ORIGINAL PAPER

Observations of the temporal variation in chemical content of decomposition fluid: A preliminary study using pigs as a model system

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In this paper we report the results of our preliminary studies into short chain fatty acids that have the potential to show reproducible patterns over certain postmortem intervals during decomposition in the absence of a soil matrix. Additional compounds that were detected, including several long chain fatty acids, were also investigated for their potential in estimating postmortem interval. Analysis of data was conducted to establish any distinct relationship between the levels of particular compounds produced with respect to time and temperature. Pork rashers (belly pork), whole stillborn piglets and whole adult pig (*Sus scrofa*) carcasses were used to model the human decomposition process in two separate locations, Western Australia (Perth) and Southern Canada (Oshawa). This enabled a comparison of components to be carried out under significantly different climatic conditions. Compounds were identified after analysis with gas-chromatography-mass-spectrometry. Preliminary observations indicate that both short chain and long chain acids followed an apparent cyclic trend. All trials showed differences with respect to rate of decomposition, both between trials and between subjects in the same trial, however, the identity of the compounds detected for the pork rasher trial (Perth) and the pig trial (Canada) remained very similar.

Keywords: Decomposition; volatile fatty acids; postmortem interval, long chain acids, geoforensics

Knowledge concerning the time since death (TSD), or postmortem interval (PMI), in investigations is essential to establish a time frame when reconstructing events. It is extremely difficult to estimate this period and while a variety of methods have been devised, most apply only to the initial hours of decay and have problems with accuracy. One promising approach is that of soil solution analysis, which attempts to age bodies by analysing the fluids released during decomposition [1].

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Vass and co-workers suggested that the use of various time dependant biomarkers, such as amino acids and neurotransmitters produced during soft tissue decomposition, might be a valuable alternative method in estimating TSD [1]. Vass used soil solution analysis to look at five particular volatile fatty acids (VFA's) (propionic, n-butyric, iso-butyric, n-valeric and iso-valeric), also known as short chain acids, and the distinct variation in the acids over time, with regards to accumulated temperature. The conclusion by Vass et al. is that VFA's can be accurate and reliable biomarkers indicating various stages of decomposition. The estimate given by Vass et al. in determining PMI using this method is currently +/- 2 days, however, to date, this research has only been studied in detail in Tennessee, and therefore, extrapolations to various edaphic and environmental conditions cannot be made.

One positive aspect in the use of a chemical biomarker for the estimation of PMI is the elimination of examiner bias. An advantage of using volatile fatty acids and other blood proteins is that they can remain biologically active and detectable for a considerable amount of time [2]. For this reason, Tuller uses VFA's in the analysis work from execution and gravesites in the former Yugoslavia [2]. However, the experimental determination of VFA's produced during decomposition is yet to be validated with a statistically sound and robust method. The effects of a controlled sample size and factors that affect decomposition and hence the appearance of VFA's is still to be investigated, particularly for forensic investigations.

Variability in temperature is the most important and significant factor influencing the rate of decomposition of a corpse [1, 3-5]. Within psychotrophic and mesotrophic temperature ranges there is a positive relationship between temperature and decomposition [6]. This finding is supported in a study by Archer, in which higher temperatures were found to increase the mass loss rates and decomposition stage progression rates of exposed neonatal remains [7]. More recent studies utilising controlled incubation temperatures with excised tissue from lamb (*Ovis aries*) and rat (*Rattus rattus*) cadavers also support this finding [6, 8]. Temperature can also affect the presence or absence of insects and bacteria as well as the breakdown of proteins and carbohydrates present in the body [1].

Vass et al state that decomposition will occur down to 0°C due to salt concentrations in the body [1]. However, this statement is not supported with experimental evidence, and currently the lowest temperature at which mammalian tissue has been shown to decompose is 2°C [6].

Meygesi et al. suggest that decomposition rates and a more accurate estimation of PMI can be achieved when accumulated temperature is taken into account rather than individual daily temperatures [9]. Accumulated degree days (ADD) are calculated by taking the sum of the average daily temperature for the length of time the corpse has been decomposing [9]. Vass suggests that the use of cumulative degree hours (CDH) is a more accurate technique [10]. CDH uses a twelve-hour temperature cycle in which the average temperature is calculated for every complete cycle rather than the average daily temperature. Both models make the assumption that the relationship between time and temperature is linear. This would indicate that they can be used to estimate PMI by succession and development of carrion insects if the temperature data is known for a specific area [11], however, experimental evidence suggests that this may not be the case [6, 8].

The second most important factor affecting decay rates and hence the resultant calculations to determine PMI is the accessibility and activity of insects [3, 4, 12, 13]. Flies and other insects are attracted to a corpse at various stages during decomposition, depending on the volatile compounds produced and associated odours that are emitted. The overwhelming majority of soft tissue destruction during decomposition is due to feeding by insects [4]. If insects are absent from the corpse, it can be assumed that decomposition will proceed at a significantly slower rate [4, 13, 14].

The cumulative effect of these two factors must be a significant consideration when examining rates and stages of decomposition. Other factors that influence the rate of decomposition of a corpse include body size and weight, the presence or absence of carnivores, humidity, clothing, trauma to the body and soil pH [4, 15].

In this paper we aim to detail preliminary observations in the chemical composition of fluids produced during decomposition in the absence of a soil matrix. The discussion is based on the observations made in these trials; however, continued extensive, replicated studies are required to confirm statistical significance and valid quantitative results. Initial steps involved development and validation of a gas chromatographic-mass spectrometric method following a simple aqueous dilution and filtration of decomposition fluid samples.

Experimental Set-up

Due to the ethical issues involved in the use of human cadavers, pig (*Sus scrofa*) carcasses have been used to model the human decomposition process [16]. They are considered to be an acceptable substitute due to their similarity to human torsos in weight, fat to muscle ratio, hair coverage, biochemistry and physiology [17, 18]. The selection of samples is described in a previous paper [19] but has been repeated here for clarification.

Initial studies were performed with pork rashers (belly pork, *Sus scrofa*) to aid in method development. Further trials were carried out with stillborn piglets and adult pig carcasses (*Sus scrofa*) to create a more realistic model of decomposition and to gain a more accurate understanding of the composition of decomposition fluid.

Samples

Pork rashers (belly pork)

Eight polystyrene cups, each with the base partially removed and replaced with fine wire mesh, contained approximately 100g of cubed pork rashers (belly pork). The rashers were obtained from a commercial retail outlet so exact levels of fat and protein are not known. Each cup was placed inside a second polystyrene cup for easy access to and collection of the decomposition fluid.

Purpose built, open mesh, metal cages were used to prevent predation by feral cats and other scavengers (ravens) but allowed flies and other insects access to the decomposing rashers. Four of the cups were covered with muslin, to prevent insect activity, and placed in one of the metal cages. The remaining four cups were left uncovered and placed in a second metal cage.

Collection vessels were monitored daily and fluid samples collected at random intervals, depending on the volume of fluid produced. Variable fluid volumes were recovered and recorded at each sampling session. Fluid was collected using a 5mL sterile syringe and placed in 20mL pre-weighed polypropylene vials for transport back to the laboratory. Syringes were discarded after each use. Samples were stored at -4°C prior to analysis.

After each sampling session, spraying with approximately 2-5mL of deionised water using a standard garden water spray gun lightly moistened the collection vessels. In the event of rain, both cages were covered with tarpaulins to prevent damage to the samples. The uncovered meat samples were disposed of on day 17, by which time they had become dry and insect activity had ceased.

Stillborn Piglets

Four stillborn piglets (approx 2-2.5kg) (supplied by PPC Linley Valley Fresh) were placed onto a custom made rack fitted into individual 15L plastic storage boxes. Two of the piglets were completely enclosed with fine wire mesh and the other two were left exposed. The 15L storage box was contained within a custom-made wire cage (80 x 70 x 60cm) to prevent access by feral animals.

The piglets were monitored daily, and fluid samples were collected from the uncovered piglets three times a week, beginning at day 14, when the first decomposition fluid was evident. Fluid collection procedures were the same as described previously for the pork

rasher (belly pork) experiment. Fluid was collected from the protected piglets beginning at day 42. Sampling for all piglets continued until day 60.

Adult pig carcasses

This study utilised two adult pig carcasses as a model for human decomposition. Carcasses (~ 55 lb, 25 kg) were euthanized by a veterinarian with an overdose of anaesthetic (sodium phenobarbital) at a local pig farm prior to experimental treatment. The effect of the anaesthetic on rate of decomposition, or the succession/development of flies, was not considered in this study. There have been a number of studies on the effects of drugs in cadavers on insect development [20-22]. The effect of residual anaesthetic in the pig carcasses is an area of potential future research. Both carcasses were wrapped in plastic and transferred to the experimental site shortly after death.

The carcasses were placed on wire racks and were covered with mesh wire to prevent scavenging by large carnivores. The mesh was sufficiently large to allow normal invertebrate activity to assist with decomposition. Each rack was placed over a small pit dug into the soil and lined with plastic. The wire racks allowed drainage of the decomposition fluids into the pit and aided sampling of the fluids without disturbing the site.

Pigs were monitored daily and fluid was collected from both pigs beginning on day 5. Fluid collection procedures were the same as described for the pork rasher (belly pork) trial. Sampling for both pigs continued until day 14 at which time the carcasses were mummified and skeletal.

Decomposition Research Facilities

Preliminary decomposition studies were carried out at two distinct locations; Perth, Western Australia and southern Ontario, Canada to provide a comparison between

compounds detected in decomposition fluid. Temperature loggers (Thermodata Pty Ltd) were used to record ambient temperature every 30 minutes in both locations.

Perth, Western Australia (March-April, June-August, 2006)

The field trial area for this project was located in, Bentley, Western Australia (32°01′S, 115°53′E), 6 kilometres southeast of Perth. It is a partially cleared scrub area, approximately 4m x 6m.

Data from the three closest official weather-recording stations (Perth metro, Perth airport and Jandakot airport) was obtained on a daily basis from the Bureau of Meteorology for comparative purposes. The pork rasher (belly pork) trial was conducted over late summer (March-April 2006) and the stillborn piglet experiment was conducted during winter (June-August 2006).

Ontario, Canada (July 2007)

The field trial was conducted in temperate woodland in southern Ontario, Canada. The study area was located approximately 51 km northeast of Toronto (43°56'N, 78°54'W) across a slight (5°) gradient, with some drainage/depression areas. Dominant vegetation at the site included eastern white cedar (*Thuja occidentalis*), maples (*Acer spp*.) and trembling aspen (*Populus tremuloides*).

The pig trial utilising adult pig carcasses was conducted over the Canadian summer (July-August 2007).

Analysis

A stock solution (80mM) of each of the five target short chain volatile fatty acids; propionic (Ajax Chemicals, Australia; Sigma-Aldrich, Canada), n-butyric (Chem Supply, Australia; Sigma-Aldrich, Canada), isobutyric (Aldrich, Australia; Sigma-Aldrich,

Canada), n-valeric (Sigma, Australia; Sigma-Aldrich, Canada) and isovaleric acid (Aldrich, Australia; Sigma-Aldrich, Canada), was prepared in deionised water. A set of standard calibration solutions in the concentration range 0.2 – 10mM was prepared by serial dilution of the stock solution. All acids used were of analytical grade (>99%). The internal standard, trimethylacetic acid (TMA, 2.5mM) (Sigma, Australia; Sigma-Aldrich, Canada), was added to each of the calibration standards in a 1:1 ratio immediately prior to analysis. Calibration and stock solutions were stored at 4^0C before and after analysis. All calibration standards were analysed in triplicate by gas chromatography-mass spectrometry (GC-MS).

The recovered fluid samples were allowed to equilibrate at room temperature. A 1:2 dilution was prepared of the fluid with deionised water. The mixture was filtered (0.8/0.2µm Acrodisc® filter, PALL Life Sciences) and 0.5mL filtrate and 0.5mL trimethylacetic acid (2.5mM) solution was prepared immediately prior to analysis by GC-MS.

In Western Australia, volatile fatty acid calibration standards and samples were analysed by Gas Chromatography (Hewlett Packard 5890 Series II) interfaced with a Mass Selective Detector (MSD) (Hewlett Packard HP5971). A 1µl aliquot was introduced into the split/splitless injector by means of a Hewlett Packard 7673A autosampler. The gas chromatograph was fitted with a 50m x 0.22mm ID x 0.25µm (df) BP20 SGE column.

In Canada, chromatographic analysis was performed on a Thermo-Finnigan Trace GC Ultra coupled with a Thermo-Finnigan Polaris Q mass spectrometer. A 1µL aliquot was introduced onto a HP-INNOWAX (J&W Scientific 19091N-133) capillary column (30 m x 0.25mm ID x 0.25um df) by means of a Thermo Triplus autosampler. Full details of the GC conditions are described in [19]. Data acquisition and analysis was performed using Chemstation software.

Results and Discussion

Pork Rasher (belly pork) Trial

Over the duration of the pork rasher trial, the average daily temperature varied between 12-23°C, with a mean of 17.4°C (Figure 1). The average daily temperature data did not show any apparent trend that correlated with trends in the production of short chain acids.

Only two of the targeted short chain acids, propionic and isovaleric acids, were detected on all collection days (Table 1). Analysis of the data from the short chain acids (C_2-C_5) showed that acetic, propionic and isobutyric acids followed an increasing trend until day 15 (ADD 359) and then a decreasing trend until the end of the trial (Figure 1). This is possibly due to their more volatile nature in comparison to butyric and isovaleric acids. Butyric and isovaleric acids also followed an increasing trend and passed through a maximum at day 15, but in contrast to the other target short chain acids, they appeared to have a cyclic nature. On day 16, the acids tended toward a second minimum value, then increased again until the end of trial on day 24. Ratios between individual acids identified in all samples were investigated. The ratios between acetic:propionic, acetic:isovaleric and propionic:isovaleric acids showed no obvious trend that might enable their ratio to be used as a reliable estimator of time since death. Semi-quantitative concentration data for target short chain fatty acids are shown in Table 1.

The initial increasing trend shown by all C_2-C_5 acids suggested the possibility that they were all produced by a common source. The second increasing trend shown by butyric and isovaleric acids would be suggestive of a possible new source during decomposition in which acetic, propionic and isobutyric acids are not generated. The patterns seen in the short chain acids can be related to initial work by Vass who suggested their abundance is at a maximum between 350 and 450 ADD [1]. Short chain fatty acids produced by the pork rashers reach a maximum at ADD 359. In contrast, short chain VFA's first appeared at 85 ADD, although Vass suggests their appearance does not occur until at least 150 ADD. Unlike the work completed by Vass, valeric acid was not detected in this trial. Acetic acid, however, appeared in abundance in the pork rasher trial but is not mentioned in Vass' previously published work [1].

The long chain fatty acids found in the pork rasher trial (oleic, myristic, palmitic, stearic, linoleic and 9-hexadecanoic acid) appeared to follow an 8-day cycle, with each acid reaching a maximum on day 15 (ADD 359) (Figure 2). This trend has not previously been reported in the literature. Similarly, the target short chain acids also reached a maximum on day 15. This observation can be tentatively related to the level of fly activity, the feeding cycle of the maggots and the adipose tissue content of the carcass (the proposed origin of the fatty acids) [1, 23]. Once larvae or eggs are laid, it can take up to 24 hours for them to hatch. During this time, production of long chain fatty acids would be expected to be relatively low, as seen on day 3. The following 5-6 days are spent feeding and growing until full size is reached [23], whereby the production of long chain fatty acids steadily increases. After this initial time period, the first wave of maggots will have grown to full size (7-8 days total), they then migrate away from the food source to an environment suitable for pupation [23] and fatty acid production will decrease. The pupation period lasts an additional 8-10 days [23]. At this time a second wave of flies can lay larvae or eggs in the decomposing meat and the cycle begins again. After pupation, the adult fly can now emerge and leave in search of a food source. A comprehensive study of the life cycle of Western Australian flies was not investigated in this work, and therefore, further trials are needed to potentially relate the feeding cycle of maggots to production of long chain fatty acids.

The long chain fatty acids, oleic and palmitic, were detected on all collection days and were analysed for possible ratios and patterns. No patterns were found between the ratios of these two acids that would indicate their effective use in estimating PMI.

Vass concluded that long chain acids were indicative of decomposition in a cold environment and could be used to verify possible movement from a cold to warm area [1]. The presence of large, water insoluble, long chain fatty acids in a warm weather environment is contrary to these findings by Vass.

Piglet Trial

The variation in the concentration levels of volatile fatty acids produced by individual piglets can be seen in Table 2. Piglet 1 showed an increasing trend in acetic acid over an 8-10 day period, however, during the course of the trial, there was a decrease in the level of acetic acid produced. No other recognisable trends were seen for this piglet. Piglets 2 and 3 showed no clear trends in the compounds found in decomposition fluid, although it should be noted that samples were collected on only 3 days for piglet 2. Piglet 4 showed a general decreasing trend over time with respect to abundance of compounds. However, samples were only collected on five separate days for piglet 4 so the validity of trends is difficult to justify. The ratio between individual short chain acids was also analysed. There appeared to be no clear trend in the ratios between volatile fatty acids that would indicate their effective use as reliable biomarkers of postmortem interval.

The range of compounds observed in the pork rasher trial was not seen in this subsequent stillborn piglet trial. The sporadic appearance of long chain fatty acids from the piglet trial eliminates their use in establishing trends in production. Widdowson [24] found that the fat content of stillborn piglets is approximately 1% of their total body weight. The lack of long chain acids in the piglet trials can be related to this since long chain acids are produced by the cleavage of triglycerides during aerobic degradation of fats and soft tissues [17].

It is interesting to note that all four pigs, whether covered or uncovered, decomposed at a different rate, and showed variation in the compounds produced, even though they were in close proximity to each other and subjected to the same environmental conditions and surroundings.

Pig Trial

All of the initial C_2-C_5 target acids were detected in each fluid sample during this trial. With the exception of isobutyric acid, target short chain acids passed through a maximum on day 6 (ADD 126), the point at which maggot activity was highest. This is in contrast to the pork rasher trial, where the short chain acids passed through a maximum on day 15

(ADD 359), possibly due to the higher level of humidity, and therefore increased insect activity, experienced in Ontario compared with Western Australia. The acids appear to follow a cyclic trend, tending towards a second minimum on day 10 (ADD 214) and then increasing again until the end of the trial on day 14 (ADD 310) (Figure 3). No fluid was collected on day 16 which would allow completion of a second cycle to confirm this theory.

The detection of long chain acids was expected due to the fat content in an adult pig. These acids (oleic, stearic, palmitic, linoleic) showed an increasing trend over the 14-day time period for which the trial was run (Figure 4). All long chain acids reached a maximum on day 14 (ADD 310) and due to time constraints and the difficulty in sampling no further fluid samples were collected to demonstrate the continuation of this trend.

In both this trial and the pork rasher trial, long chain acids were first detected on day 6 (maximum maggot activity) with calculated ADD values of 126 and 192 respectively. In both trials, the long chain acids peak around day 14-15, again showing comparable ADD values of 310 and 359. This apparent 8-day increase in the level of long chain acids can again be tentatively related to the feeding and life cycle of the fly [23]. If this trial had continued for an extended period of time, the level of long chain fatty acids would be expected to decrease on the next sampling session and then show another cyclic trend.

The ratio between individual acids was analysed for patterns but no information was found that would indicate their use as an effective indicator of PMI.

Future Directions

Decomposition fluids were confirmed to have a complex and highly variable chemical composition. Further development and validation of analytical methodologies is required for complete characterisation of the fluid produced both in the absence and presence of soil matrices under controlled and field conditions. Techniques such as capillary

electrophoresis and liquid chromatography-mass spectrometry, for analysis of biogenic amines and amino acids respectively, are currently being investigated. In order to properly compare between studies by different groups there is also a need to establish harmonised analytical protocols to enable direct comparison of results.

While this preliminary study, like others [1, 3, 4, 16, 17], has focussed on field trials there is a need to investigate significant climatic variables using controlled experiments [6, 8]. This is important in order to evaluate effectively the relevance of trends observed for the various chemical markers in the field. Two distinct locations were utilised in the field trials to provide a comparison between compounds detected during the analysis of decomposition fluid. All three trials showed differences with respect to rate of decomposition, both between trials and between subjects in the same trial, however, the identity of the compounds detected for the pork rasher trial (Perth) and the pig trial (Canada) remained very similar.

Future work will require the use of larger sample sets with increased replication to provide a more realistic approach for the characterisation of decomposition fluid, and to ensure appropriate significances are detected. The lack of production of long chain fatty acids in the piglet trial highlighted the importance in the source of fluid with this trial showing considerable variation in type and abundance of compounds. Further investigations with samples where the various levels of fat and protein are known are required. Approaches to standardising the test subjects would also therefore be worthy of study, but this may be logistically difficult in any transnational studies.

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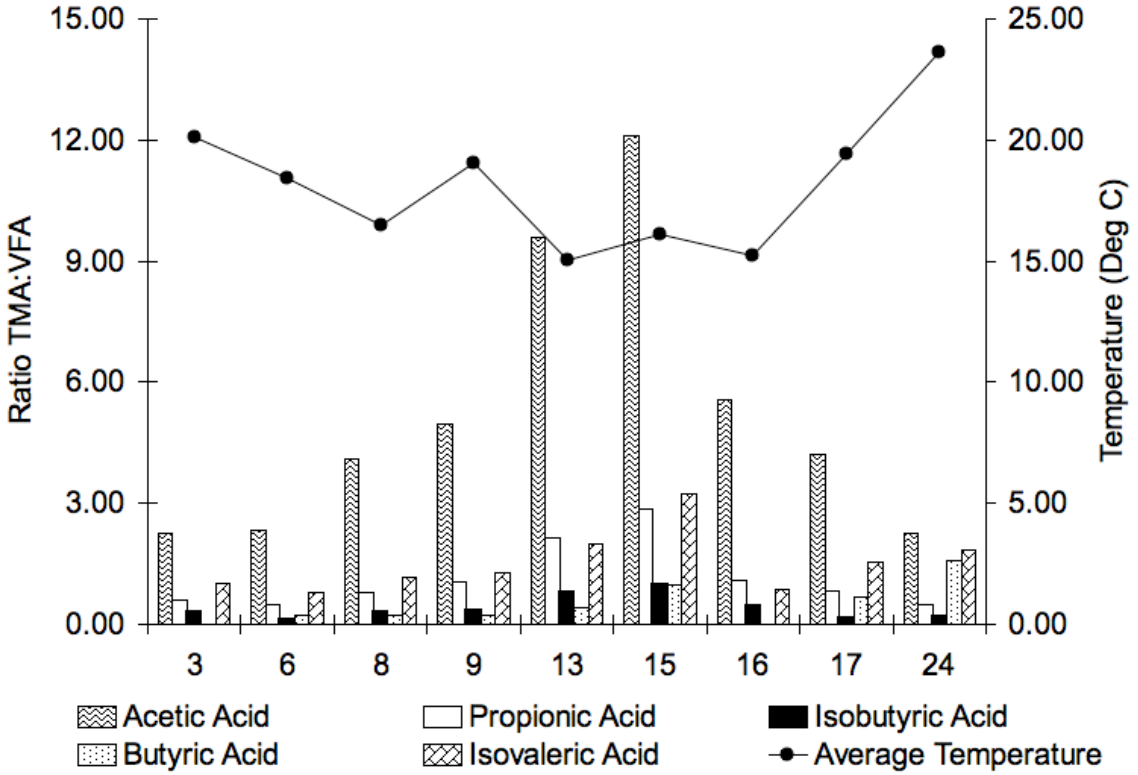
Figures

Tables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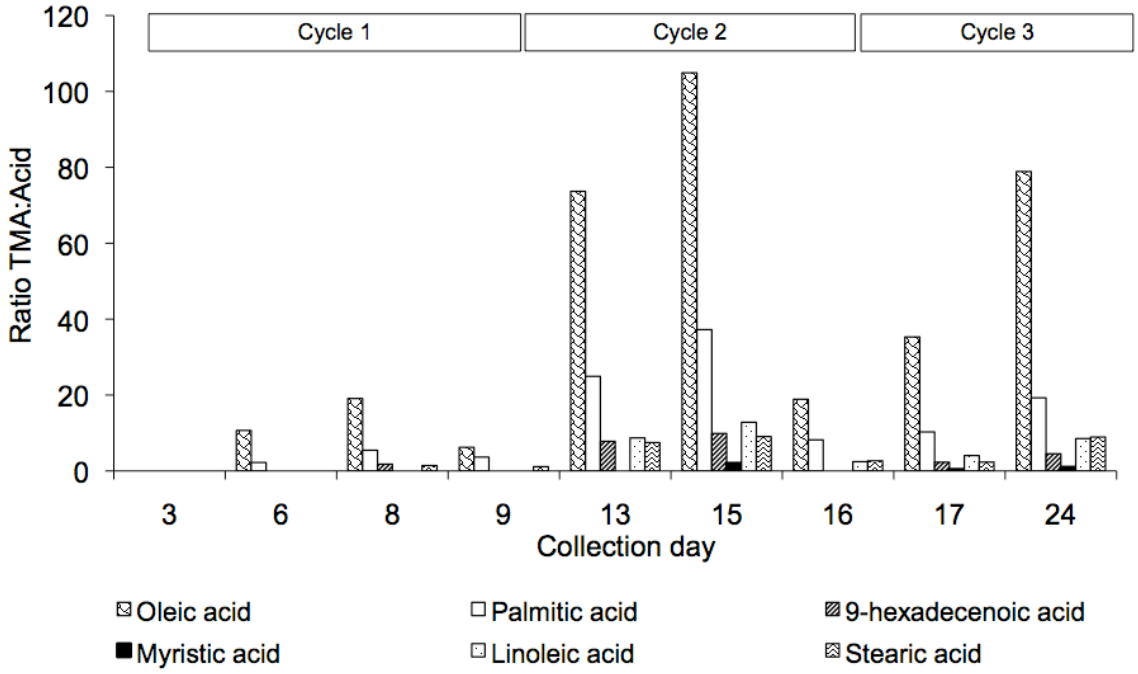
Acetic Acid
Propionic Acid
Isobutyric Acid
Butyric Acid
Isovaleric Acid
Average Temperature

Fig 1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Ratio TMA:Acid
Cycle 1
Cycle 2
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6
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15
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17
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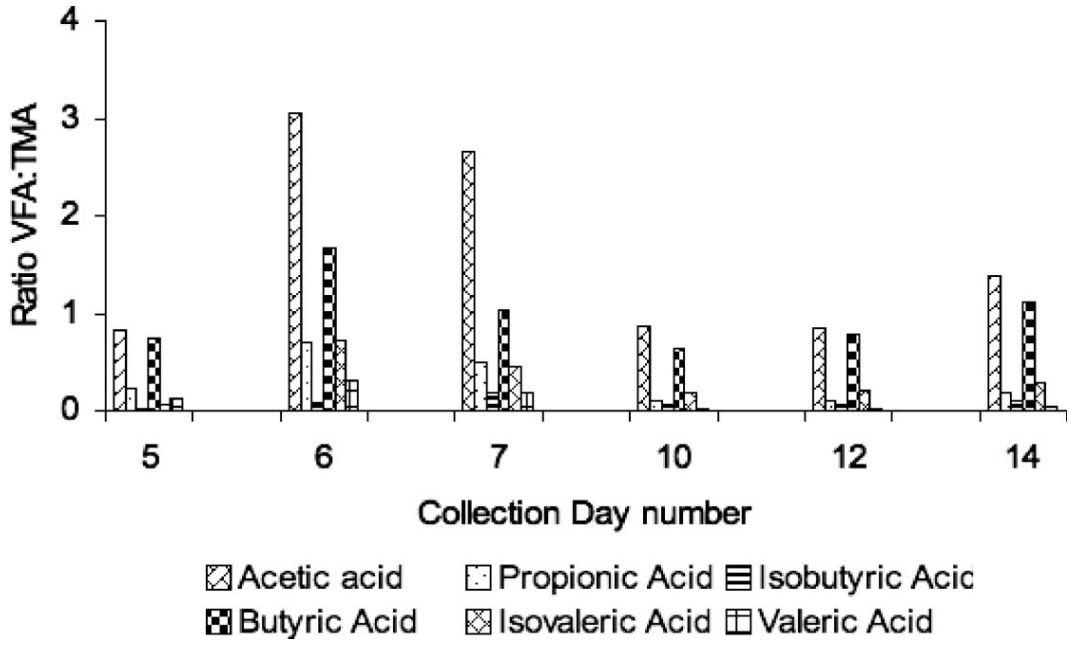
Collection day
Oleic acid
Palmitic acid
9-hexadecenoic acid
Myristic acid
Linoleic acid
Stearic acid

Fig 2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12
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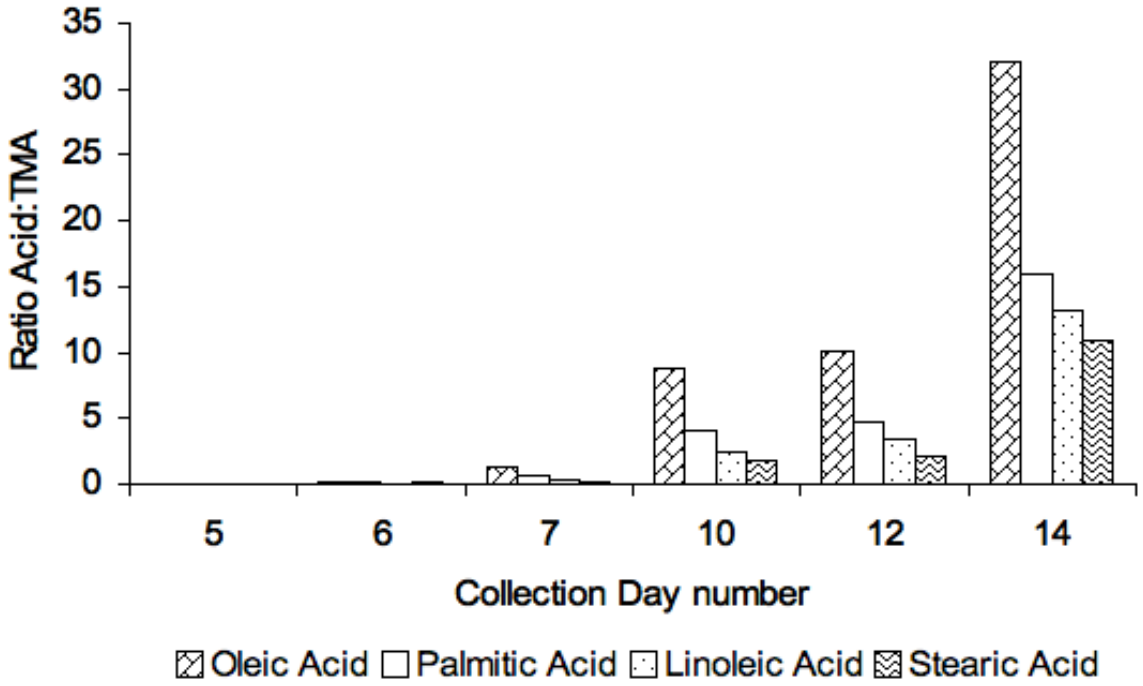
Collection Day number
Acetic acid
Propionic Acid
Isobutyric Acic
Butyric Acid
Isovaleric Acid
Valeric Acid

Fig 3


Ratio Acid:TMA
35
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Collection Day number
Oleic Acid
Palmitic Acid
Linoleic Acid
Stearic Acid

Fig 4

Table 1

	Collection Day								
Compound	**3**	**6**	**8**	**9**	**13**	**15**	**16**	**17**	**24**
Acetic acid	✓	✓	✓	✓	✓	✓	✓	✓	✓
Propionic acid	0.27	0.2	0.38	0.52	1.11	1.52	0.54	0.4	0.21
Isobutyric acid	0.18		0.16	0.2	0.53	0.69	0.29	0.06	0.07
Butyric acid		0.11	0.11	0.09	0.26	0.7		0.44	1.14
Isovaleric acid	0.92	0.7	1.04	1.17	1.83	3.05	0.76	1.42	1.72
Piperidone	✓	✓	✓	✓	✓	✓	✓	✓	✓
Isocaproic acid			✓	✓	✓		✓		✓
Phenylacetic acid		✓		✓		✓	✓	✓	✓
Phenylpropanoic acid		✓		✓		✓	✓	✓	✓
Palmitic acid		✓	✓	✓	✓	✓	✓	✓	✓
Myristic acid						✓		✓	✓
Oleic acid		✓	✓	✓	✓	✓	✓	✓	✓
Linoleic acid					✓	✓	✓	✓	✓
Stearic acid			✓	✓	✓	✓	✓	✓	✓
Palmitoleic acid			✓		✓	✓		✓	✓

Table 2

Collection Day	Volatile Fatty Acid				
	Propionic Acid	**Isobutyric Acid**	**Butyric Acid**	**Isovaleric Acid**	**Valeric Acid**
14	1 (1.27)	1 (2.23)	1 (2.94)	1 (4.16)	1 (4.58)
16				1 (0.15)	
20				1 (0.14)	
22	3 (0.18)	3 (0.33)	3 (0.30)	3 (1.3)	3 (0.77)
26	3 (0.08)	3 (0.15)		3 (1.04)	
28	1 (0.14) 3 (0.09)	1 (0.06) 3 (0.28)		1 (0.2) 2 (0.82)	
30	3 (1.21)	3 (0.28)		3 (0.96)	
36	1 (0.43) 3 (0.12)	1 (0.69) 3 (0.17)	1 (1.15)	1 (2.09) 3 (0.66)	1 (1.19)
40				1 (0.09) 3 (0.06)	
42	2 (0.52) 3 (0.07) 4 (0.34)	2 (0.07) 4 (0.4)	2 (0.37) 3 (0.09) 4 (0.09)	1 (0.22) 2 (0.51) 3 (0.2) 4 (1.3)	3 (0.07)
44	2 (0.82) 4 (0.23)	2 (0.25) 4 (0.31)	2 (0.12)	2 (0.63) 4 (1.28)	
47	1 (0.15) 2 (0.06)	1 (0.29) 2 (0.26)	1 (0.79) 2 (0.16) 4 (0.07)	1 (1.19) 2 (0.75) 4 (0.21)	