8	27	1.49 (0.89–2.81)	15	19	0.93 (0.50–1.44)	10	26	0.57 (0.32–1.43)	12	22	0.45 (0.29–0.65)
Acetone	1	34	140.46 (99.71–189.06)	2	32	165.71 (95.15–288.56)	—	36	163.21 (98.85–315.15)	—	34	123.12 (89.42–191.01)

n_{tot}—total number of exhaled breath samples.
n_1—number of samples with concentrations below the limit of detection.
n_2—number of samples with concentrations above the limit of detection.
[a] median (minimum–maximum).

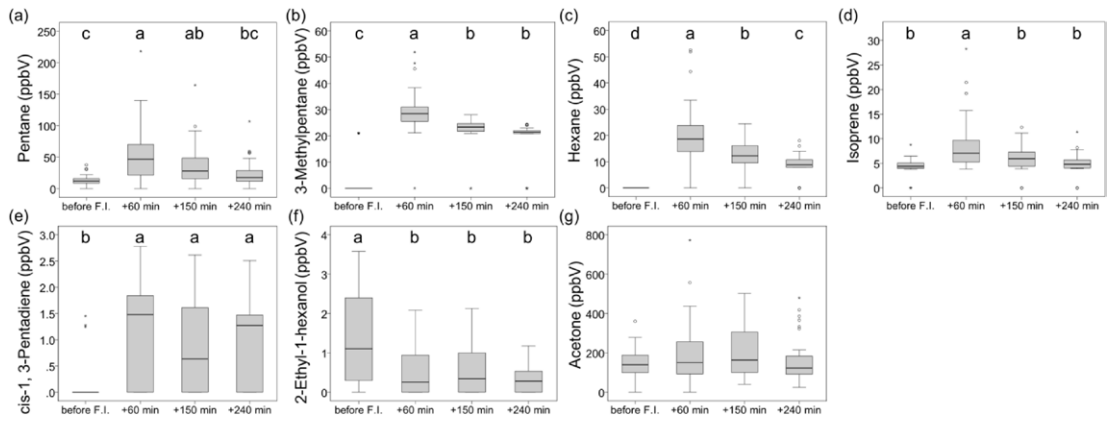

Figure 2. Concentration courses of selected volatile organic compounds in exhaled breath of healthy goats ($n = 9$) before and after food intake. Box-and-whisker plots represent summarized data of two measurement campaigns (4-week interval). At each campaign, two samples of exhaled breath were collected consecutively per goat and time point. Different letters indicate significant differences between time points of collection of breath gas samples before and after food intake (Multifactorial ANOVA, Tukey's HSD post hoc test, the significance level of 0.05 was adjusted to the Bonferroni correction). ppbV—parts per billion by volume; F.I.—food intake.

Table 5. Effects of different factors on exhaled VOC concentrations (*P*-values; multifactorial ANOVA).

VOCs	Food intake[a]	Goat[b]	Duplicate[c]	Measurement campaign[d]	Respiratory rate	Eructation
Pentane	<0.001	n.s.	n.s.	<0.05	n.s.	n.s.
3-Methylpentane	<0.001	n.s.	n.s.	<0.05	n.s.	n.s.
Hexane	<0.001	<0.01	n.s.	<0.05	n.s.	n.s.
Isoprene	<0.001	<0.001	n.s.	<0.001	n.s.	n.s.
cis-1, 3-Pentadiene	<0.001	<0.01	<0.05	<0.05	n.s.	n.s.
2-Ethyl-1-Hexanol	<0.001	<0.001	n.s.	<0.01	<0.05[e]	n.s.
Acetone	n.s.	<0.001	n.s.	<0.001	n.s.	n.s.

VOC—volatile organic compound.
n.s.—not significant.
[a] all time points related to food intake (before food intake; +60 min, +150 min, +240 min after food intake).
[b] individual goat.
[c] duplicate—the collection of breath gas samples of one goat was immediately repeated.
[d] measurement campaign—the collection of breath gas samples was repeated after four weeks.
[e] Additional evaluations by analyses of regression did not support any biologically significant relationship between the concentration of 2-ethyl-1-hexanol and the respiratory rate.

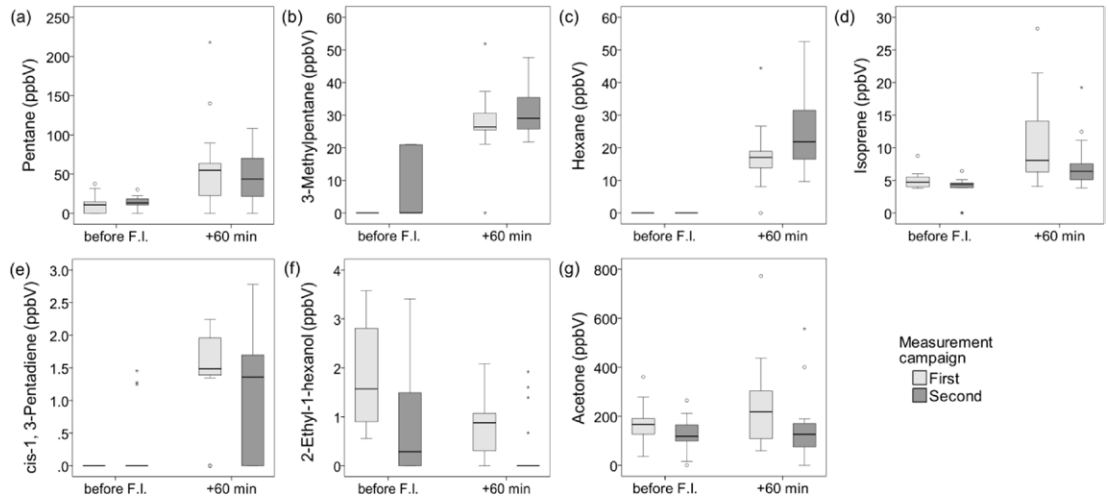

Figure 3. Concentration courses of selected volatile organic compounds in exhaled breath of healthy goats ($n = 9$) before and 60 min after food intake at the two different measurement campaigns. Box-and-whisker plots represent data measured within the same group of animals, but at two different campaigns at a 4-week interval. At each of the two campaigns, two samples of exhaled breath were collected consecutively per goat and time point. ppbV—parts per billion by volume; F.I.—food intake.

4.2. Impact of food intake on VOC concentrations

Breath gas analysis in nine healthy goats using NTME-GC-MS revealed six out of more than 100 substances showing interesting time courses after food intake. The six selected VOCs that belonged to the chemical classes of saturated hydrocarbons (pentane, 3-methylpentane, hexane), unsaturated hydrocarbons (isoprene, cis-1, 3-pentadiene), and alcohols (2-ethyl-1-hexanol), fulfilled the selection criteria of being affected by food intake.

In most samples collected before the morning feeding, 3-methylpentane, hexane, and cis-1, 3-pentadiene were not detectable, whereas pentane and isoprene were already detectable. Interestingly, the concentration patterns of these five hydrocarbons were almost the same, i.e. significant postprandial increases occurred with highest concentrations 60 min after the food intake.

Peroxidation of lipids related to oxidative stress, and to a lesser extent, protein oxidation and colonic bacterial metabolism, are described as potential pathways for endogenous generation of straight chain and methylated saturated hydrocarbons in humans (Kneepkens *et al* 1994, Phillips *et al* 2000). Increasing lipid peroxidation may be due to growing oxidative activity in the liver or to increased bacterial metabolism in the gut of the ruminants after food intake.

Isoprene can be formed endogenously during cholesterol biosynthesis (Deneris *et al* 1985, Stone *et al* 1993) or through bacterial metabolism (Kuzma *et al* 1995). Exhaled isoprene concentrations markedly increased in the goats after feeding. In contrast, other studies in healthy humans did not report any significant differences in exhaled isoprene concentration associated with different diet, or fasting and non-fasting states (Gelmont *et al* 1981). To what extend these contradicting findings are related to the differences in digestive metabolism in humans and ruminants has yet to be defined.

Dienes, such as cis-1, 3-pentadiene, were previously found in the exhaled breath of healthy human volunteers (Phillips 1997, Filipiak *et al* 2012). The pathway for endogenous generation of cis-1, 3-pentadiene is still unknown.

In contrast to concentration patterns of the hydrocarbons, concentrations of 2-ethyl-1-hexanol in exhaled breath were highest before food intake and decreased after food intake. 2-Ethyl-1-hexanol is an aroma component of plants (e.g. wheat and laminaceae) (Birkett *et al* 2004, Li *et al* 2014) and has been detected in the breath of cattle in a previous study (Ellis *et al* 2014). Our study, however, does not provide evidence for a significant reflection of 2-ethyl-1-hexanol intake via plant-based food in exhaled breath. Hypothetically, decreasing concentrations after food intake could be the result of changes in distribution between different compartments or an increase in metabolism, when e.g. splanchnic perfusion is enhanced due to digestive activity.

Acetone is one of the most prominent volatile compounds in exhaled breath in humans (de Lacy Costello *et al* 2014), and ruminants (Dobbelaar *et al* 1996, Spinhirne *et al* 2004, Ellis *et al* 2014, Fischer *et al* 2015). It is stated that acetone is generated endogenously during the fat and carbohydrate metabolism (Miekisch *et al* 2004). Many studies have attempted to use acetone as a volatile marker for diabetes mellitus summarized by Turner (2011) and Wang and Wang (2013), but a number of confounding factors considerably influence the concentration of breath acetone even in the healthy population, e.g. age, diet and diurnal variability (Schwarz *et al* 2009, Spanel *et al* 2011). In contrast to other studies, our results do not prove a significant effect of food intake on the concentration of exhaled acetone. In a study with healthy humans, the acetone concentration in exhaled breath was initially high during fasting and decreased postprandial (Smith *et al* 1999). In another study, breath acetone gradually increased several hours after a ketogenic diet in healthy humans (Spanel *et al* 2011). One reason for the different outcome could be that goats assessed in our study were not totally fasted overnight and their metabolism did not switch to conditions of starvation. Therefore, an increase in acetone in blood and exhalate due to lipolysis and ketogenesis was lacking at the initial VOC measurements before morning feeding. Furthermore, differences in the physiology of digestion and metabolism in ruminants compared to monogastrics like humans can be reflected in different patterns of acetone exhalation.

Although, impact on exhaled VOC concentrations due to diurnal exhalation patterns—as seen for H_2O_2 in exhaled breath condensate (Knobloch *et al* 2008)—cannot be fully excluded, significant changes of exhaled VOCs assessed in this study were most likely caused by either adsorption of food ingredients, or changes in the endogenous production as a result of food intake and subsequent digestive and metabolic processes. The accurate determination of pathways for endogenous generation of selected volatile markers that resulted in a significant increase in exhaled breath remains to be elucidated in future studies.

4.3. Impact of methodological and biological sampling conditions

This study, although focused on the effects of food intake on exhaled concentration patterns of volatiles, provides new insights into inter- and intra-individual variability in breath gas analysis. On the one hand, variation can be induced if food intake is not taken into account, as exhalation of some substances was markedly influenced through food ingestion. On the other hand, there was a non-negligible intra-individual variation and some inter-day variation, when animals were analyzed a second time, 4 weeks after the first analyses, although all experimental conditions were identical. Nevertheless, results of immediate intra-individual repetition were highly reproducible.

The fact that respiratory rates did not affect exhaled VOC concentrations in our study indicates successful collection of the alveolar portion of exhaled breath by means of an adapted automated CO_2-controlled

sampling method (Schubert *et al* 2001, Miekisch *et al* 2008). Alveolar gas sampling is an appropriate approach to monitor volatile compounds in exhaled breath originating from blood, as they can pass the blood-air barrier, appear in alveolar gas composition, and will be eliminated from the lung by exhalation (Schubert *et al* 2001).

During digestive processes in ruminants, gases (mostly carbon dioxide and methane, with traces of nitrogen, oxygen, hydrogen and hydrogen sulphide) are continuously produced during microbial fermentation in their forestomach system, and must be expelled via eructation in order to prevent bloating (Dougherty 1968). These gases could potentially affect exhaled VOCs when exhaled via upper airways. We did not detect any significant influence of eructation on exhaled concentrations of the selected VOCs.

5. Conclusions

Food intake generated significant physiological effects on exhaled VOC concentrations in healthy goats. Particularly VOCs belonging to the chemical classes of saturated and unsaturated hydrocarbons and alcohols showed characteristic changes in alveolar concentration patterns that could be attributed to previous intake of plant-based food. Consequently, time after food intake needs to be considered as an important confounding factor affecting VOC composition in breath gas analyses. Hence, standardized sampling protocols for VOC analyses require that time of food intake as well as characteristics of food composition need to be taken into account in any species.

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

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