Laboratory Procedure Manual

Analyte: **Aldehydes**

Matrix: **Serum**

Method: **Solid Phase Microextraction (SPME), Gas Chromatography (GC) and High-resolution Mass Spectrometry (HRMS) with selected ion mass detection and isotope-dilution techniques**

As performed by:

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Important Information for Users

The Centers for Disease Control and Prevention (CDC) periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table

Data File Name	Variable Name	SAS Label
ALD_H & ALDS_H	LBXBZAL	Benzaldehyde (ng/mL)
	LBXCRAL	Crotonaldehyde (ng/mL)
	LBXI5AL	Isopentanaldehyde (ng/mL)
	LBXTLAL	o-Tolualdehyde (ng/mL)
	LBX3AL	Propanaldehyde (ng/mL)
	LBX4AL	Butyraldehyde (ng/mL)
	LBX5AL	Pentanaldehyde (ng/mL)
	LBX6AL	Hexanaldehyde (ng/mL)
	LBX7AL	Heptanaldehyde (ng/mL)
	LBX8AL	Octanaldehyde (ng/mL)
	LBX9AL	Nonanaldehyde (ng/mL)
	LBX10AL	Decanaldehyde (ng/mL)

1. Clinical Relevance and Summary of Test Principle.

a. Clinical Relevance

Aldehydes are formed as thermal degradation byproducts released into the atmosphere from the burning of organic matter such as gasoline, food flavorants, and tobacco. Aldehydes are potentially carcinogenic and mutagenic; therefore, exposure to aldehydes has raised concerns about adverse health effects in humans. To support studies exploring the relationship between aldehyde exposure and adverse health effects, an automated analytical method was developed using solid phase microextraction (SPME) gas chromatography (GC) and high-resolution mass spectrometry (HRMS) with selected ion mass detection and isotope-dilution techniques. This analytical method quantifies trace levels of nineteen aldehydes (acetaldehyde, acrolein, propanal, butanal, isobutanal, isopentanal, crotonaldehyde (2-butenal), pentanal, hexanal, furaldehyde, *trans*-2-hexenal, heptanal, benzaldehyde, octanal, *trans*-2-octenal, *o*-tolualdehyde, nonanal, *trans*-2-nonenal, and decanal) from protein adducts in human serum.

b. Test Principle

Aldehydes tend to react with biological molecules to form various products, including Schiff base protein adducts. Our method analyzes free aldehydes released into the headspace of biological samples from the Schiff base protein adducts at low pH (~3). Our method reproducibly hydrolyzes aldehyde adducts covalently bound to proteins by individually incubating samples prior to analysis with hydrochloric acid (Yeo 2004).

The incubation of samples is controlled by two PAL auto-sampler arms (Combi-PAL and Prep-PAL) using the software program Chronos (Axel Semrau GmbH & Co.). The automated processing of samples is described below:

1. Prep-PAL with a 1000 µL syringe obtains 330 µL of 0.1 M hydrochloric acid from reservoir and adds acid into SPME vial containing sample and internal standard cocktail.
2. Prep-PAL then takes the vial with acid addition and places the vial into the agitator (agitatorMx) for 20 minutes at 30°C and 350 rpm.
3. PrepPAL moves SPME vial from agitatorMx into agitator1.
4. Combi-PAL with SPME fiber attachment then extracts analytes from the headspace onto the fiber during the 10 minute extraction at 50°C and 350 rpm.

The analytes are in equilibrium between the serum matrix and the headspace above the sample. Free aldehydes are captured by the SPME fiber, desorbed into the heated GC injector. Extracted aldehydes are focused at the head of the

GC column using a cryogenically cooled trap (cryo-trap). Analytes are separated on a DB-624 capillary column and quantified using multiple ion detection (MID) with mass spectrometric (high resolution at 10,000 mass resolution using a Thermo Fisher Scientific DFS mass spectrometer). This method employs isotope dilution to precisely quantify trace amounts of aldehydes with limits of detection in the low parts per trillion range. The linear calibration curve spanned over 3 orders of magnitude.

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

ALL OF THE COMPOUNDS USED IN THIS STUDY ARE HAZARDOUS CHEMICALS! Use a high draft fume hood and lower all the sashes to recommended operating height when working with neat (undiluted) materials or highly concentrated solutions since a number of these compounds are toxic. Wear nitrile gloves when handling hazardous chemicals to prevent absorption through the skin. Some of the compounds used in this study are known or suspected carcinogens, mutagens and/or teratogens.

b. Radioactive Hazards

None.

c. Microbiological Hazards

Follow Universal Precautions. Because of the possibility of exposure to various microbiological hazards, appropriate measures should be taken to avoid any direct contact with the human serum specimens. A Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues.

d. Mechanical Hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Laboratorians should read and follow the manufacturer's information regarding safe operation of the equipment. Avoid direct contact with the mechanical and electronic components of the gas chromatograph or mass spectrometer unless all power to the instrument is off. Generally, mechanical and electronic maintenance and repair should only be performed by qualified technicians. The autosampler and the mass spectrometer contain a number of areas which are hot enough to cause burns. Precautions should be used when working in these areas.

e. Protective Equipment

Standard safety precautions should be followed when performing this procedure, including the use of a lab coat/disposable gown, safety glasses, appropriate gloves, and chemical fume hood. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

f. Training

Formal training in the use of the gas chromatograph, mass spectrometer, and Chronos autosampler software is necessary. Users are required to read the operation manuals and should demonstrate safe techniques in performing the method. Users should be trained in use of all other potentially hazardous equipment, including centrifuges, chemical fume hoods, etc.

g. Personal Hygiene

Follow Universal Precautions. Care should be taken when handling chemicals or any biological specimen. Routine use of gloves and proper hand washing should be practiced. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

h. Disposal of Wastes

Waste materials must be disposed of in compliance with CDC laboratory, federal, state, and local regulations. Solvents and reagents should always be disposed in an appropriate container clearly marked for waste products and temporarily stored in a chemical fume hood. Disposable plastic, glass, and paper (pipette tips, vacutainers, gloves, etc.) that contact human serum are to be placed in a biohazard autoclave bag. The biohazard autoclave bags should be kept in appropriate containers until sealed and autoclaved. Wipe down all surfaces with fresh 70% ethanol solution when work is finished. Disposable pipet tips used to remove serum from the sample container should be placed immediately into a sharps container and autoclaved when the sharps container becomes full. All other non-disposable glassware that contact serum should be decontaminated with a freshly prepared bleach solution (a 10% dilution of commercial sodium hypochlorite (bleach) or equivalent) before re-use or disposal.

Observe Universal Precautions. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis according to CDC/DLS guidelines for disposal of hazardous waste.

All non-disposable glassware that contacts human serum should be decontaminated with a freshly prepared bleach solution (a 10% dilution of

commercial sodium hypochlorite (bleach) or equivalent) before re-use or disposal.

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

This method has been validated using the solid phase microextraction technique coupled with a gas chromatograph and DFS mass spectrometer run with the Thermo Fisher Scientific Xcalibur 2.2 software. Results are exported from Xcalibur software to Microsoft Excel files and imported into the STARLIMS relational database. Knowledge of and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

b. Sample Information

Information pertaining to particular specimens is entered into the database either manually or electronically transferred. Results from each analysis batch are processed into a single file using data analysis software that provides serum level results along with corresponding calibration curve, QC, blank data. The result file is transferred electronically into the database. No personal identifiers are used, and all samples are referenced to a blind coded sample identifier.

c. Data Maintenance

Integrity of specimen and analytical data generated by this method is maintained by proofreading all transcribed data, storage of data in multiple computer systems, and redundantly archiving data. Original data files contain traceable header information (date, run number, sample type and sample identification) and are stored in duplicate on two separate external hard drives. Data is transferred through an encrypted flash drive to the shared network drive along with relevant meta-data (including peak integrations, calibration curves, blanks, and isotope corrections). Processed results files are transferred electronically into the local area network (LAN) and stored in a shared directory. Data is loaded into the STARLIMS database system using an automated data import module. The data files are backed up onto the CDC network.

Routine backup procedures include: 1) weekly backup of hard disks; 2) daily backup of share drive database files; 3) weekly backup of database files onto an encrypted hard drive. A separate, backup tape database is stored off site. Either the supervisor or the local area network manager should be contacted for emergency assistance.

d. Information Security

Information security is managed at multiple levels. The information management systems that contain the final reportable results are restricted through user ID and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided at multiple levels through restricted access to the individual laboratories, buildings, and site. Confidentiality of results is protected by referencing results to blind coded sample IDs (no names or personal identifiers).

4. Procedures for Collecting, Storing, and Handling Samples; Criteria for Sample Rejection

a. Special Instructions

No special instructions such as fasting or special diets are required.

b. Sample Collection

The specimen type is human serum collected into blood collection tubes specifically designed to separate serum from other blood products. The serum is aliquoted into Nalgene cryogenic vials or assorted other tubes like a 2 mL sterile, screw-cap Nalgene cryogenic vial. The 2 mL cryogenic vials are labeled with a self-laminating barcoded label that contains the Sample ID, which is a 9-digit number that represents the unique 6-digit SP ID concatenated with the 3-digit vessel ID. Processed vessels are generally stored in 2-inch, 3-inch or 5-inch tall cardboard boxes with 9x9, 7x7, or 5x5 inch cardboard grids. Some boxes contain foam inserts. A storage box is referred to as a “container.” Each container is labeled with a 6-digit “container ID” number where the first three digits represent the stand number. The last three digits are sequentially numbered. Each vessel is generally assigned to a separate container.

After a vessel is processed, it is stored in the container in a -70 °C freezer, according to test requirements.

Each laboratory is assigned a unique 3-digit identification number. This identification number is used in correspondence and in the database to link and track the samples throughout the processing, testing, result, and QC submission processes.

c. Sample Handling

The vacutainers used for the blood collection are sterile and contain no anticoagulants. The collected blood is allowed to sit for a period of 15 to 30 minutes, not to exceed 30 minutes, for the blood to clot. Allowing samples to clot over 30 minutes can lead to artifactual formation of aldehyde. Therefore to minimize artifactual formation of aldehydes, it is important to carefully control maximum clotting time. The blood sample is then separated by micro-centrifuge into serum and other blood products. The blood tube is spun for ten minutes at 3000 rpm to separate out the serum. Additional ten minute intervals in the centrifuge can be utilized as needed for proper separation of the serum from the other blood products. A disposable glass pipet tip is used to transfer the separated serum into a sterile 5.0 mL screw-cap Nalgene cryogenic vial. Since VOCs are highly volatile, care must be taken to insure that samples are kept at cold temperatures during storage and shipment. All samples should be placed on dry ice or in a -70 °C freezer within 30 min of sample collection. In addition, samples should be shipped with enough dry ice or equivalent cooling material to insure that the samples will remain frozen throughout the shipment process. Samples should be shipped to insure that the samples will arrive at the CDC on normal business days to insure the proper processing of the samples upon arrival. Samples should be shipped within 1 to 2 days of collection.

d. Sample Quantity

The optimal amount of specimen required for this method is 0.60 mL. Required amount of sample for analysis is 0.250 mL. This optimum amount (0.60 mL) allows us to do one repeat if necessary.

e. Unacceptable Specimens

The criteria for unacceptable specimen are a low volume (<0.25 mL), failure to control clotting time, failure to maintain sample as frozen before sample analysis, and suspected contamination.

Failure to obtain adequate sample volume is obvious when the samples are received. Visual inspection of the cryogenic vials reveals the estimated serum volume is less than the required 0.25 mL. Maintenance of temperature during shipment is verified by examining the shipment temperature upon receipt. A description of reasons for each rejected sample is recorded in the STARLIMS relational database as the samples are logged into the lab.

5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides

Not applicable for this procedure.

6. Reagents, Standards, and other Materials and Supplies; Equipment and Instrumentation

a. Reagent Sources

1) Solvents

Reagent	Grade	Source*
Water	HPLC	Mallinckrodt Baker, Phillipsburg, NJ
Hydrochloric acid	HPLC	Fluka Analytical, St. Louis, MO
Methanol	P & T	Honeywell, Burdick & Jackson, Muskegon, MI

* Or equivalent

Purge and Trap grade methanol is required for all dilutions of native standards and labeled analogs. Other grades of methanol typically contain unacceptable levels of aldehyde contamination.

HPLC grade water of acceptable purity has been acquired from Baker-Mallinckrodt. Variability in the contaminant levels in this product requires the testing of product lots. Once an acceptable lot has been found, a 1-year supply of water may be purchased to ensure an adequate supply. The purchased water is further processed by helium sparging and distillation to reduce VOCs before use. Directions for this procedure are given below under Section 6.b, and are based on previously published techniques for removing residual VOCs from reagent water (3).

b. Reagent Preparation

1. Preparation of Blank water

a. Apparatus

Distillation of the raw water is accomplished using a Fuchs continuous reflux apparatus which has been modified to run with helium stripping during the distillation process.

b. Procedure

1. Water distillation

   Fill the 12,000 mL 3-neck flask with approximately 10,000 mL of HPLC grade water. Adjust the helium flow to produce an active flow through the sparger. Allow the helium to bubble through the raw water for approximately 12-18 hrs. After sparging, turn on the heating mantle to bring the water to a boil. Allow the water to reflux for approximately 2 hrs. At the end of the reflux period, begin collecting the finished blank water. Dispense the finished water into 100 mL glass stoppered Pyrex bottles and cap immediately. If more blank water is needed, allow the storage head to refill and repeat the process.

2. Water storage

   The blank water is either used directly from the glass stoppered Pyrex bottles or stored in 5 and 20 mL flame sealable Pyrex ampoules. Water is aliquoted for storage by transfer from the Pyrex bottles using a Portapet Pipetter equipped with a 10 mL long tip glass serum pipette. A torch (methane and oxygen fuel) is used to melt the ampoule neck to produce a gas-tight seal. A tight seal is checked by tapping the sealed end on a hard surface and looking for a water leak. The sealed ampoules are stored in the dark at room temperature.

c. Standard Materials

1) Native Standards

Purchase native aldehyde standards from a commercial vendor. One such vendor is Sigma-Aldrich Company (Milwaukee, WI). Prepare one combined native standard (except acetaldehyde) stock solution in methanol in a 50 mL volumetric flask (Solution VII). Acetaldehyde stock Solution VIIa is to be prepared separately in methanol in a 10 mL volumetric flask. Store the two stock solutions in a -20ºC freezer. Make the individual calibration standard solutions from the combined native standard stock solution(s) as described below.

a) Standard Stock Solution VII (mg/ml)

Add about 40 mL purge and trap grade methanol to one 50 mL volumetric flask. Label the 50 mL flask “Stock Solution VII”. Sequentially add the following neat compounds listed in the table

below to the flask labeled “Stock Solution VII" and determine the total weight, to 0.0001 g, between each addition. Keep the lid tightly screwed onto the flask unless volume is being added, minimizing the loss of methanol and/or analyte due to evaporation.

Formulation of Stock Solution VII (mg/mL)

Analyte	Volume (µL)	Density (g/mL)	Expected Wt. (g)
Acrolein	500	0.839	0.4195
Propanal	150	0.805	0.1208
Isobutanal	60	0.790	0.0474
Butanal	50	0.800	0.0400
Isopentanal	60	0.804	0.0482
Crotonaldehyde	30	0.853	0.0256
Pentanal	200	0.810	0.1620
Hexanal	400	0.815	0.3260
Furaldehyde	100	1.16	0.1160
trans-2-Hexenal	50	0.846	0.0423
Heptanal	30	0.817	0.0245
Benzaldehyde	40	1.045	0.0418
Octanal	75	0.820	0.0615
trans-2-Octenal	150	0.846	0.1269
o-Tolualdehyde	8	1.039	0.0083
Nonanal	200	0.827	0.1654
trans-2-Nonenal	150	0.846	0.1269
Decanal	100	0.830	0.0830

Acetaldehyde Standard Stock Solution VIIa (mg/mL)

Add about 8 mL purge and trap grade methanol to one 10 mL volumetric flask. Label the 10 mL flask “Stock Solution VIIa”. Add neat acetaldehyde, as listed in table below to the flask labeled “Stock Solution VIIa” and determine the total weight, to 0.0001g, between each addition. Keep the lid tightly screwed onto the flask unless volume is being added, minimizing the loss of methanol and/or analyte due to evaporation.

Formulation of Acetaldehyde Stock Solution VIIa

Analyte	Volume (µL)	Density (g/mL)	Expected Wt. (g)
Acetaldehyde	1700	0.785	1.3345

Once all of the neat standards are added to the flasks, fill the flasks to the mark with purge and trap grade methanol. Invert flask 5 times and mix via sonication for 3 min to insure complete mixing. Stock Solutions VII and VIIa

are now prepared and ready to be further diluted for the preparation of standards.

b) Standard Stock Solution VI

Use the flask labeled Stock Solution VI to dilute 750 µL of Stock Solution VII and 1000 µL of Stock Solution VIIa in 25 mL of purge and trap grade methanol. Invert flask Stock Solution VI 5 times and mix via sonication for 3 min to insure complete mixing. Stock Solution VI is now prepared and ready to be further diluted for the preparation of standards.

c) Standard Calibration Standards Stock Solutions (ng/ml)

Add about 20 mL purge and trap grade methanol to nine 25 mL volumetric flasks. Prepare ampoule standards 1 - 9 by preparing dilutions of Stock Solutions VII, VIIa, and VI as listed in the table below.

Formulation of ampoule calibration standards using Stock Solution VII, VIIa, and VI as follows.

Standard number	Stock Solution Used	Volume Added (µL)	Final Volume (mL)
1	VI	40	25
2	VI	100	25
3	VI	200	25
4	VII, VIIa	15, 15	25
5	VII, VIIa	60, 30	25
6	VII, VIIa	160, 60	25
7	VII, VIIa	300, 100	25
8	VII, VIIa	400, 200	25
9	VII, VIIa	800, 400	25

Mix all solutions for 3 min. Label the ampoules with the standard level (1 - 9) and a standard batch identifier. Dispense the standard solutions into glass ampoules and flame seal as described in Appendix A. Make at least 100 sealed ampoules of each standard, 1 - 9. Label each ampoule with a sequential 3 digit number indicative of the preparation order of that aliquot and sealed (e.g. 005 is the fifth aliquot prepared for the above standard 2. After leak checking the flame sealed ampoules as described in Appendix A, place the ampoules in a 10 x 10 grid box and store in a -70 °C freezer.

d) Daily Standards (ng/ml)

Daily standards are made by taking particular concentrations of the above ampoules and diluting in helium sparged/distilled water. For example standard 5 is created by taking 40 µL from the flame sealed ampoule and adding into a 25-mL volumetric flask with water along with 350 µL of internal standard working solution (described below). Concentrations can be found in Section 2b.

2) Isotopically Labeled Internal Standards

Purchase ^{13}C-labeled and deuterium labeled aldehyde internal standards from a commercial vendor. One such vendor is Cambridge Isotope Laboratories, Inc. (Andover, MA).

Compound	Formula	Safety	Source
Acetaldehyde-d_4	$C^2H_3C^2HO$	a, c, d, e	f
Acetaldehyde-$^{13}C_2$	$^{13}CH3^{13}CHO$	a, c, d, e	f
Acrolein-$^{13}C_3$	$^{13}CH_2=^{13}CH^{13}CHO$	b, c, d, e	f
Propanal-^{13}C	$CH_3CH_2{}^{13}CHO$	e, d	h
Isobutanal-^{13}C	$(CH_3)_2CH^{13}CHO$	e, d	h
Butanal-^{13}C	$CH_3CH_2CH_2{}^{13}CHO$	e, d	i
Isopentanal-^{13}C	$(CH_3)_2CHCH_2{}^{13}CHO$	e, d	f
Crotonaldehyde-$^{13}C_4$	$^{13}CH_3{}^{13}CH=^{13}CH^{13}CHO$	e, c	h
Crotonaldehyde-d_6	$C^2H_3C^2H=C^2HC^2HO$	e, c	h
Pentanal-^{13}C	$CH_3(CH_2)_3{}^{13}CHO$	e, b	h
Hexanal-^{13}C	$CH_3(CH_2)_4{}^{13}CHO$	e, d	h
Furaldehyde-d_4	$C_4{}^2H_3OC^2HO$	e, b, a	i
Furaldehyde-C	$C_4H_3O^{13}CHO$	e, b, a	i
trans-2-Hexenal-^{13}C	$CH_3(CH_2)_2$ $CH=CH^{13}CHO$	e, d	j
Heptanal-^{13}C	$CH_3(CH_2)_5{}^{13}CHO$	e, d	h
Benzaldehyde-$^{13}C_7$	$^{13}C_6H_5{}^{13}CHO$	e, d	f
Octanal-^{13}C	$CH_3(CH_2)_6{}^{13}CHO$	e, d	h
trans-2-Octenal-^{13}C	$CH_3(CH_2)_4$ $CH=CH^{13}CHO$	e, d	j
o-Tolualdehyde-^{13}C	$C_6H_5CH_3{}^{13}CHO$	e, d	i

Nonanal-^{13}C	$CH_3(CH_2)_7{}^{13}CHO$	e, d	h
trans-2-Nonenal-^{13}C	$CH_3(CH_2)_5$ $CH{=}CH{}^{13}CHO$	e, d	j
Decanal-^{13}C	$CH_3(CH_2)_8{}^{13}CHO$	e, d	h

Key: a - Cancer suspect agent; b - Toxic; c - Lachrymator; d - Flammable liquid; e - Irritant; f - Aldrich Chem.(Milwaukee, WI); g – Fluka Chemicals; h – Can SynChem Corp; i – CDN Isotopes; j – ISOSCIENCES, LLC

a) Primary analog L stock solutions

Primary analog L stock solutions are made by initial dilution of the neat compound into 10 mL of purge and trap grade methanol. L solutions were made for each labeled analog. This provides a consistent source of these compounds for further dilutions. For each analog add approximately 9 mL purge and trap grade methanol to a 10-mL volumetric flask. Keep the flask sealed when not directly adding standards. Structures can be found in Appendix B. Label the flask and dilute the compounds according to the scheme described below:

Aldehyde labeled internal L stock solution

Standard	**Compound**	**Approximate Concentration (ppm)**
L	Acetaldehyde-d_4	41236
L	Acetaldehyde-$^{13}C_2$	67220
L	Acrolein-$^{13}C_3$	7000.0
L	Propanal-^{13}C	23170
L	Isobutanal-^{13}C	27800
L	Butanal-^{13}C	9736.0
L	Isopentanal-^{13}C	12456
L	Crotonaldehyde-$^{13}C_4$	17280
L	Crotonaldehyde- d_6	3676.0
L	Pentanal-^{13}C	30241
L	Hexanal-^{13}C	15242
L	Furaldehyde-d_4	47200
L	Furaldehyde-C_1	8100.0

L	*trans*-2-Hexenal-^{13}C	27200
L	Heptanal-^{13}C	19181
L	Benzaldehyde-$^{13}C_6$	12142
L	Octanal-^{13}C	38070
L	*trans*-2-Octenal-^{13}C	14724
L	*o*-Tolualdehyde-^{13}C	4313.0
L	Nonanal-^{13}C	15580
L	*trans*-2-Nonenal-^{13}C	6036.0
L	Decanal-^{13}C	21270

Fill the flasks to the 10 mL mark with purge and trap grade methanol. Seal approximately 0.250 mL of these solutions in ampoules as described above. Repeat these steps until as many sealed ampoules (at least 30) as possible are made. Label and place the sealed ampoules in an appropriate holder and store in a -70 °C freezer.

b) Secondary analog stock solutions (Ampouled) (SLVII)

Label and fill a 50 mL volumetric flask with approximately 45 mL of purge and trap grade methanol. Label the flask and dilute the compounds according to the scheme described in the table below:

Aldehyde labeled internal stock solution (Ampouled) SLVII.

Compound	**Volume of Stock L (µL)**	**Approximate Concentration (ppm)**
Acetaldehyde-d_4	80	65.98
Acetaldehyde-$^{13}C_2$	50	67.22
Acrolein-$^{13}C_3$	600	84.00
Propanal-^{13}C	30	13.90
Isobutanal-^{13}C	20	22.24
Butanal-^{13}C	160	31.16
Isopentanal-^{13}C	40	9.960
Crotonaldehyde-$^{13}C_4$	20	6.910
Crotonaldehyde-d_6	100	7.350
Pentanal-^{13}C	12	7.260

Hexanal-^{13}C	200	60.97
Furaldehyde-d_4	150	141.6
Furaldehyde-^{13}C	800	129.6
trans-2-Hexenal-^{13}C	100	54.04
Heptanal-^{13}C	40	15.34
Benzaldehyde-$^{13}C_6$	40	9.710
Octanal-^{13}C	40	30.46
trans-2-Octenal-^{13}C	200	58.90
o-Tolualdehyde-^{13}C	80	6.900
Nonanal-^{13}C	300	93.48
trans-2-Nonenal-^{13}C	1400	169.0
Decanal-^{13}C	360	153.1

Fill to the mark with purge and trap grade methanol and mix the solution by sonicating for 3 min. Seal approximately 150 µL of these solutions in ampoules as described above in Appendix A. Repeat these steps until as many sealed ampoules as possible are made (at least 100). Label and place the sealed ampoules in an appropriate holder and store in a -70 °C freezer.

c) Working ISTD solutions

This method uses two ISTD working solutions. One is used for standards and one is used for unknown samples, QCs, and water blanks. Prepare the ISTD working solution for standards by adding 100 µL from the ampoule solution SLVII into a 10-mL flask containing VOC free water. This solution will be used to add 350µL to each working standard solution (STDs 1-9). From the same ampoule solution SLVII take 25 µL and add to a 25-mL flask containing VOC free water. This solution is used to spike 40 µL of ISTD into each sample (serum, QC, and water blanks).

3) Quality Control Materials

QC Materials are prepared with the final concentrations (ppb) given in the table below. These concentrations allow for the loss of some compounds by volatilization during the mixing step.

Approximate concentrations of aldehydes for low and high QC pools.

Analyte	Low QC (ppb)	High QC (ppb)
Acetaldehyde	165	212
Acrolein	30.4	49.8
Propanal	18.4	24.3
Isobutanal	4.95	5.69
Butanal	4.34	6.54
Isopentanal	4.70	6.91
Crotonaldehyde	0.850	1.77
Pentanal	18.6	27.5
Hexanal	90.7	108
Furaldehyde	13.7	23.5
trans-2-Hexenal	2.54	4.99
Heptanal	5.70	7.23
Benzaldehyde	10.9	16.0
Octanal	2.84	3.91
trans-2-Octenal	12.6	18.5
o-Tolualdehyde	0.700	1.20
Nonanal	10.7	16.2
trans-2-Nonenal	34.5	47.4
Decanal	4.83	6.86

a) Serum Collection

Two non-smoker human serum lots (approximately 300 mL each) were used as quality control pools. Collect blood in an appropriate serum separator tube. Invert 5 to 10 times, and let sit for 15-30 minutes at room temperature to allow blood to clot. Within two hours of collection, spin tube at 10000 rpm for 10-15 minutes. Recover serum with a transfer pipette. If red blood cells are visible in bottom of collection tube, re-spin tube at 10000rpm for an additional 10 min. Before further processing, screen stock serum for aldehydes.

b) Spiking serum for QC preparation

Thoroughly mix the two nonsmoker serum lots. When homogeneous, divide into two lots of equal volume. Both lots were spiked with a known concentration of aldehydes for the low and high QC pools.

c) Preparation of stock solution Q1 for low and high QC

Add 50 and 75 µL of Stock VI to 300 g serum lots to obtain the concentrations shown below for low and high QC pools, respectively.

Calculations of approximate concentrations of spike for high QC

Analyte	**Low QC (ppb)**	**High QC (ppb)**
Acetaldehyde	165	212
Acrolein	30.4	49.8
Propanal	18.4	24.3
Isobutanal	4.95	5.69
Butanal	4.34	6.54
Isopentanal	4.70	6.91
Crotonaldehyde	0.850	1.77
Pentanal	18.6	27.5
Hexanal	90.7	108
Furaldehyde	13.7	23.5
trans-2-Hexenal	2.54	4.99
Heptanal	5.70	7.23
Benzaldehyde	10.9	16.0
Octanal	2.84	3.91
trans-2-Octenal	12.6	18.5
o-Tolualdehyde	0.700	1.20
Nonanal	10.7	16.2
trans-2-Nonenal	34.5	47.4
Decanal	4.83	6.86

d. Other Materials and Supplies

Materials, supplies and sources used during the development and validation of this method are listed below. Materials and supplies for use with this method should be equivalent to those listed if obtained from other sources.

- Pipettes and disposable tips capable of accurately dispensing the following volumes: 0.5 µL to 250 µL, 500µl, 1mL
- Gilson Variable positive displacement microliter pipettes 20-100 µL, 50-250 µL
- Disposal Pasteur pipettes and rubber bulbs
- Clear pre-scored ampoules; 2 mL, 5 mL, and 20 mL
- Clear glass vacuoles, 1 mL and 10 mL
- Graduated 10 mL glass pipette
- Portapet pipette, 10 mL volume
- Heavy duty desiccator and molecular sieve
- VWR positive displacement micro-dispensors, 40 µL, 50 µL, 100 µL, 250 µL
- VWR replacement glass capillaries, 40 µL, 50 µL, 100 µL, 250 µL
- SMI positive displacement micro-pipettors, 20-100 µL, 50-250 µL
- SMI replacement glass capillaries, 20-100 µL, 50-250 µL
- Pyrex volumetric flasks with Teflon-lined screw caps, 25 mL and 50 mL
- Headspace Vials, 10 mL
- Septa, Teflon Beige/White silicone, Level IV
- Stainless steel washers, metric size 10 cm
- Hand-operated 20-mm Crimper
- Open Center seals 20-mm
- Monoject brand sterile blood collection tubes, 10-mL draw, 16 X 100-mm
- Ultrasonic cleaner with heater and timer

- Fisher hematology mixer
- Orbital shaker
- Research-grade helium gas, 99.999%
- Research-grade methane gas, 99.99%
- Oxygen, 99.99%, 200-300 cu. ft.
- Liquid nitrogen, 160-L, 22-psi
- Flow tube, 150-mm, for helium 0 to 100 cc/min
- Adapter 1/8" to 1/8" MPT
- DB-624 Capillary Column, 0.20-mm I.D., 25-m, 1.12-µm film
- Assorted laboratory glassware, racks, trays, and containers

e. Equipment

Equipment used during the development, validation, and application of this method are listed below. Equipment procured from other sources should meet or exceed these listed requirements.

- Gas Chromatograph system with sub ambient cooling such as Thermo 1300 (or comparable)
- High Resolution Double-Focusing Magnetic Sector (DFS) mass spectrometer such as Thermo-Fisher DFS (or comparable)
- Dell OPTIPLEX 960 w/ 40.0 GB hard disk, 8.0 GB RAM, flat screen monitor, and Pentium(IV) 2.4 GHz processor (or comparable)
- Squaroid vacuum oven, 2.3-cu. ft.
- Direct-drive vacuum pump
- Sartorius analytical balance
- Bar code scanner
- Datamax label matrix label printer and Brady printer labels

- Ultra-low temperature freezer
- Refrigerator
- Standard laboratory freezer
- Sterilized hood biological safety cabinet
- Laboratory Information Management System (LIMS) database
- PC SAS statistical software
- Distillation equipment for helium-sparged distilled water
- Purging equipment for QC material

f. Instrumentation

1) DFS high resolution mass spectrometer (Thermo Fisher Scientific) combined with a Combi-PAL auto-sampler (CTC Analytics) using SPME-fiber technology (Supelco) and with gas chromatographic separation (Thermo 1300 GC) uses a 25-m DB-624 capillary column (J&W Scientific) with 1.12-µm film thickness and 200-µm internal diameter. The carrier gas is helium.

a) Thermo 1300 GC Parameters

Event Run Table

Time (min)	Switching Valve #	Set Point	Condition Initiated
0.00	1	ON	Cryo-Trap Cools to -100°C
2.00	1	OFF	Cryo-Trap Heats to 220°C
24.00*	1	ON	Cryo-Trap Cools to -100°C

* Valve #1 must close (ON) before the end of GC run.

Event RUN Table (only exists in the GC microprocessor)

Injector type: Split/splitless inlet (front inlet)
- Injection mode: Splitless
- Purge flow: 70 mL/min
- Purge time: 2.00 min
- Inlet temperature: 200 °C
- Carrier gas: Helium
- Carrier gas control mode: Constant flow at 0.70 mL/min

Front Detector: 240 °C GC/MS transfer line

GC column: DB-624 (J&W Scientific)
- Length: 25 m
- Diameter: 200 µm
- Film thickness: 1.12 µm

GC Oven temperature program:
- Initial Temp: -1 °C for 3 min
- Ramp 1: 50 °C /min to 30 °C; hold at 30 °C for 1 min
- Ramp 2: 10 °C /min to 200 °C; no hold at 200 °C
- Ramp 3: 50 °C /min to 220 °C; hold at 220 °C for 3 min
- Equilibrium time: 0.05 min
- Run time: 24.71 min

Cryogenic cooling for GC Oven:
- Liquid nitrogen at 22 psi
- Quick cooling enabled
- Ambient temp: 25 °C
- Cryo timeout: 60 min
- Shutdown enabled

b) Chronos- PAL Auto-sampler (Axel Semrau)

i) Chronos Method: SPME Twin Single Arm Thermo System

Note: This Chronos method is able to utilize both functions of either one or two auto-sampler PAL arms. If the “Hydrolysis” function is selected as “Yes” then both PAL arms will be used (twin arm). If “No” is selected, then only the fiber PAL arm will be operated (single arm).
Parameters:
- Acid volume: 330 µL
- Acid Source: Res1
- Mixing time: 1200 sec
- Incubation Time: 10 sec
- Enrichment Time: 600 sec
- Desorption Time:1780 sec
- GC Runtime + Cooling Down: 1800 sec
- Injector: GC Inj1
- Auto-sampler Combi-PAL
- Prep Auto-sampler: PrepPAL
- Xcalibur Method: Aldehyde_2016_KOEN_717_WIN7
- Vial Penetration: 25 mm

- Injector Penetration: 54 mm

*Tasks 1-7 only apply for twin arm (when Hydrolysis function is “Yes”)

	Task	**Description**
1	Repeat	CleanCycles if Hydrol is YES else False
2	Transfer	Rinse
3	RepeatEnd	
4	Transfer	Add acid to sample
5	Transport	Move Sample to Mixer
6	WaitOverlapped	Wait for Sample to Mix
7	Transport	Move Sample to Agitator for Dual Arm
8	Transport	Move Sample to Agitator for Single Arm
9	MoveToHome	Put the Arm Home
10	WaitOverlapped	Incubation
11	FiberExposure	Expose Fiber in Sample
12	Repeat	
13	Transfer	Rinse
14	RepeatEnd	
15	MoveToHome	Put the Arm Home
16	Wait	Enrichment for Dual Arm
17	Wait	Enrichment for Single Arm
18	SetOutputSignal	Send Prepare Signal
19	Wait	Allow the method to download to the GC
20	FiberAspiration	Pull Fiber out of Sample
21	FiberExposure	Expose Fiber in Injector
22	SetOutputSignal	Send extra injected injected signal to ensure GC start program
23	Transport	Move the sample back with prep sampler
24	MoveToHome	Put the Arm Home
25	WaitOverlapped	Desorption in injector
26	Wait	
27	Fiber Aspiration	Remove the fiber from the Injector
28	Wait	
29	MoveToHome	Put the SPME Arm Home
30	WaitOverlapped	GC-remaining time

Peltier Tray (CTC Analytics)

Peltier Tray uses solid state technology to cool the metal sample tray (also called cooler 1 and 2)
- Temp set point: 15 °C
- Actual cooling range: 15-16 °C

(a) Cryo-Trap (Scientific Instrument Services, Inc.)

Cryo-Trap used to trap volatiles on head of GC column by cryogenic cooling
- Temp set point: -100 °C
- Liquid nitrogen at 22 psi

Cryo-Trap desorbs trapped volatiles by ballistic heating
- Temp set point: 220 °C
- Electric Heater

The cooling and heating cycles of the Cryo-Trap are controlled by the Thermo 1300 GC through Switching Valve Driver #1 in the RUN Table.

(b) DFS high resolution mass spectrometer (Thermo Fisher Scientific).
Parameters
Lua Procedure: sleep.lua 4
Tune File: tune1
MCAL File: mcal1 (for small mass range)
Calibrate the EDAC (electric scan) using the Lua command:
electric calibration 100 131 5 (done once a week or after resetting the DFS)

Acquisition START is controlled by the Thermo 1300 GC through Switching Valve Driver #1 in the RUN Table.

Calibration and Lock: PFK (high boiling range)
PFK Lock Masses used: 39.9618 for Time Segments 1 and 2, 68.9947 for Time Segments 3-8, and 99.9931 for Time Segments 9-11.

PFK Cali Masses used: 68.9947 for Time Segment 1 and 2, 99.9931 for Time Segments 3-8, 118.9915 for Time Segment 9, and 130.9915 for Time Segments 10-11.

- Starting intensities for the Lock and Cali masses (at 10000 mass resolution [5% valley definition] at beginning of sample queue at the operational voltage of the electron multiplier): 130.9915 signal at 1.5 to 2 volts (used to set ion signals from the PFK). Neat PFK (about 1.0 µL) is added by a 10µL syringe to a heated reservoir of the Reference Gas Inlet and the needle valve of the reservoir is adjusted to deliver the above signal intensities. Over a 24-hr period the signals of the Lock and Cali masses will decrease slowly as the gaseous mixture pumps away.

Multiple Ion Detection (MID) Method Global Settings:

- MID file: aldehyde_2013_all_final01.mid
- Mass resolution: 10000
- MID mode: Lock
- Offset: 20 µV
- Measure/Lock ratio: 1
- Width first lock: 0.20 amu
- Acquisition mode: Centroid
- Sweep peak width: 3.00
- Electric Delay: 8 ms
- Magnetic Delay: 150 ms
- Electric range: 300%
- Number of MID Sections: 11
- Ionization mode: EI Positive
- Acquisition Time: 4.00 to 21.00 min

MID Segment Time Settings for MID file aldehyde_2013_all_final01 (11 sections):

Typical Section 1 Time Settings.

Section 1	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	4.00	Lock	39.9618	1	9	5	PFK Ar^+
Measure Time	2.00		41.0022	1	1	51	Acetaldehyde Confirm $CHCO^+$
End Time	6.00		42.0100	1	1	51	Acetaldehyde Quant CH_2CO^+
			46.0324	1	1	51	Acetaldehyde $^{13}CH_3{}^{13}CHO^+$
CycleTime 0.40 sec			48.0508	1	1	51	Acetaldehyde $CDOCD_3{}^+$
		Cali	68.9947	1	9	5	PFK $CF_3{}^+$

Typical Section 2 Time Settings.

Section 2	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	6.00	Lock	39.9618	1	9	9	PFK Ar^+
Measure Time	1.20		55.0178	1	1	56	Acrolein Confirm $CH_2{=}CHCO^+$
End Time	7.20		56.0257	1	1	56	Acrolein Quant $CH_2{=}CHCHO^+$
Cycle Time 0.50 sec			57.0335	1	1	56	Propanal Confirm $CH_3CH_2CO^+$
			58.0279	1	1	56	Acrolein $^{13}CH_2{=}{}^{13}CH^{13}CO^+$
			58.0413	1	1	56	Propanal Quant $CH_3CH_2CHO^+$

			59.0447	1	1	56	Propanal $CH_3CH_2{}^{13}CHO^+$
		Cali	68.9947	1	9	9	PFK CF_3^+

Typical Section 3 Time Settings.

Section 3	Min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	7.20		57.0335	1	1	51	Isobutanal/Butanal Confirm $(CH_2)_2CHO^+$
Measure Time	2.30	Lock	68.9946	1	10	5	PFK CF_3^+
End Time	9.50		72.0570	1	1	51	Butanal Quant $CH_3(CH_2)_2CHO^+$
			72.0570	1	1	51	Isobutanal Quant $(CH_3)_2CHCHO^+$
Cycle Time 0.40 sec			73.0603	1	1	51	Butanal $CH_3CH_2CH_2{}^{13}CHO^+$
			73.0603	1	1	51	Isobutanal $(CH_3)_2CH{}^{13}CHO^+$
		Cali	99.9931	1	10	5	PFK $C_2F_4^+$

Typical Section 4 Time Settings.

Section 4	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	9.50		58.0413	1	1	56	Isopentanal Confirm $CH_3CH_2CHO^+$
Measure Time	0.85		68.0257	1	1	56	Crotonaldehyde Confirm $CH_2CH{=}CHCO^+$
End Time	10.35	Lock	68.9947	1	9	6	PFK CF_3^+
Cycle Time 0.50 sec			70.0413	1	1	56	Crotonaldehyde Quant $CH_3CH{=}CHCHO^+$
			71.0491	1	1	56	Isopentanal Quant $CH_3CHCH_2CHO^+$
			72.0525	1	1	56	Isopentanal $CH_3CHCH_2\ {}^{13}CHO^+$
			74.0547	1	1	56	Crotonaldehyde $^{13}CH_3{}^{13}CH{=}{}^{13}CH{}^{13}CHO^+$
		Cali	99.9931	1	9	6	PFK $C_2F_4^+$

Typical Section 5 Time Settings.

Section 5	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	10.35		57.0335	1	1	57	Pentanal Quant

							$CH_3CH_2CO^+$
Measure Time	1.65		58.0413	1	1	57	Pentanal Confirm $CH_3CH_2CHO^+$
End Time	12.00		59.0447	1	1	57	Pentanal $CH_3CH_2{}^{13}CHO^+$
Cycle Time 0.50 sec		Lock	68.9947	1	10	5	PFK CF_3^+
		Cali	99.9931	1	10	5	PFK $C_2F_4^+$

Typical Section 6 Time Settings.

Section 6	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	12.00	Lock	68.9947	1	10	5	PFK CF_3^+
Measure Time	1.80		72.0570	1	1	57	Hexanal Confirm $CH_3CH_2CH_2CHO^+$
End Time	13.80		82.0777	1	1	57	Hexanal Quant $CH_3(CH_2)_3CH{=}C^+$
Cycle Time 0.38 sec			83.0811	1	1	57	Hexanal 13C $CH_3(CH_2)_3CH{=}{}^{13}C^+$
		Cali	99.9931	1	10	5	PFK $C_2F_4^+$

Typical Section 7 Time Settings.

Section 7	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	13.80		67.0178	1	1	43	Furaldehyde Confirm $C_4H_3O^+$
Measure Time	1.00	Lock	68.9947	1	10	4	PFK CF_3^+
End Time	14.80		83.0491	1	1	43	Hexenal Quant $CH_2{=}CHCH{=}CHCHO^+$
Cycle Time 0.40 sec			84.0525	1	1	43	Hexenal 13C $CH_2{=}CHCH{=}CH^{13}CHO^+$
			96.0206	1	1	43	Furaldehyde Quant $C_4H_7OCHO^+$
			98.0316	1	1	43	Furaldehyde-D_4 $C_4D_3OCDO^+$
			97.0648	1	1	43	Hexenal Confirm $CH_3(CH_2)_2CH{=}CHCO^+$
		Cali	99.9931	1	10	4	PFK $C_2F_4^+$

Typical Section 8 Time Settings.

Section 8	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	14.80	Lock	68.9947	1	10	6	PFK CF_3^+
Measure Time	1.20		81.0699	1	1	62	Heptanal Quant $(CH_2)_4CHC^+$
End Time	16.00		82.0732	1	1	62	Heptanal ^{13}C $(CH_2)_4CH^{13}C^+$
Cycle Time 0.38 sec			86.0726	1	1	62	Heptanal Confirm $CH_3(CH_2)_3CHO^+$
		Cali	99.9931	1	10	6	PFK $C_2F_4^+$

Typical Section 9 Time Settings.

Section 9	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	16.00		83.0587	1	1	51	Benzaldehyde $^{13}C_6H_5^+$
Measure Time	1.50		99.0804	1	1	51	Octanal Confirm $(CH_2)_5CHO^+$
End Time	17.50	Lock	99.9931	1	10	5	PFK $C_2F_4^+$
Cycle Time 0.40 sec			101.0916	1	1	51	Octanal $CH_3(CH_2)_4{}^{13}CHO^+$
			105.0335	1	1	51	Benzaldehyde Confirm $C_6H_5CO^+$
			106.0413	1	1	51	Benzaldehyde Quant $C_6H_5CHO^+$
			110.1090	1	1	51	Octanal Quant $CH_3(CH_2)_5CHC^+$
		Cali	118.9915	1	10	5	PFK $C_2F_5^+$

Typical Section 10 Time Settings.

Section 10	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	17.50		97.0648	1	1	34	Octenal Quant $CH_3(CH_2)_2CH=CHCHO+$
Measure Time	1.60	Lock	99.9931	1	8	4	PFK $C_2F_4^+$
End Time	19.10		108.0934	1	1	34	Octenal Confirm $CH_3(CH_2)_3CHCHCCH^+$
			109.0967	1	1	34	*trans*-2-Octenal $CH_3(CH_2)_4{}^{13}CHO^+$
Cycle Time 0.50 sec			114.1039	1	1	34	Nonanal Quant $CH_3(CH_2)_5CHO^+$

			115.1073	1	1	34	Nonanal $CH_3(CH_2)_5{}^{13}CHO^+$
			119.0491	1	1	34	*o*-tolualdehyde Quant $C_6H_4CH_3CO^+$
			120.057	1	1	34	*o*-tolualdehyde Confirm $C_6H_4CH_3CHO^+$
			121.0603	1	1	34	*o*-tolualdehyde $C_6H_4CH_3{}^{13}CHO$
			124.1247	1	1	34	Nonanal Confirm $CH_3(CH_2)_7CHC^+$
		Cali	130.9915	1	8	4	PFK $C_3F_5{}^+$

Typical Section 11 Time Settings.

Section 11	min	L/C	Mass m/z	Gain	Int.	Time (ms)	Ion Type
Start Time	19.10	Lock	99.9931	1	9	5	PFK $C_2F_4{}^+$
Measure Time	1.90		109.1012	1	1	50	Decanal Quant $(CH_2)_6CHC^+$
End Time	21.00		111.0804	1	1	50	Nonenal Quant $(CH_2)_7CHO^+$
Cycle Time 0.45 sec			122.1090	1	1	50	Nonenal Confirm $CH_3(CH_2)_5CHC+$
			112.0838	1	1	50	Nonenal_^{13}C $(CH_2)_7{}^{13}CHO^+$
			128.1196	1	1	50	Decanal Confirm $CH_3(CH_2)_6CHO^+$
			129.1229	1	1	50	Decanal_^{13}C $CH_3(CH_2)_6{}^{13}CHO^+$
		Cali	130.9915	1	9	5	PFK $C_3F_5{}^+$

2.) Typical retention time and mass for aldehydes for a level 3 water calibrator are listed below.

GC retention times for the analytes and labeled analogs:

Analyte Native	RT Native (min)	ISTD Label	RT ISTD (min)	Code ANALYTE
Acetaldehyde	4.52	Acetaldehyde-$^{13}C_2$	4.52	2AL
Acetaldehyde	4.52	Acetaldehyde-d_4	4.52	2AL
Acrolein	6.05	Acrolein-$^{13}C_3$	6.05	ACAL

Propanal	6.12	Propanal-^{13}C	6.12	3AL
Isobutanal	7.32	Isobutanal-^{13}C	7.32	I4AL
Butanal	8.07	Butanal-^{13}C	8.07	4AL
Isopentanal	9.40	Isopentanal-^{13}C	9.40	I5AL
Crotonaldehyde	9.58	Crotonaldehyde-$^{13}C_4$	9.58	CRAL
Crotonaldehyde	9.58	Crotonaldehyde-d_6	9.58	CRAL
Pentanal	10.33	Pentanal-^{13}C	10.33	5AL
Hexanal	12.46	Hexanal-^{13}C	12.46	6AL
Furaldehyde	13.64	Furaldehyde-d_4	13.64	FRAL
Furaldehyde	13.64	Furaldehyde-^{13}C	13.64	FRAL
trans-2-Hexenal	13.79	*trans*-2-Hexenal-^{13}C	13.79	T6AL
Heptanal	14.45	Heptanal-^{13}C	14.45	7AL
Benzaldehyde	15.99	Benzaldehyde-$^{13}C_6$	15.99	BZAL
Octanal	16.32	Octanal-^{13}C	16.32	8AL
trans-2-Octenal	17.54	*trans*-2-Octenal-^{13}C	17.54	T8AL
o-Tolualdehyde	17.84	*o*-Tolualdehyde-^{13}C	17.84	TLAL
Nonanal	18.04	Nonanal-^{13}C	18.04	9AL
trans-2-Nonenal	19.18	*trans*-2-Nonenal^{13}C	19.18	T9AL
Decanal	19.62	Decanal-^{13}C	19.62	10AL

Ion masses used for analysis:

Analyte Native	RT Native (min)	Mass 1[a]	Mass 2 [a]	RT ISTD (min)	Mass ISTD
Acetaldehyde	4.52	42.0100	41.0022	4.52	48.0508
Acrolein	6.05	56.0257	55.0178	6.05	58.0279
Propanal	6.12	58.0413	57.0335	6.12	59.0447
Isobutanal	7.32	72.057	57.0335	7.32	73.0603
Butanal	8.07	57.0335	72.0570	8.07	73.0603
Isopentanal	9.40	71.0491	58.0413	9.40	72.0525
Crotonaldehyde	9.58	70.0413	68.0257	9.58	75.0727
Pentanal	10.33	57.0335	58.0413	10.33	59.0447
Hexanal	12.46	72.0570	82.0777	12.46	83.0811
Furaldehyde	13.64	96.0206	67.0178	13.64	98.0316
trans-2-Hexenal	13.79	83.0491	97.0648	13.64	84.0525
Heptanal	14.45	81.0699	86.0726	14.45	83.0732
Benzaldehyde	15.99	106.0413	105.0335	15.99	83.0587
Octanal	16.32	110.1090	99.0804	16.32	101.0916
trans-2-Octenal	17.54	97.0648	108.0934	16.32	109.0967
o-Tolualdehyde	17.84	97.0542	119.0491	17.84	121.0603
Nonanal	18.04	114.1039	124.1247	18.04	115.1073
trans-2-Nonenal	19.18	111.0804	122.1090	19.18	112.0838
Decanal	19.62	109.1012	128.1196	19.62	129.1229

7. Calibration

a. Creation of Calibration curve

1) Data Collection

A full set of 9 calibrators is analyzed with each batch of data and used for the quantification of analytes in all samples from that batch. The calibration curves are constructed for each analyte from the relative response factors for each of the 9 calibrators.

2) Calculation of curve statistics

The slope, intercept and R-squared value for the nine point calibration curves are generated using a 1/x-weighted linear regression using the Thermo Fisher Scientific Xcalibur Quan Browser software.

3) Evaluation of curve statistics

The R-squared values for each analyte calibration curve must in all cases be greater than 0.98. More than 90% of the time the R-squared values are greater than 0.98. Linearity of standard curves should extend over the entire standard range, three orders of magnitude. Intercepts calculated from the least squares fit of the data are typically not significantly different from 0.

b. Usage of Curve

All calibration standards are created in water because it proved impractical to consistently reduce the background VOC levels in serum below detectable levels. Matrix spike experiments established that calibration curves in serum and water have the same slope (shown in the table below). This validates the use of water-based calibrators for quantifying VOCs in serum.

Typical Slopes of calibration curves in water and serum

Analyte Code	Serum Calibration		Water Calibration	
	Slope	Y-intercept	Slope	Y-intercept
2AL	0.0213	1.1238	0.0267	4.3162
ACAL	0.5899	0.2197	0.6500	6.1594
3AL	0.4436	0.5831	0.4875	3.0516
I4AL	0.3215	0.0791	0.3347	0.0881
4AL	0.9110	0.4460	0.9554	2.3777
I5AL	0.6404	0.0301	0.6357	1.3838
CRAL	1.0738	0.0453	1.0473	0.4366
5AL	0.2304	0.3678	0.2501	0.7299
6AL	1.1125	1.2874	1.2056	5.6015
FRAL	0.0251	-0.0044	0.0523	0.0738
T6AL	0.1017	-0.0065	0.2107	0.0422
7AL	1.0406	0.5502	0.9830	1.0299
BZAL	0.1771	0.0185	0.1821	0.6835
8AL	0.7219	0.4129	0.6999	0.7152
T8AL	0.2162	4.0380	0.1420	0.1085
TLAL	1.1769	0.0404	1.0216	0.2119
9AL	0.1224	0.3106	0.0915	0.5716
T9AL	0.0264	-0.0166	0.0212	-0.0064
10AL	0.3560	2.0949	0.4161	2.4541

The lowest point on the calibration curve is at or below the measurable detection limits and the highest point is above the expected range of results. The remaining points in this curve are distributed between these two points with more calibrators in the concentration range at which more unknowns are determined. The calibration curve spans three orders of magnitude. The values of these standards are given below in the table.

c. Calibration Verification

Calibration is performed as part of each analytical run and a calibration curve is constructed from the nine calibration standards. Additional verification is conducted by quantitating Quality Control samples of known value against the calibration curve and statistically comparing the calculated results to known values.

Analyte	Typical concentration in final 25 mL water standard (ppb)								
	STD 9	STD 8	STD 7	STD 6	STD 5	STD 4	STD 3	STD 2	STD 1
Acetaldehyde	3542	1771	886	531	266	133	70.8	35.4	14.2
Acrolein	418	209	157	83.7	31.4	7.84	3.14	1.57	0.627
Propanal	124	62.2	46.6	24.9	9.324	2.33	0.932	0.466	0.186
Isobutanal	47.4	23.7	17.78	9.48	3.56	0.889	0.356	0.178	0.071
Butanal	41.1	20.5	15.4	8.21	3.08	0.770	0.308	0.154	0.062
Isopentanal	50.2	25.1	18.8	10.0	3.76	0.941	0.376	0.188	0.075
Crotonaldehyde	21.6	10.8	8.10	4.32	1.62	0.405	0.162	0.081	0.032
Pentanal	170	85.1	63.8	34.0	12.8	3.19	1.28	0.638	0.255
Hexanal	338	169	127	67.7	25.4	6.34	2.54	1.27	0.507
Furaldehyde	121	60.4	45.3	24.2	9.06	2.27	0.906	0.453	0.181
trans-2-Hexenal	43.3	21.7	16.2	8.66	3.25	0.812	0.325	0.162	0.065
Heptanal	26.4	13.2	9.91	5.28	1.98	0.495	0.198	0.099	0.040
Benzaldehyde	40.9	20.4	15.3	8.17	3.06	0.766	0.306	0.153	0.061
Octanal	62.8	31.4	23.5	12.6	4.71	1.18	0.471	0.235	0.094
trans-2-Octenal	134	66.8	50.1	26.7	10.0	2.51	1.00	0.501	0.200
o-Tolualdehyde	8.50	4.25	3.19	1.70	0.637	0.159	0.064	0.032	0.013
Nonanal	170	85.0	63.8	34.0	12.8	3.19	1.28	0.638	0.255
trans-2-Nonenal	136	68.1	51.1	27.2	10.2	2.55	1.02	0.511	0.204
Decanal	85.2	42.6	31.9	17.0	6.39	1.60	0.639	0.319	0.128

8. Procedure; Calculations; Interpretation of Results

a. Sample Preparation

Samples are transferred to the 10 mL SPME vials via a GILSON 250 µL positive displacement pipette.

1) Sample Handling

(a) All samples (both non-biological and biological) are transferred to a preassembled 10 mL SPME vial (using a Supelco open center seal, a steel washer, and a Supelco PTFE/Silicone faced septum) using a GILSON 250 µL positive displacement pipette. Rinse the pipette tip once with water (Baker, HPLC grade).

(b) Transferring sample (water or serum) into SPME vial. Discharge 40 µL of the internal standard solution into the aliquoted sample using the fixed 40 µL micro-pipettor. Place the used disposable glass micropipettes in a sharps container and rinse the plunger tip with HPLC grade water before replacing with a new glass capillary pipette. Crimp and seal the SPME vial using a hand crimper.

b. Sample Analysis

1) Setting the samples in Xcalibur

a. From the Xcalibur home page, click File New (Ctrl N). A New Sequence Template Dialog box will appear. Enter the Base File Name, data path, instrument method, and processing method. It is important to create a new sequence for each run. DO NOT use previous sequence as template.

b. Type the sample type (e.g. Blank, QC, Standard bracket, or unknown).

c. For each sample, fill in the sample comments, sample ID, vial position, and levels of standard or QC.

d. For NHANES study, the instrument method is C:\Xcalibur\methods\Aldehyde_2014_KOEN_717_Win7 and processing method is C:\Xcalibur\methods\Aldehyde_2016M_KOEN2_717.

e. All above steps can set up in Excel (csv) and import to Xcalibur.

f. Import the same sequence (csv) used in Xcalibur to Chronos.

    i. For NHANES study, the Chronos method is: SPME Twin Single Arm Thermo System

g. Once the sequence set up is complete, samples can be run.

2) Analyzing samples

    i. Create schedule using Chronos by highlighting samples and clicking **Create Schedule**. Chronos controls the timing of auto preparation. This will only schedule the sample preparation, it will not start Chronos (see step 3).
    ii. On Xcalibur, highlight samples to be run. On the Actions Menu, select **Run this Sample** or **Run Sequence.** Xcalibur is used to send the start signal to the GC and Mass Spectrometer.
    iii. Go back to Chronos tab. Click **Run** from the Run Tab. Chronos will begin sample prep.

iv. The last sample to be run should be the **shutdown** procedure.

Note: Make sure the large capacity reservoir contains adequate liquid nitrogen and that the liquid access valve is open.

c. Processing of Data

The first “sample” is a fiber cleaning procedure, and the second one is the blank water sample (Bench QC). The calculated ion ratios (native to internal standard area per analyte) are compared with historical ratios to check for possible contamination.

1) If the blank water passes the contamination check, run either the low or high QC serum sample and calculate the ion ratios (native to internal standard area per analyte). Using these ratios, ensure that the sample meets the QC Criteria described in section 10.b.2. If the QC sample passes, all the unknowns will be run and the second aliquot of the same QC will be run at the end of the sequence.

d. Morning startup procedure

1) Data System Startup

The data system PC may need to be reset.

(a) Turn off the data system PC by using the START menu and restart.

(b) After the system has restarted, log into Windows 7 by entering the user id and password.

2) Mass Spectrometer Setup

First double-click the Xcalibur Icon from desktop of the PC to connect the PC with mass spectrometer (DFS). If the DFS is connected to the PC, then the Xcalibur road map and tune parameters window will be appear. If not, switch the F1 lever on the rear section of the DFS. NOTE: If a black target icon appears in top left of tune page, close all windows on PC and switch F1 lever once more. Once the connection is established, a yellow standby icon will appear in top left. Click the yellow icon and it will turn to green triangle; the DFS is ready for tuning. Second double-click the Chronos Icon from desktop of the pc to connect the PAL auto-sampler.

3) Mass Spectrometer (DFS) Tuning

The tuning of the DFS is usually done once each day as follows.

(a) Add enough Lock mass calibrant (high boiling PFK) to achieve 2 to $4x10^6$ µV for mass131.
(b) Select mass 131 and obtain 10,000 resolution by changing the entrance and exit slit widths and also optimizing the beam rotation and focus quad on the Source Focusing Setting Dialog box. Select tune fork icon from menu. An Auto-tune dialog box will appear. All required parameters are on the Auto-tune Tab. Click Start to execute Auto-tune procedure. Auto-tune should be repeated until the reported change in intensity is smaller than +/-5%. Save tune and print report. This report is saved with archived raw chromatographic data.
(c) Click on the icon with the calibration curve. The calibration dialog box will appear. Select the advanced tab. Click on the box. This will open the reference editor. Search and select Reference mass 1 as 69 and reference mass 2 as 131. Click Calibrate to start the procedure. This will reset the EDAC Zero and EDAC Gain values.
(d) Run a short MID program to ensure that the masses will lock properly during the run by selecting the **aldehyde_2016_all_final01-short** mid program in the MID window. This method can be selected and started from the Define Scan Dialog. Select MID Tab. Select the MID Lock Test method aldehyde_2016_all_final01-short in the mode scroll down. Click start to run method. Observe the Lock process in the Tune View.
(e) Change the MID program to the proper MID for the study. (e.g. aldehyde_2016_all_final01 for the NHANES aldehyde method).

e. Data Analysis

1) Delete the fiber cleaning and shutdown entries from the Xcalibur sequence and save the file with an appropriate run name. (IYYMMDD.sld, where I is an alphabetic instrument identifier, YY is the 2 digit year identifier, and MM is the 2 digit month identifier, DD is the 2 digit date identifier, and .sld is the Xcalibur sequence file type).

2) Process the run by selecting the **Batch Process** button on the Tool Bar and then open the file by selecting **Quan Browser** from the Xcalibur road map page.

3) Resave the file as IYYMMDD.XQN, where .XQN is the Xcailibur quantitative file type.

4) For each analyte in turn, examine the chromatographic peaks to verify that the automated peak detection routine has correctly identified the peak for both the native compound and the internal standard.

5) Inspect the integration of all peaks and the baseline for all analytes; reintegrate manually where needed.

6) Inspect the calibration curve at the low concentration end and perform the correction to maximize the correlation coefficient. Due to the limited mass difference between native and labeled compounds, the native analytes have some response at the mass used for the labeled isotope. Likewise, the labeled isotope often contributes some response at the mass being used for analyte quantitation. In these cases, corrections must be made for the contribution of the labeled analyte to the native ion and vice versa. In order to properly determine the relative response factors between the analyte and analog, these effects must be taken into account. Xcalibur Quan Browser allows for these adjustments using the “Isotope%” function under “Calibration Settings”.

7) After data has been processed, resave the .XQN file.

8) To create the Excel long report, select File, Export data to Excel, Export Long Excel Report. The Excel file will automatically be saved to local drive.

9) Save all the data to an external USB to transfer to a CPU with network access.

10) Make a duplicate copy of the same data on a different DVD.

f. Transfer Data to the Network Drive

1) Transfer all the data created in the above steps and other files associated with the run to a secured shared network drive.

g. Transfer Data to the STARLIMS Database

1) Log onto the STARLIMS data base from your PC.

2) Import the Long Excel sheet for the particular data set by selecting the proper instrument, analyst, assay, study reference number, and run number.

3) Inspect the data and change the dates for all runs so that they all are the same date (if your run goes over two days) and transfer the data by selecting transfer button. This is to allow for the proper logic in evaluating which unknown results correspond to a particular QC sample.

4) Finally, review the run by using the review command to make sure data are transferred.

h. Evaluation of QA/QC Data

Two QC materials are included in each analytical run (QC Low and QC High). Acceptability of results for the entire analytical run is dependent upon the agreement of the results from these QC materials with established ranges.

For QC processing, examine each QC result and compare to the statistical data from the characterization runs (described in Section 10.b). If a QC result is "out-of-control," the cause of the failure must be determined and corrected. No results from the associated batch may be reported.

The following additional conditions will also necessitate elimination of results:

Low QC signal
Possible or suspected contamination
Outside of standard curve range
Unconfirmed result
Ion Ratio out of limits
Saturated signals

9. Reportable Range of Results

a. Limit of Detection

A total of 60 runs containing water blanks and 4 varying dilute analyte concentrations in serum samples labeled LOD00, LOD01, LOD02, and LOD03 were used to calculate the LOD limits for this assay. A 4 point plot of the LOD mean concentration vs LOD standard deviation gives a linear y=mx+b relationship, where m is the LOD slope and b is the LOD intercept. Using these two variables and the blank sample mean concentration and standard deviation, the LOD for each analyte can be calculated using the following equation:

$$\mathrm{LOD} = \frac{\mathrm{BL}_{\mathrm{mean}} + 1.645\left(\mathrm{BL}_{\mathrm{stdev}} + \mathrm{LOD}_{\mathrm{Int}}\right)}{1 - \left(1.645 * \mathrm{LOD}_{\mathrm{slope}}\right)}$$

The calculated LOD for each analyte is given in the table below

Typical LOD and Reportable range of results.

Analyte	Method LOD (ppb)	Lowest calibration standard (ppb)	Lower reportable limit (ppb)	Upper reportable limit (ppb)
Acetaldehyde	50.6	14.2	36.1	3540
Acrolein	2.16	0.627	1.19	418
Propanal	1.16	0.186	0.841	124
Isobutanal	0.109	0.071	0.141	47.4
Butanal	0.313	0.062	0.100	41.1
Isopentanal	0.119	0.070	0.075	50.2
Crotonaldehyde	0.147	0.032	0.155	21.6
Pentanal	0.316	0.255	0.436	170
Hexanal	0.693	0.507	0.830	338
Furaldehyde	1.24	0.181	0.219	121
trans-2-Hexenal	0.290	0.065	0.101	43.3
Heptanal	0.312	0.040	0.109	26.4
Benzaldehyde	0.461	0.061	0.544	40.9
Octanal	0.660	0.094	0.597	62.8
trans-2-Octenal	1.12	0.200	0.507	134
o-Tolualdehyde	0.142	0.013	0.074	8.50
Nonanal	2.63	0.255	1.08	170
trans-2-Nonenal	2.68	0.204	0.567	136
Decanal	3.90	0.128	2.36	85.2

b. Accuracy

No standard reference material is available for aldehydes in serum. The accuracy for this method in human serum was established by analyzing three levels of human serum spiked with known amount of aldehyde. The actual determined values for all analytes based on analytical recoveries range from 52-102%. Samples were run as triplicates. Low percent recovery of unsaturated aldehydes in serum (acrolein, crotonaldehyde, *trans*-2-hexenal, *trans*-2-octenal, and *trans*-2-nonenal) may be contributed to formation of non-Schiff base adducts (i.e. Michael addition)

Typical aldehyde recoveries from water

Analyte	Low spike				Medium Spike				High Spike			
	Target	Mean	CV %	Recovery %	Target	Mean	CV %	Recovery %	Target	Mean	CV %	Recovery %
Acetaldehyde	459	387	5.13	84.4	906	842	5.13	92.9	1308	971	5.88	74.2
Acrolein	24.2	24.1	0.548	99.4	50.5	50.3	1.26	99.7	74.8	74.6	4.62	99.8
Propanal	9.10	8.97	3.03	98.5	19.3	19.2	2.50	99.3	23.2	23.0	1.97	99.4
Isobutanal	-	-	-	-	7.41	7.73	0.55	104	13.7	13.61	0.33	99.3
Butanal	2.65	2.43	2.60	91.8	5.27	5.52	1.21	105	7.19	7.01	1.36	97.5
Isopentanal	2.21	2.21	1.79	100	4.34	4.43	1.55	102	6.26	5.49	3.02	87.7
Crotonaldehyde	0.549	0.479	3.68	87.3	1.10	1.12	3.32	102	1.59	1.40	1.87	88.2
Pentanal	10.9	11.2	3.17	103	21.6	24.9	1.90	115	31.3	30.5	0.03	97.6
Hexanal	13.6	13.4	1.93	98.5	27.1	25.5	0.253	94.0	39.1	41.7	10.2	107
Furaldehyde	7.43	7.74	2.49	104	14.8	16.7	1.83	113	21.3	22.9	0.893	107
trans-2-Hexenal	2.80	2.74	1.36	98.1	5.57	6.10	4.02	109	8.07	8.05	7.87	99.7
Heptanal	1.11	1.12	4.20	101	2.17	2.36	2.17	109	3.13	2.94	1.12	94.2
Benzaldehyde	2.72	2.77	0.891	102	5.37	5.92	1.84	110	7.75	7.80	3.12	101
Octanal	1.46	1.85	10.8	127	2.87	3.69	8.98	129	4.15	4.77	8.10	115
trans-2-Octenal	8.74	9.29	1.69	106	17.5	22.0	12.2	126	25.4	28.1	10.9	111
o-Tolualdehyde	0.719	0.900	2.74	125	1.44	1.82	1.69	126	2.08	2.27	2.26	109
Nonanal	4.20	4.96	20.8	118	8.36	9.06	4.61	108	12.1	11.4	1.68	94.3
trans-2-Nonenal	8.87	10.6	2.46	120	17.7	21.0	7.92	118	25.7	29.7	19.2	116
Decanal	1.81	1.86	14.4	103	3.18	3.48	4.76	109	4.40	3.87	2.54	87.8

Typical aldehyde recoveries from human serum

Analyte	Low spike				Medium Spike				High Spike			
	Target	Mean	CV %	Recovery %	Target	Mean	CV %	Recovery %	Target	Mean	CV %	Recovery %
Acetaldehyde	647	575	13.3	89.0	1009	929	1.57	92.1	1274	1192	2.90	93.6
Acrolein	93.6	95.2	8.56	102	102	100	7.37	98.5	109	108	9.38	99.7
Propanal	40.9	39.4	3.29	94.7	46.3	43.8	3.05	93.0	49.1	47.0	2.32	94.3
Isobutanal	3.38	3.25	4.27	96.2	7.45	7.52	0.42	101				
Butanal	4.79	4.39	0.364	91.6	7.33	6.80	9.07	92.0	12.5	19.5	5.26	155
Isopentanal	9.56	8.77	1.35	85.9	12.5	10.9	0.286	97.2	14.5	12.2	2.00	80.7
Crotonaldehyde	0.98	0.643	3.21	62.1	1.50	0.867	0.747	54.7	1.92	1.03	4.63	52.3
Pentanal	40.4	40.7	2.51	94.3	53.0	53.5	3.18	95.8	62.4	64.4	1.36	98.7
Hexanal	837	830	8.71	98.9	850	774	12.4	90.9	859	710	5.64	82.6
Furaldehyde	10.1	8.95	6.29	89.1	19.2	17.8	1.83	92.4	36.2	36.0	0.168	100
trans-2-Hexenal	7.37	5.98	8.31	78.1	9.84	6.61	1.79	65.3	12.1	7.76	7.68	62.8
Heptanal	14.7	13.5	0.848	90.5	15.8	13.6	1.57	84.4	16.5	14.0	0.590	85.4
Benzaldehyde	10.0	9.65	1.08	93.1	12.5	11.8	0.872	92.0	14.2	13.4	3.11	92.5

Octanal	5.88	4.20	2.35	73.3	7.48	6.91	4.18	89.7	8.34	8.40	3.45	98.0
trans-2-Octenal	33.1	25.2	5.35	77.9	42.2	24.5	7.34	59.5	48.0	28.8	9.92	60.9
o-Tolualdehyde	1.18	0.800	3.76	79.1	1.94	1.49	0.500	86.6	2.34	1.97	1.77	77.2
Nonanal	26.2	24.4	0.798	89.6	30.2	28.8	1.61	92.2	33.9	32.7	2.66	93.7
trans-2-Nonenal	41.0	32.7	5.86	85.0	51.2	33.4	10.7	70.6	59.5	38.0	7.93	71.1
Decanal	2.79	2.45	13.7	82.0	3.46	3.52	1.17	96.1	4.33	4.49	2.23	99.0

The absolute accuracy for calibrators was established by analyzing independently prepared and characterized proficiency testing (PT) solutions as unknowns. The PT solutions were made from neat aldehydes from a different lot than standard solutions. Purity of standards and PT materials were verified as follows: A concentrated liquid sample in methanol was run using full scan method. The identities of the desired compounds and any known impurities were confirmed using mass spectrometer library. The ratio of area counts of the desired analyte to the total area was used to calculate the percent purity for each compound. For labeled material, ion ratios of labeled to native signal were used to determine isotope purity.

Typical accuracy results for all analytes for all PT levels

Analyte	PT22301		PT22302		PT22303		PT22304	
	Conc. (ng/mL)	Stdev (ng/mL)	Conc. (ng/mL)	Stdev (ng/mL)	Conc. (ng/mL)	Stdev (ng/mL)	Conc. (ng/mL)	Stdev (ng/mL)
Acetaldehyde	99.9	5.12	252	13.0	397	20.8	607	30.4
Acrolein	12.4	0.656	30.3	1.58	78.9	4.16	200	10.6
Propanal	3.10	0.176	7.40	0.400	19.6	1.09	49.9	2.72
Isobutanal	1.25	0.067	3.02	0.160	8.04	0.432	20.1	1.10
Butanal	1.25	0.066	2.99	0.157	8.03	0.432	20.1	1.09
Isopentanal	2.50	0.134	6.00	0.320	12.1	0.640	19.9	1.07
Crotonaldehyde	0.504	0.027	1.00	0.054	3.02	0.160	6.02	0.320
Pentanal	5.03	0.272	12.0	0.640	31.7	1.60	81.2	4.16
Hexanal	5.01	0.272	12.1	0.656	31.5	1.60	80.8	4.16
Furaldehyde	3.72	0.192	8.98	0.496	23.8	1.25	60.1	3.20
trans-2-Hexenal	4.99	0.272	9.97	0.544	15.1	0.848	24.9	1.34
Heptanal	0.999	0.056	3.02	0.160	5.99	0.320	11.9	0.640
Benzaldehyde	4.99	0.272	10.0	0.544	15.1	0.800	19.9	1.04
Octanal	5.02	0.272	10.0	0.544	15.0	0.848	19.9	1.09
trans-2-Octenal	4.97	0.288	9.94	0.592	30.1	1.76	60.1	3.52
o-Tolualdehyde	0.200	0.011	0.780	0.042	1.60	0.085	3.03	0.160
Nonanal	5.01	0.272	9.92	0.528	30.1	1.57	56.0	3.04
trans-2-Nonenal	3.78	0.208	8.93	0.496	24.1	1.33	59.8	3.20
Decanal	5.01	0.272	10.0	0.544	25.5	1.33	34.4	1.76

c. Precision

The precision for this method is based on the measurement of ten individual serum samples at three different concentrations (blank serum without spike, level 1 spike: S1 and level 2 spike: S2). Relative standard deviations are in most cases less than 10%. As expected, most exceptions were found in the low spike samples. These standard deviation results are actually higher than would be encountered in typical serum determinations since they include variation in the serum both before and after spiking.

Typical assay precision

Analyte	Spiked level	Measured Concentration (ng/mL)	%CV
Acetaldehyde	Blank serum	340 ± 57	17
	S1	153 ± 15	10
	S2	2461 ± 319	13
Acrolein	Blank serum	153 ± 15	10
	S1	274 ± 30	11
	S2	323 ± 36	11
Propanal	Blank serum	42.0 ± 4.3	10
	S1	66.4 ± 3.6	14
	S2	112 ± 6.5	5.8
Isobutanal	Blank serum	1.70 ± 0.30	17
	S1	5.60 ± 0.32	6.5
	S2	6.00 ± 0.47	7.9
Butanal	Blank serum	2.36 ± 0.63	27
	S1	7.53 ± 0.37	5.0
	S2	14.5 ± 0.92	6.3
Isopentanal	Blank serum	2.52 ± 0.36	14
	S1	8.12 ± 0.43	5.3
	S2	15.3 ± 0.68	4.5
Crotonaldehyde	Blank serum	0.623 ± 0.70	9.0
	S1	1.20 ± 0.05	3.8
	S2	3.98 ± 0.17	4.2
Pentanal	Blank serum	19.1 ± 1.3	6.8
	S1	33.2 ± 1.2	3.6
	S2	51.1 ± 3.1	6.0
Hexanal	Blank serum	26.2 ± 2.5	10
	S1	164 ± 9.9	6.0
	S2	105 ± 5.8	5.5
Furaldehyde	Blank serum	16.5 ± 1.4	8.4
	S1	15.9 ± 3.1	20
	S2	80.2 ± 7.7	9.6
trans-2-Hexenal	Blank serum	5.83 ± 0.63	11

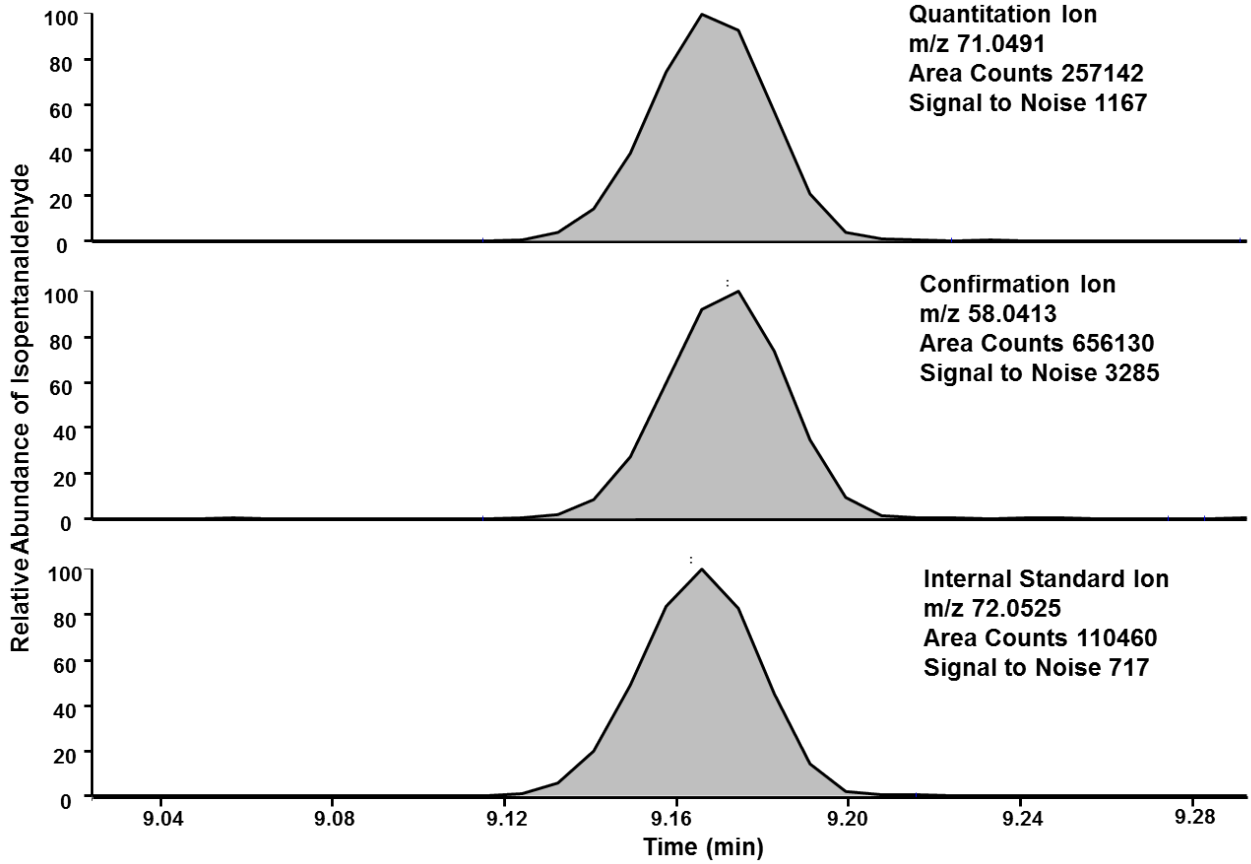
	S1	12.4 ± 3.2	26
	S2	50.0 ± 3.9	7.9
Heptanal	Blank serum	11.3 ± 0.75	6.6
	S1	22.7 ± 0.63	2.8
	S2	30.8 ± 1.6	5.1
Benzaldehyde	Blank serum	7.69 ± 0.51	6.7
	S1	11.9 ± 0.46	3.8
	S2	31.5 ± 2.2	7.0
Octanal	Blank serum	2.44 ± 0.36	15
	S1	4.18 ± 0.19	4.6
	S2	12.7 ± 0.7	5.5
trans-2-Octenal	Blank serum	32.6 ± 2.3	7.1
	S1	36.8 ± 1.3	3.5
	S2	61.7 ± 3.7	5.9
o-Tolualdehyde	Blank serum	0.100 ± 0.02	15.0
	S1	0.932 ± 0.03	3.1
	S2	3.60 ± 0.13	3.6
Nonanal	Blank serum	13.4 ± 1.2	9.0
	S1	22.8 ± 1.4	6.1
	S2	39.1 ± 2.6	6.6
trans-2-Nonenal	Blank serum	1.14 ± 0.12	10.6
	S1	41.4 ± 2.1	5.2
	S2	70.8 ± 4.0	5.6
Decanal	Blank serum	0.94 ± 0.21	22.3
	S1	5.09 ± 0.70	14
	S2	10.3 ± 1.3	13

NA= “Not Applicable” because blank serum did not contain measurable analyte levels

d. Analytical Specificity

Analytical selectivity is a measure of the extent to which a method can determine a particular compound in the matrix of interest without interference from matrix components. Because this method utilizes high resolution mass spectrometry (10,000 resolution) it is highly selective for the method analytes. This selectivity is shown in the figure, a typical extracted ion chromatogram of isopentanal in human serum.
Method selectivity was further assessed by evaluation of the ratio of confirmation ion peak areas to quantitation ion peak areas.

Extracted ion chromatogram of isopentanal in human serum.



e. Recovery

See accuracy section 9 b above.

f. Linearity Limits

The aldehyde calibration curves established are linear over the concentration ranges from the low and high standard with R^2 values greater than or equal to 0.98. The lower reportable limit is either the LOD or the lowest standard concentration, whichever is higher. The upper reportable limit is the highest standard concentration. A residual plot of the calibrators may be used to confirm linearity.

g. Ruggedness testing

Ruggedness testing was performed to assess the potential of important analytical variables to affect results. The variables which are found to have a substantial

influence on the final analytical results include extraction time, internal standard volume addition, and acid extraction volume. Each of these variables was varied to examine their influence, if any, on the analytical results and was optimized to achieve sensitivity and high throughput. Specific test and results are listed in Appendix C.

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

Quality assurance and quality control procedures follow standard practices (4). Daily experimental checks are made on the stability of the analytical system. Four QC samples are included in each day's run (QC low and QC high are run before and after serum samples). A water blank containing the internal standard is run at the beginning of each day to check for the presence of contamination. In addition, determination of labeled ion counts for this material is used to check daily method sensitivity. Relative retention times are examined for each analyte to ensure the choice of the correct chromatographic peak.

After the completion of a run, the quality control limits are consulted to determine if the run is "in control". The results of the analysis of the quality control samples measured at the beginning and the end of the analytical run are averaged and the quality control rules are applied to this average. The quality control results are evaluated according to Westgard (5) rules:

Standard Shewhart QC charts are maintained for this internal QC specimen. A separate QC chart is to be maintained for each QC material used for this internal QC specimen. Standard criteria for run rejection based on statistical probabilities are used to declare a run either in control or out-of-control. These rules are:

Analytical run with 2 QC results are evaluate according to DLS Policies and Procedures Manual (Pirkle 2012).

Quality Control procedures implemented in this method are defined by the Division's Policies and Procedures Manual. For more information see: Caudill et al. 2008.

b. Establishing QC Limits

Quality control limits are established by characterizing assay precision with 20 distinct analyses of each QC pool. Two different pools of serum quality control material are used: QC low, and QC high. Different calibration materials are

included in the analysis (e.g. different sets of standards and internal standards and 20 different sets of QC low and high) to capture realistic assay variation over time. The mean, standard deviation, coefficient of variation, and confidence limits are calculated from this QC characterization data set. Individual quality control charts for the characterization runs are created, examined, and quality control limits are used to verify assay precision and accuracy on a daily basis. Typical QC characterization statistics for aldehydes are listed below.

Typical means and Limits for Quality Control sera.

Analyte Code	QC material	%CV	Mean - 3σ	Mean - 2σ	Mean	Mean + 2σ	Mean + 3σ
2AL	QL223	12.5	84.2	101	135	168	187
	QH223	9.02	157	201	245	289	341
ACAL	QL223	14.9	10.8	21.9	31.2	40.5	56.8
	QH223	9.87	33.7	45.6	56.8	68.0	86.4
3AL	QL223	8.60	4.45	6.38	7.70	9.03	11.3
	QH223	8.20	11.3	14.2	16.7	19.7	24.1
I4AL	QL223	4.30	2.74	2.97	3.25	3.53	3.78
	QH223	4.00	6.81	7.24	7.88	8.53	9.08
4AL	QL223	7.00	1.92	2.11	2.46	2.80	3.08
	QH223	5.60	4.46	4.94	5.57	6.20	6.92
I5AL	QL223	5.10	2.76	3.07	3.41	3.76	4.10
	QH223	5.20	7.34	7.90	8.83	9.76	10.6
CRAL	QL223	21.5	0.047	0.149	0.261	0.374	0.506
	QH223	21.2	0.163	0.283	0.492	0.701	0.802
5AL	QL223	11.8	7.15	8.81	11.5	14.3	16.2
	QH223	6.60	20.4	22.4	25.8	29.3	31.5
6AL	QL223	4.70	28.3	32.2	35.6	39.0	43.8
	QH223	5.20	63.3	68.2	76.1	84.0	90.5
FRAL	QL223	5.00	8.46	9.43	10.5	11.5	12.4
	QH223	4.20	24.4	25.7	28.0	30.3	31.5
T6AL	QL223	15.3	2.04	2.65	3.82	4.99	5.79
	QH223	11.8	3.56	6.62	8.67	10.7	12.4
7AL	QL223	6.30	2.57	2.85	3.27	3.68	3.99
	QH223	4.50	5.68	6.19	6.80	7.41	7.98
BZAL	QL223	7.60	3.10	3.62	4.27	4.92	5.54
	QH223	6.00	8.61	9.34	10.6	11.9	13.0
8AL	QL223	5.60	4.30	4.71	5.31	5.90	6.38
	QH223	5.40	11.2	12.1	13.6	15.1	16.3
T8AL	QL223	12.4	6.24	9.89	13.2	16.5	19.9
	QH223	13.2	13.6	19.7	26.8	33.8	41.1
TLAL	QL223	5.90	0.565	0.653	0.741	0.828	0.907
	QH223	5.50	1.68	1.80	2.02	2.25	2.38
9AL	QL223	9.00	16.0	18.4	22.3	26.4	28.7

	QH223	7.10	34.1	42.0	49.0	56.0	62.1
T9AL	QL223	6.89	10.3	12.7	14.8	16.8	20.3
	QH223	7.82	16.7	19.2	22.7	26.3	29.5
10AL	QL223	10.7	3.79	4.20	5.40	6.57	7.36
	QH223	9.80	10.0	11.4	14.2	17.0	18.6

c. Proficiency Testing

The proficiency testing (PT) scheme for this method is administered, analyzed, and evaluated by an in-house Proficiency Testing Coordinator. Five samples of unknown PT concentrations are analyzed twice a year using the same method described for unknown samples.

Analytical PT results are reviewed by the analyst and laboratory supervisor, and submitted to the in-house PT Coordinator electronically. The PT results are evaluated by the PT Coordinator; the analysis passes proficiency testing if $\geq$ 80% of the results deviate $\leq$ 25% from the known value. A summary report of the PT evaluation is maintained by the laboratory supervisor. If the assay fails proficiency testing then the sample preparation and instrumentation are thoroughly examined to identify and correct the source of assay error. Unknown specimens are not analyzed until the method successfully passes proficiency testing.

11. Remedial Action if Calibration or QC System Fails to Meet Acceptable Criteria

If the calibration or QC fails, all operations are suspended until the source or cause of failure is identified and corrected. Analytical results are not reported. After calibration and /or quality control have been reestablished, analytical runs may be resumed.

a. Internal Standard Response

If the labeled ion counts of the blank samples fall below 20% of the median of these values, this indicates that the instrumental sensitivity has fallen below acceptable limits. The following steps should be taken and the instrument sensitivity rechecked after each is performed. Once sensitivity has been reestablished and calibration verification checked, further steps are not necessary.

1) Check for an air leak in the system.

2) Remove and clean the mass spectrometer ion volume. Replace the filament and any ceramics which may be conducting.

3) Test the electron multiplier gain and replace if electron energy is 2.5 kV or higher.

4) Remove and clean the mass spectrometer outer source. Replace any ceramics which may be conducting.

b. Calibration Regression

If the linearity of the calibration curve criterion of 0.95 is not met, check to see if the standards are prepared correctly or if an instrument malfunction has occurred. If an inordinately large amount of analyte is measured in the blank, this could greatly affect the lower end of the calibration curve. However, if this is not seen in the remainder of the standard samples, this indicates a temporary contamination of the blank. The source of this incident should be investigated to prevent repeat occurrences but, no further action is required.

If no standard preparation is found in error, check to the sensitivity of the mass spectrometer. Other instrument specific factors that could cause calibrations problems, such as a clog in the injection inlet could cause a shift in the retention times of the eluting peaks should be checked and corrective action taken as needed.

c. Analytes in Standards or QC Materials

If an unexpectedly large amount of analyte is measured in one of the calibration standards or QC materials, but is not seen in the remainder of the samples, this indicates a contamination of this particular sample. The source of this incident should be investigated to prevent repeat occurrences, but no further action is required.

d. Analytes in All Samples

If an inordinately large amount of analyte is present in all measurements for a particular day, either the labeled analog solution is contaminated or there is a continual source of contamination. The following steps should be taken until the contamination is removed.

1) Check the immediate area of the mass spectrometer and the laboratory where standards are made for use of the contaminating agent.

2) Discard the purge and trap grade methanol used for dilution of the internal standard. For further analyses use a new bottle of purge and trap grade methanol (or new lot of methanol).

3) Check all glassware which has been used.

e. QC Sample Outside of Control Limits

If one or more of the quality control sample concentration results fall outside the 99% limits, one of the above is the most likely cause. Follow the steps outlined above to isolate and correct the problem.

In all cases the supervisor should be consulted for the appropriate corrective actions. No analytical results will be reported for runs not in statistical control. After corrective actions are carried out, calibration verification and quality control materials must be analyzed before proceeding with unknown analyses.

12. Limitations of Method; Interfering Substances and Conditions

This method is an isotope dilution mass spectrometry method, widely regarded as the definitive method for the measurement of organic toxicants in human body fluids. Alteration of particular aspects of this method can result in major interferences. Care is required in order to produce non-contaminated blanks, vacutainers, and quality control materials. The range of linearity and limits of detection are given above in Sections 9.a. and 9.b., respectively.

13. Reference Range (Normal Values)

No reference ranges exist for serum aldehyde levels in the US population.

14. Critical-Call Results (“Panic” Values)

The health effects resulting from exposure to low levels of volatile organic compounds is currently unclear. The method described here is designed for the measurement of low level exposure to VOCs.

15. Sample Storage and Handling During Testing

Specimens may reach and maintain ambient temperature during analysis. If the measurement is delayed to the next day, samples should be refrigerated and not left at ambient temperature overnight. Most sample queues run for extended time periods of up to 24-hr duration. As a precaution biological samples (unknowns and QC) are racked into a chilled tray (15 $\pm$ 5 °C) while awaiting analysis.

16. Alternative Methods for Performing Test and Storing Specimens if Test System Fails

The analysis of VOCs in serum at parts-per-billions levels is an extremely complex measurement. There are no acceptable alternative methods for this analysis. If the analytical system fails, storage of unprepared and partially prepared specimens at 4 °C is recommended up to 24 hr.

a. Length of Time Samples may be banked

Repeat measurements of samples stored in a -70°C freezer have shown that serum samples may be banked for at least 7 weeks. Longer storage times result in samples that are harder to manipulate and produce additional analytical problems. Thus, even though analytical results may not change over this time, samples may be less amenable to analysis. Volatile organic compounds occur naturally in the body, and metabolism may alter the concentration of some of these analytes with storage.

b. Proper banking procedures

Serum samples for VOC measurement should be stored in the dark in a -70°C freezer. Since VOCs are lost whenever the containers in which they are stored are opened, it is not appropriate to transfer the serum samples to another container which would be more resistant to breaking.

17. Test-Result Reporting System; Protocol for Reporting Critical Calls (if applicable)

Results are generally reported to 2 significant digits. In addition, reports of reference range means and medians should also accompany all reports since these values are not available elsewhere.
The health effects resulting from exposure to low levels of volatile organic compounds is currently unclear. Therefore no critical call levels are set.

18. Procedures for Specimen Accountability and Tracking

If greater than 0.25 mL of sample remains after analysis, this material should be returned to storage in a -70 °C freezer.

Samples locations are tracked using a STARLIMS. It is recommended that records be maintained for 3 years, including related QA/QC data, and that duplicate records be kept off-site in electronic format. All personal identifiers should be available only to the medical supervisor to maintain confidentiality.

Due to the complex nature of the analyses and the unique testing capabilities of this laboratory, it is not expected that specimens will be referred to other laboratories for testing. Should such a need arise, the laboratory supervisor will

consult with local subject matter experts to establish an appropriate mechanism and work process.

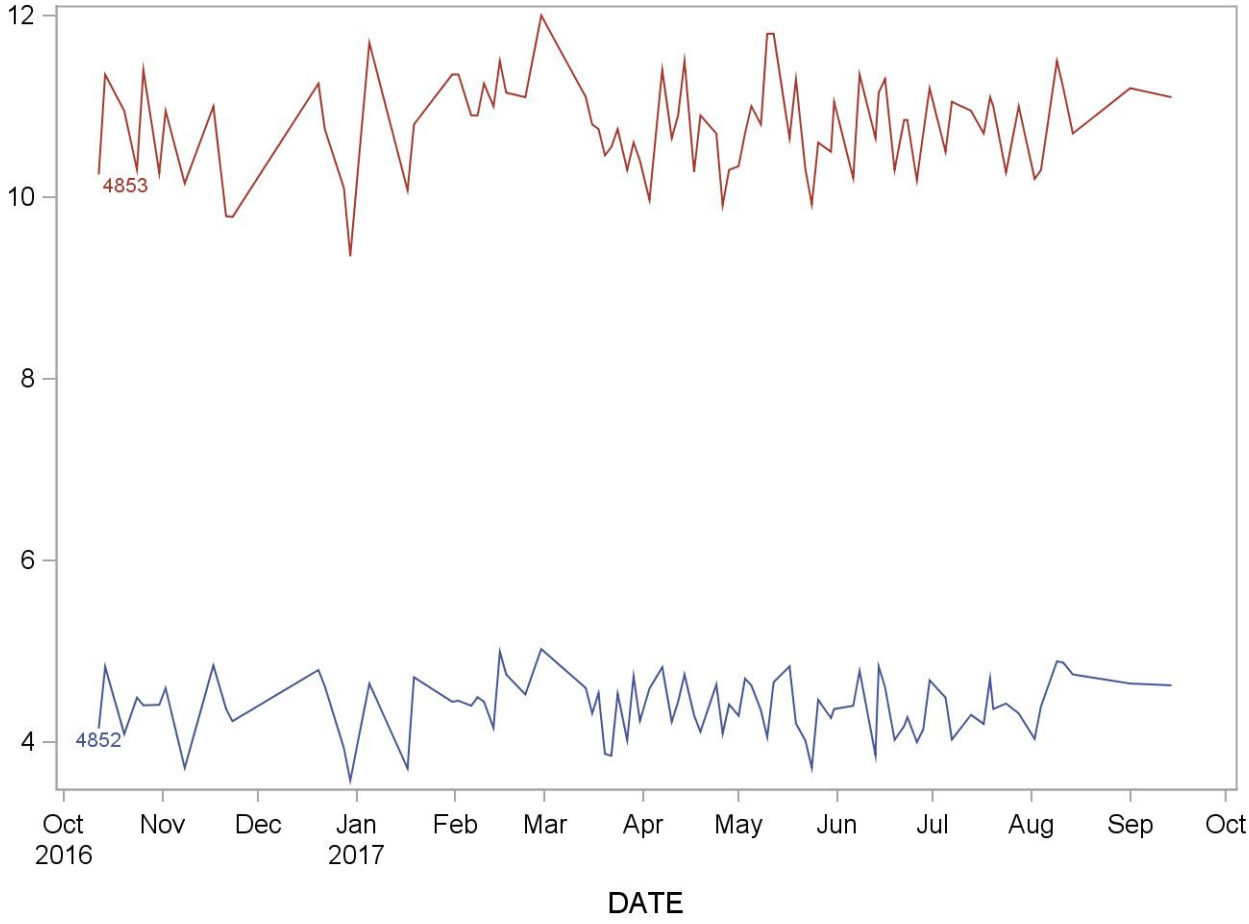
19. SUMMARY STATISTICS

See following pages.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

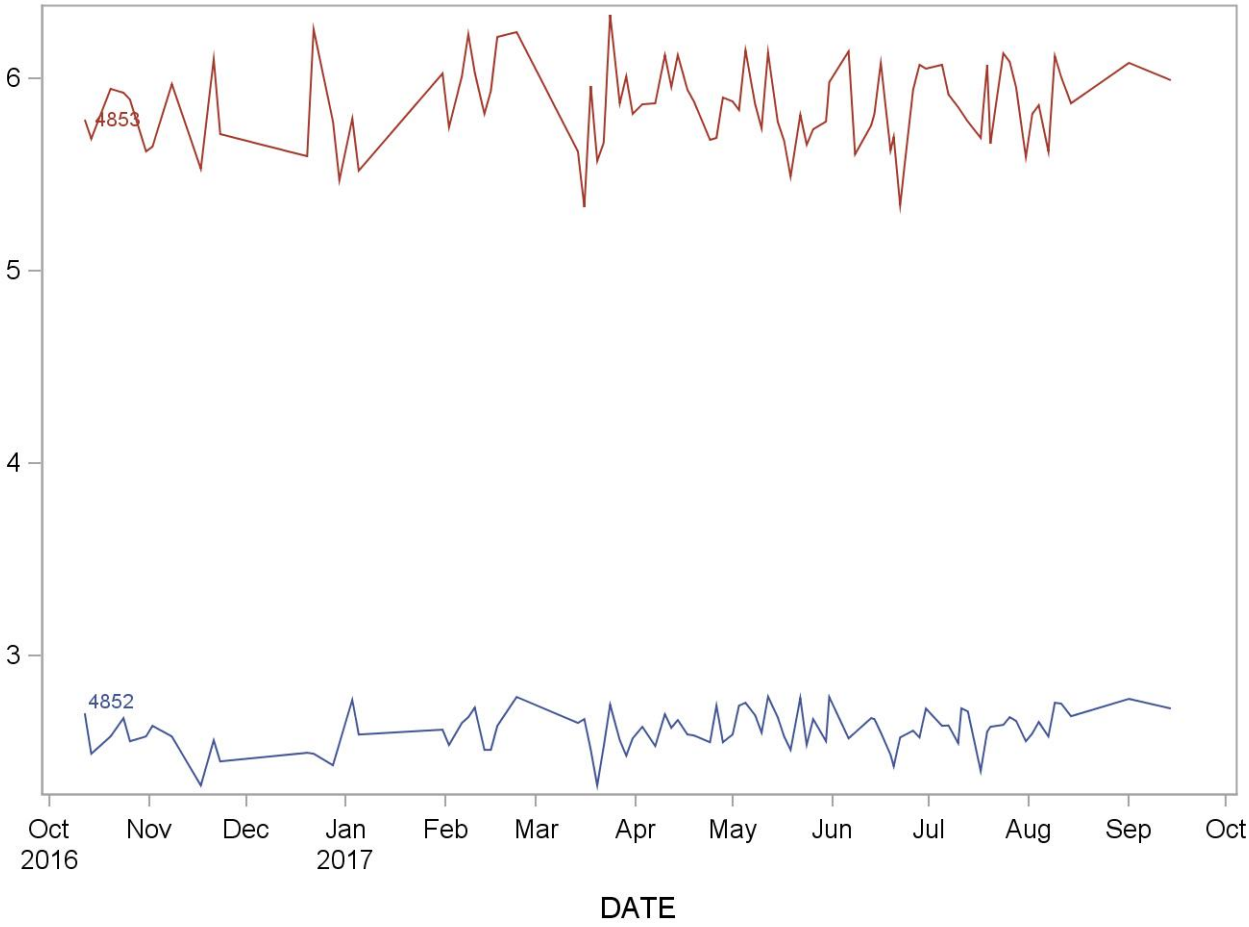
2013-2014 Summary Statistics and QC Chart for Benzaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	86	12OCT16	14SEP17	10.78140	0.51530	4.8
4852	86	12OCT16	14SEP17	4.39295	0.32273	7.3



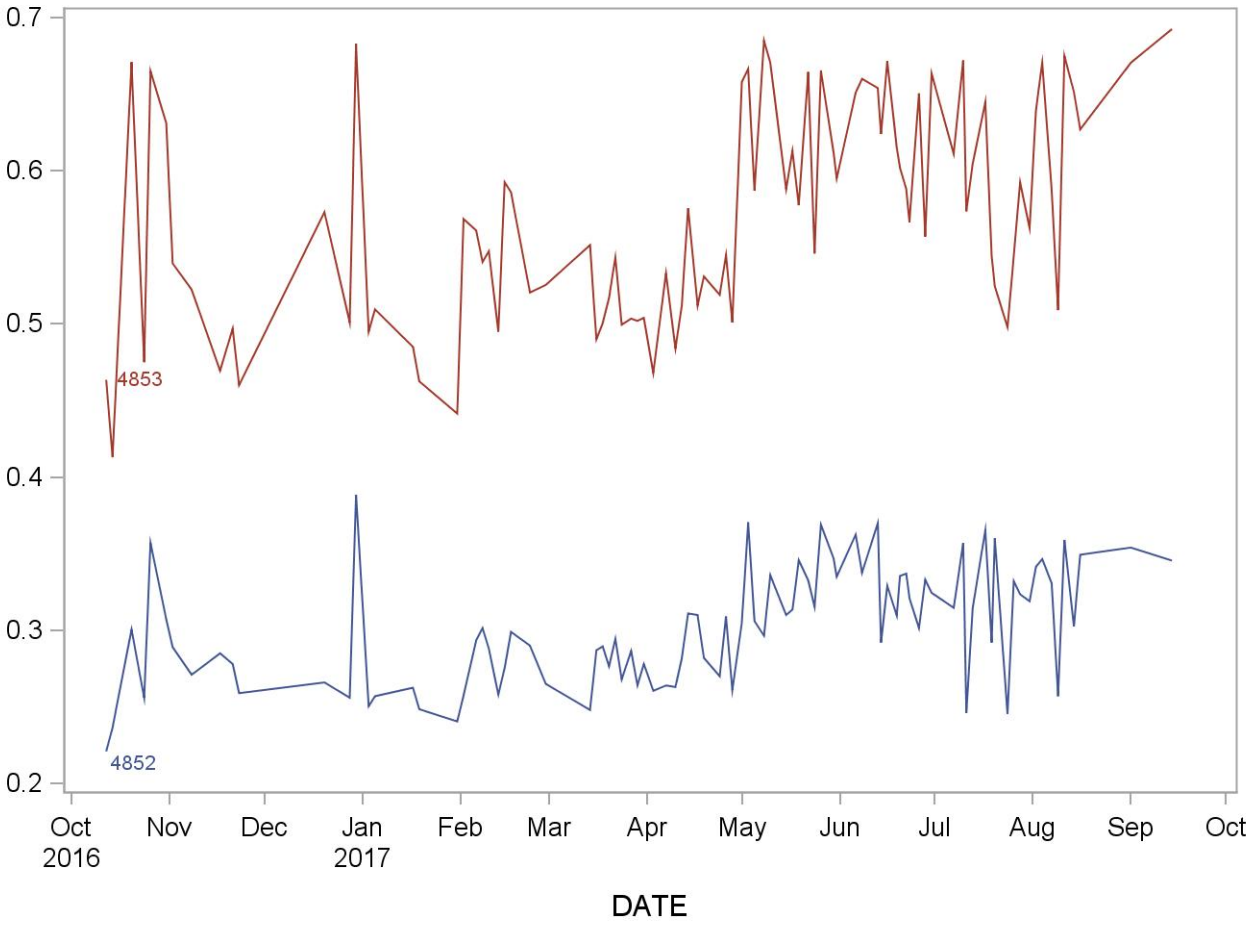
2013-2014 Summary Statistics and QC Chart for Butyraldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	89	12OCT16	14SEP17	5.85837	0.21203	3.6
4852	90	12OCT16	14SEP17	2.61118	0.10105	3.9



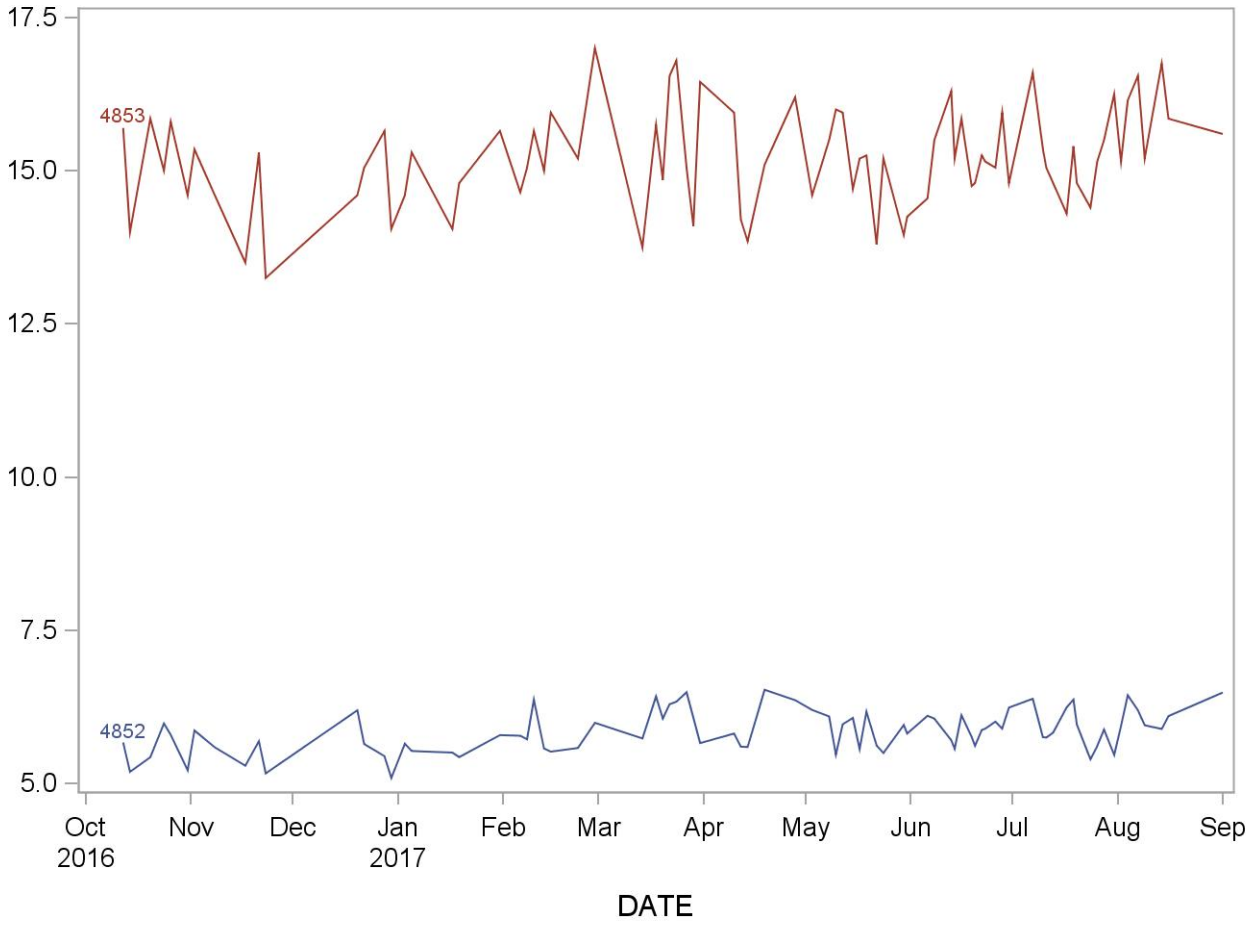
2013-2014 Summary Statistics and QC Chart for Crotonaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	92	12OCT16	14SEP17	0.56969	0.07053	12.4
4852	92	12OCT16	14SEP17	0.30165	0.03815	12.6



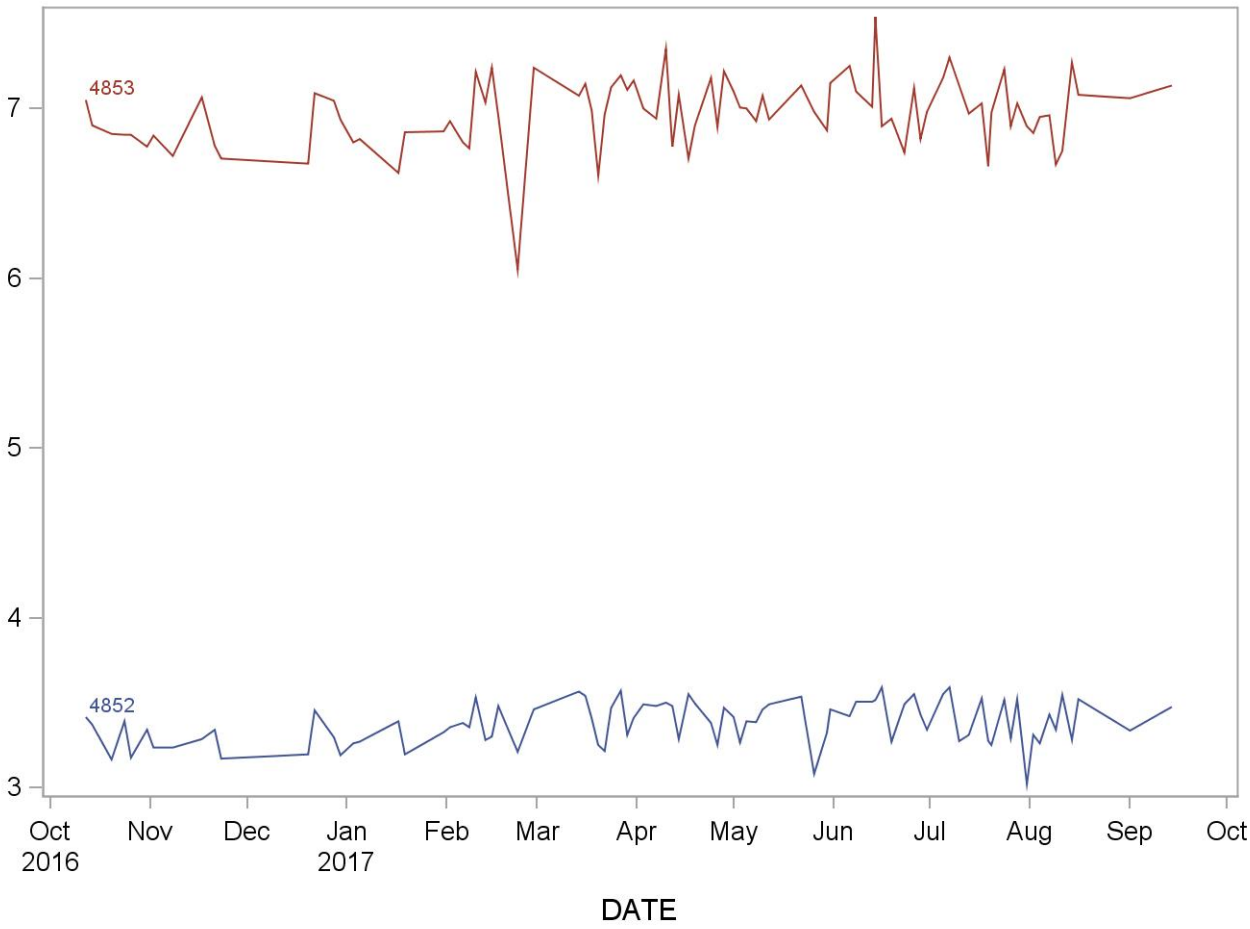
2013-2014 Summary Statistics and QC Chart for Decanaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	81	12OCT16	01SEP17	15.18765	0.81154	5.3
4852	80	12OCT16	01SEP17	5.84354	0.34440	5.9



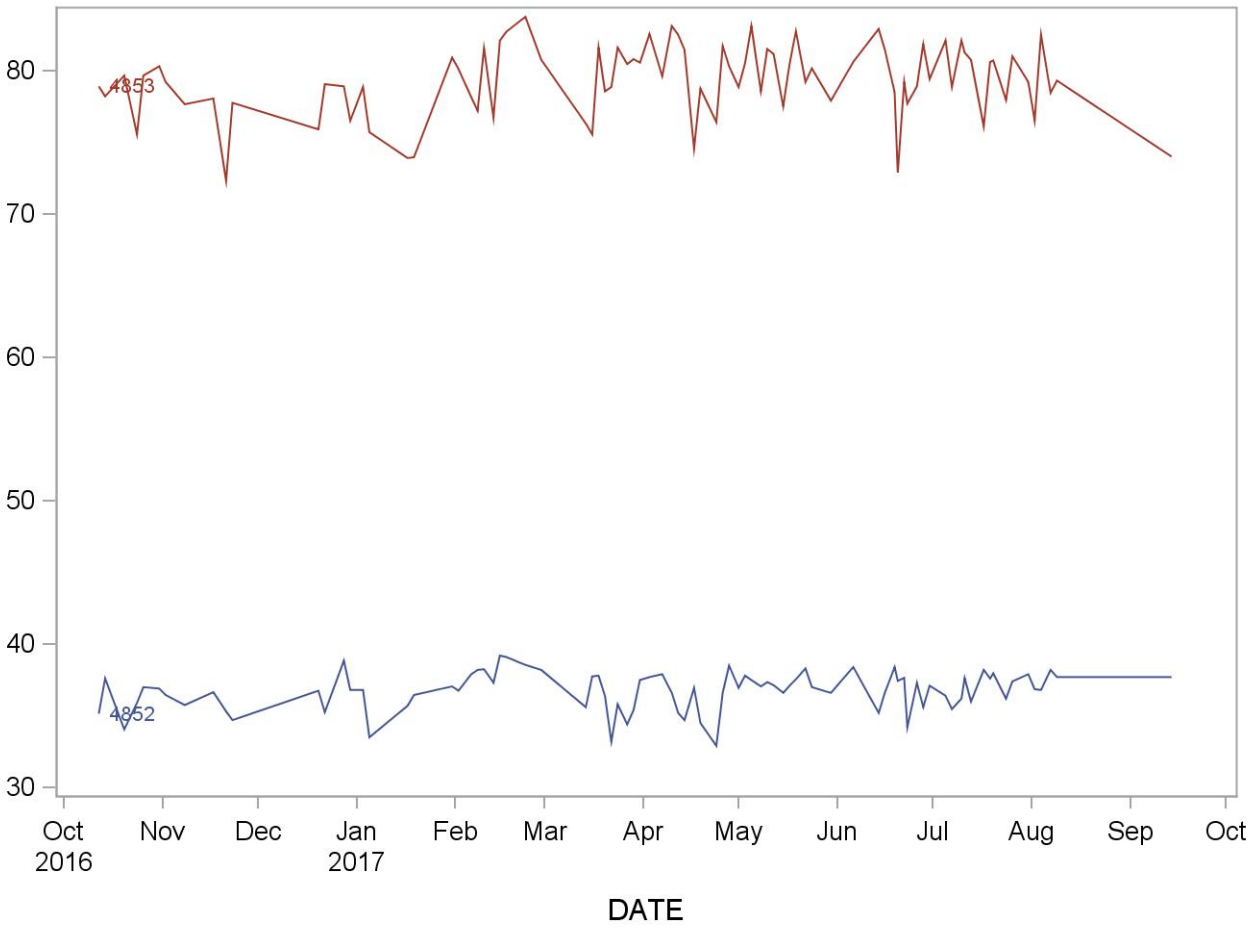
2013-2014 Summary Statistics and QC Chart for Heptanaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	87	12OCT16	14SEP17	6.96764	0.20658	3.0
4852	88	12OCT16	14SEP17	3.37720	0.12735	3.8



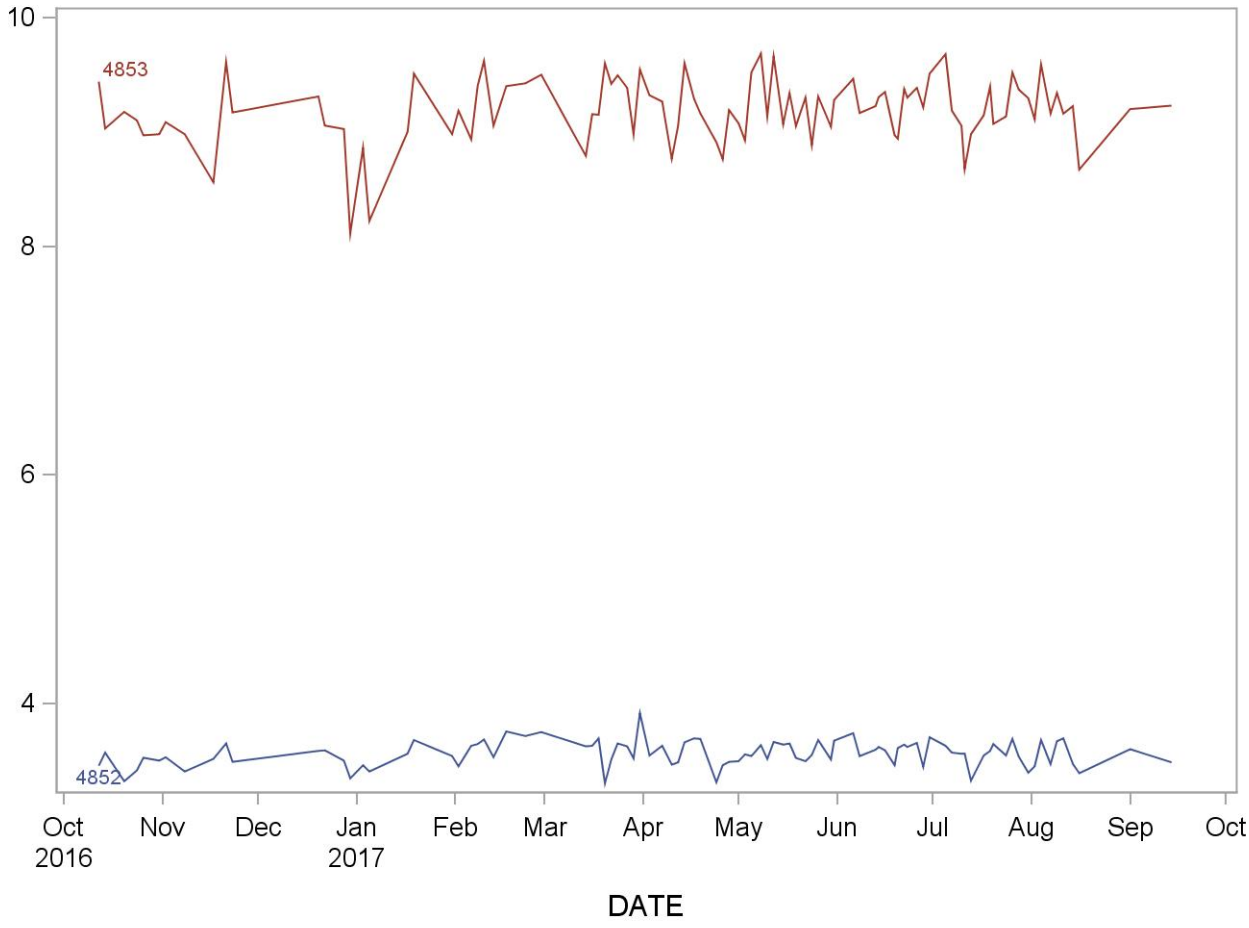
2013-2014 Summary Statistics and QC Chart for Hexanaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	86	12OCT16	14SEP17	79.24767	2.52880	3.2
4852	86	12OCT16	14SEP17	36.75029	1.34424	3.7



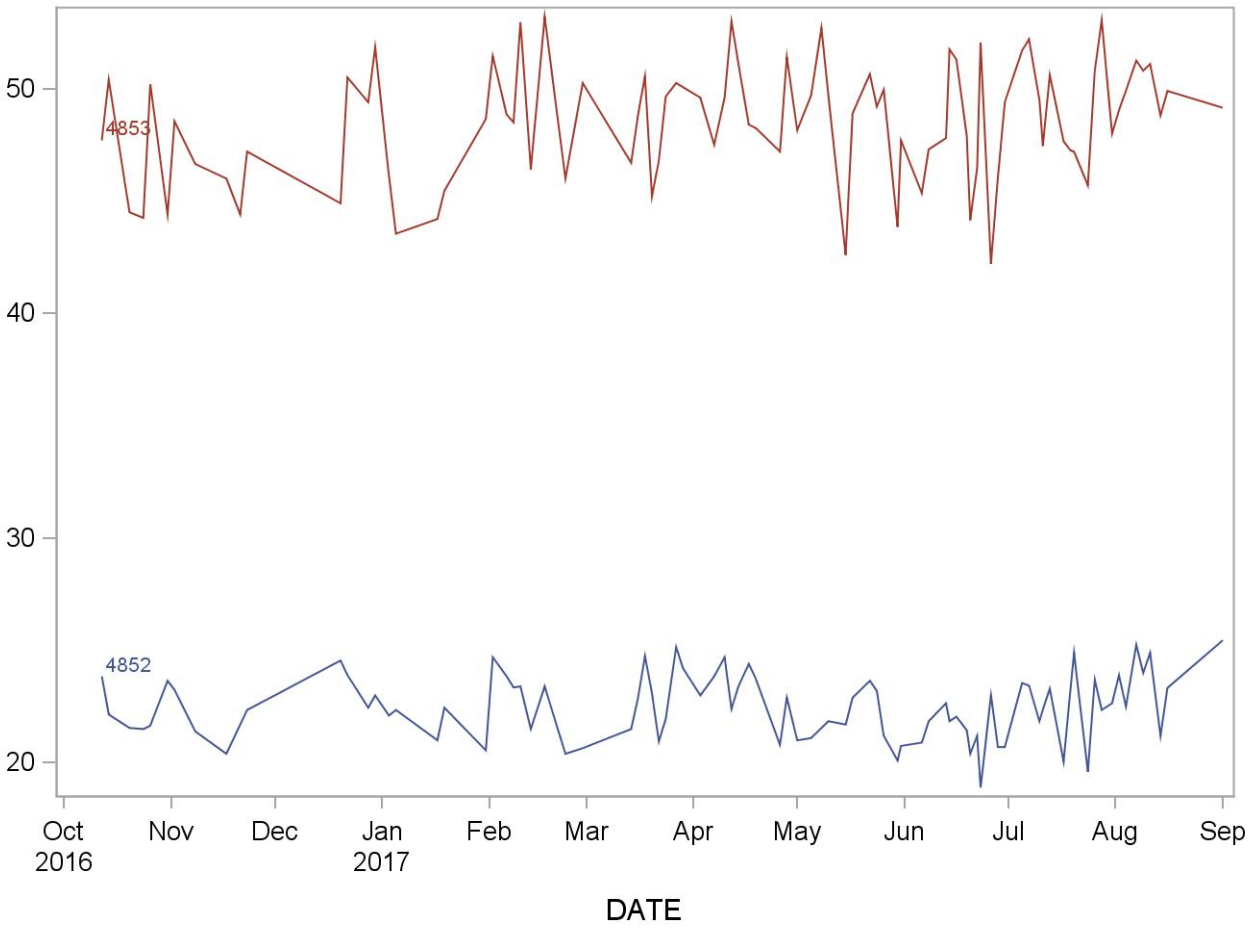
2013-2014 Summary Statistics and QC Chart for Isopentanaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	94	12OCT16	14SEP17	9.1829	0.2869	3.1
4852	94	12OCT16	14SEP17	3.5632	0.1113	3.1



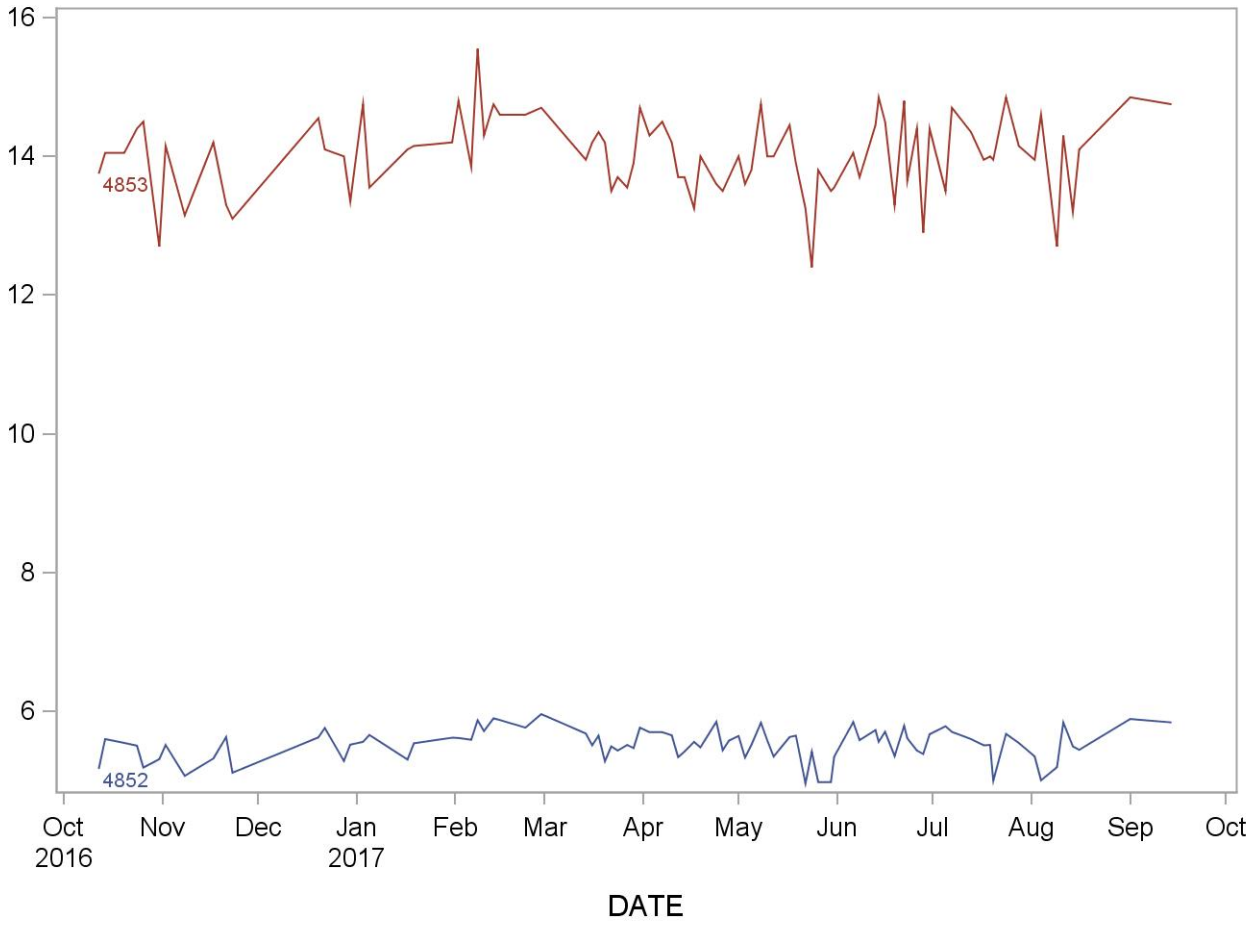
2013-2014 Summary Statistics and QC Chart for Nonanaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	87	12OCT16	01SEP17	48.46379	2.62966	5.4
4852	86	12OCT16	01SEP17	22.48527	1.45511	6.5



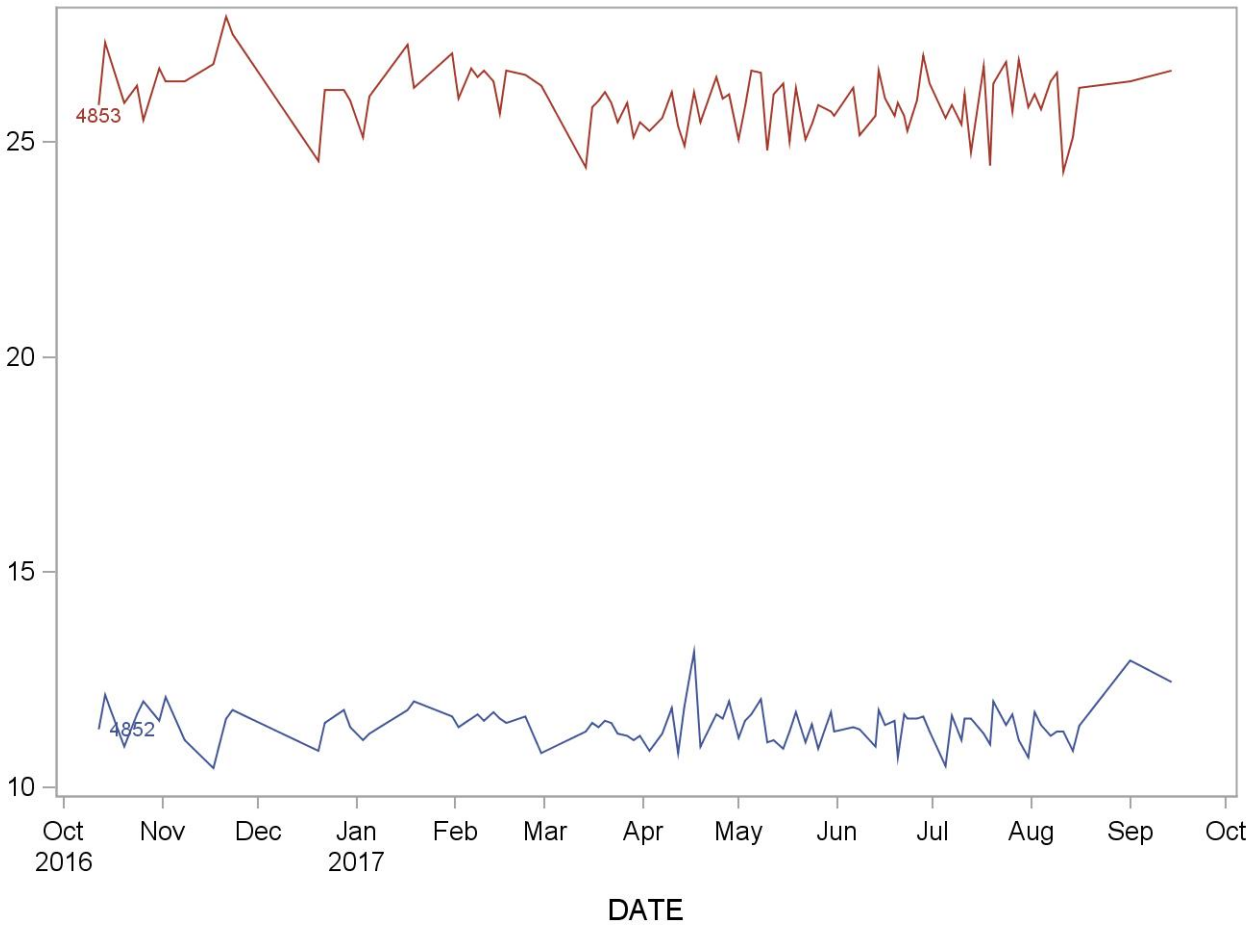
2013-2014 Summary Statistics and QC Chart for Octanaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	87	12OCT16	14SEP17	14.01897	0.56979	4.1
4852	87	12OCT16	14SEP17	5.52807	0.23285	4.2



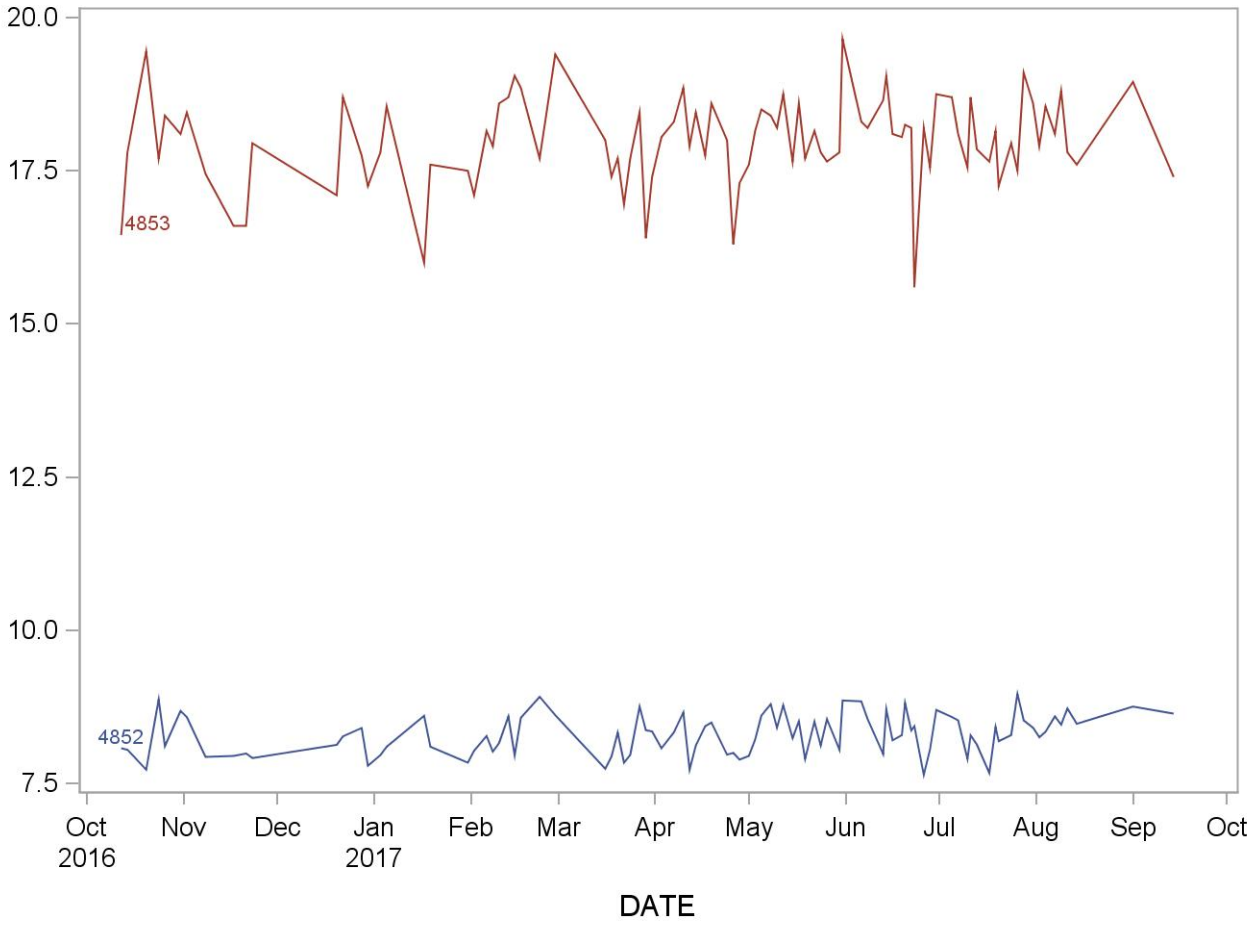
2013-2014 Summary Statistics and QC Chart for Pentanaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	95	12OCT16	14SEP17	25.97421	0.69561	2.7
4852	95	12OCT16	14SEP17	11.45386	0.44702	3.9



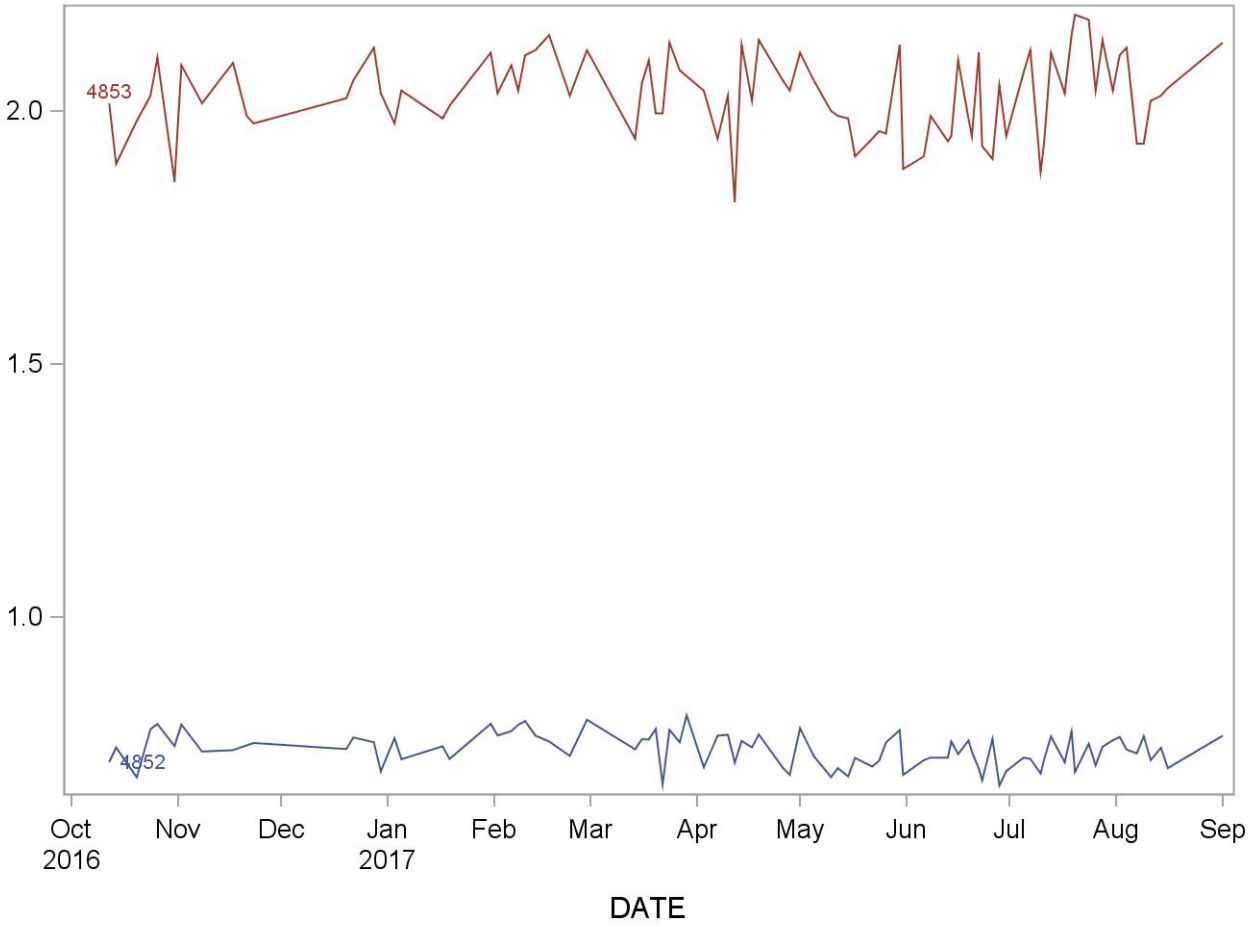
2013-2014 Summary Statistics and QC Chart for Propanaldehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	93	12OCT16	14SEP17	17.97688	0.73900	4.1
4852	93	12OCT16	14SEP17	8.29561	0.33168	4.0



2013-2014 Summary Statistics and QC Chart for o-Tolualdehyde (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4853	87	12OCT16	01SEP17	2.02977	0.08090	4.0
4852	87	12OCT16	01SEP17	0.73677	0.03300	4.5



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Appendix A: Flame Sealing Procedure

1) Preparation of glassware

All glassware used in this study is carefully cleaned to be certain to remove possible nonspecific VOC contamination. To remove possible analytical interferences, rinse glassware (volumetric flasks, ampoules, and storage bottles) with reagent-grade methanol, and heat at 150°C in a vacuum oven with an independent vacuum source for at least 8 hr. to remove adsorbed VOCs. The independent vacuum source is necessary to prevent cross-contamination from other laboratory operations. There is the risk of changing the calibration of volumetric glassware by heating, but the error resulting from this is small compared to other sources of error in the VOC method. Cool the glassware to room temperature under vacuum and restore pressure using nitrogen (UHP grade). Remove treated glassware from the oven and store sealed with Teflon-lined caps until used in standard preparation.

2) Preparation of native analytical standards

a. Procedure for handling neat compounds

All analytes are purchased as neat liquids, most are in flame sealed ampoules. After opening the ampoule the remaining (unused) material is discarded. A few of the most expensive analytes (custom synthesis products) are aliquoted into an individual amber borosilicate glass ampoule and flame sealed for future use. After transferring the compounds, flame seal the ampoule and store in an explosion-proof freezer. Properly label the ampoule(s) and store in a covered box to reduce light exposure to all neat compounds. Store neat standards in a separate location from serum samples, blanks, and quality control materials.

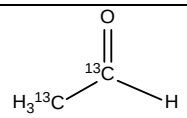
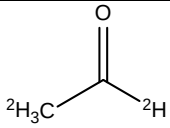
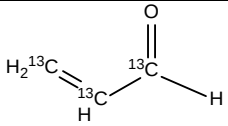
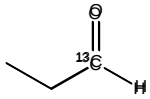
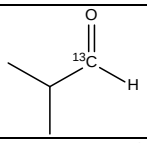
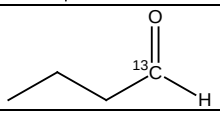
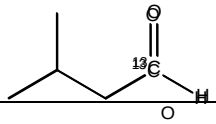
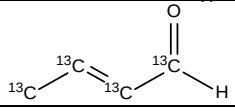
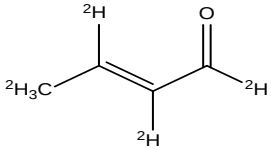
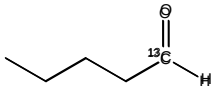
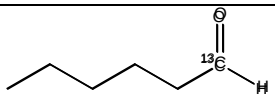
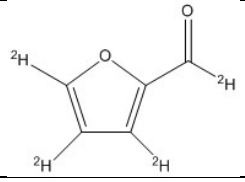
b. Procedure for filling and sealing glass ampoules

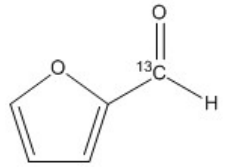
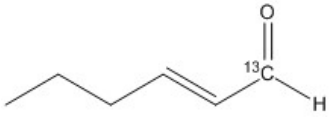
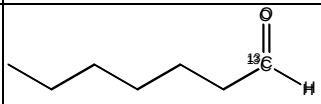
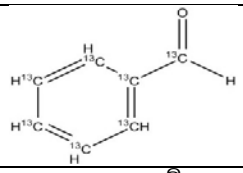
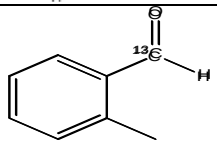
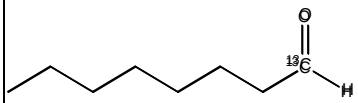
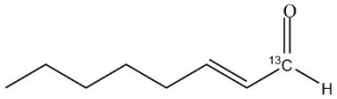
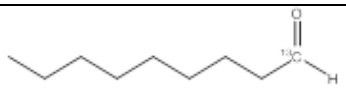
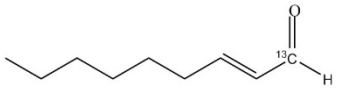
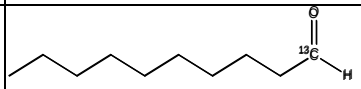
Aliquot about 0.2 mL of the neat standard into a liquid nitrogen cooled 1 mL borosilicate glass vacuole. Place the vacuole in a metal rack that has been cooled with liquid nitrogen. Always wear cryo-gloves and a face shield when handling liquid nitrogen. If there are a substantial number of vacuoles to be flame sealed (over 25), then the metal rack should be continuously cooled in an insulated container that is filled with liquid nitrogen. The cooling of the vacuole will require approximately 1 min. If the vacuole gets too cold and builds up frozen condensate, then oxygen and/or nitrogen will dissolve in the liquid and can cause the vacuole to shatter or develop leaks that can compromise the standard. Remove the vacuole from the cooled metal rack. Use a glass capillary and zero headspace micro-dispenser to transfer the liquid. Before using, rinse the

pipette by initially filling with the neat standard and expelling to waste. (NOTE: There may not be enough neat standards to perform this rinse step). Make sure the liquid is placed in the bottom of the vacuole and is not adhering to the neck. Otherwise, during the sealing procedure, ignition of the liquid will produce a loud pop and could shatter the vacuole. Use a torch that is fueled with ultra-high purity grade methane and oxygen to seal the vacuole by melting and pulling the molten neck to affect a seal. Dark filter protective eyewear is required during flame sealing to shield eyes from the arc of the flame. Allow the sealed vacuole to come to room temperature in a separate storage rack. In a chemical fume hood, invert the vial and gently tap the sealed end on a lab wipe-lined hard surface. If the vacuole does not leak, the vacuole is ready to label and store. If a leak does occur, do not attempt to reseal the vacuole. Dispose of the vacuole and make a new one. Repeat the above steps until at least 12 sealed vacuoles are made. Place the sealed vacuoles in an appropriate holder and store in a -70 °C freezer.

Appendix B: Structures of internal standards analyzed in this method

Structures of Aldehyde Internal Standards

Analyte	Structure
$^{13}C_2$-2AL	
d_4-2AL	
$^{13}C_3$-ACAL	
^{13}C-3AL	
^{13}C-I4AL	
^{13}C-4AL	
^{13}C-I5AL	
$^{13}C_4$-CRAL	
d_6-CRAL	
^{13}C-5AL	
^{13}C-6AL	
d_4-FRAL	

^{13}C-FRAL	
^{13}C-T6AL	
^{13}C-7AL	
$^{13}C_6$-BZAL	
^{13}C-TLAL	
^{13}C-8AL	
^{13}C-T8AL	
^{13}C-9AL	
^{13}C-T9AL	
^{13}C-10AL	

Appendix C: Ruggedness

1) Testing Results

Ruggedness testing for analytical method to detect and quantify aldehyde in human serum

After the analytical method is developed and/or updated the ruggedness of the accuracy of the method is assessed by evaluating the following three variables.

a. Effect of amount of internal standard addition to sample

This method is only designed to add 40 µL of internal standard (IS) to the sample. Following Table shows the variation of amount of internal standard to the sample. According to these data less than 4 µL differences from target volume (40 µL) in internal standard have not significantly changed the concentration of analytes.

Typical effects of amount of internal standard addition to sample

Analyte Code	Mean ± stdev (ng/mL)				
2AL	207 ± 31	229 ± 37	**246 ± 53**	762 ± 185	972 ± 197
ACAL	70.6 ± 7.8	78.3 ± 6.5	**78.4 ± 2.9**	93.4 ± 7.4	91.6 ± 7.1
3AL	35.0 ± 4.3	37.0 ± 1.8	**35.3 ± 1.4**	43.2 ± 1.8	41.9 ± 3.5
4AL	3.09 ± 0.29	3.42 ± 0.12	**3.29 ± 0.14**	2.98 ± 0.18	3.30 ± 0.09
I5AL	4.27 ± 0.46	4.54 ± 0.07	**4.45 ± 0.06**	5.15 ± 0.11	5.47 ± 0.85
CRAL	0.461 ± 0.046	0.509 ± 0.053	**0.461 ± 0.009**	0.430 ± 0.026	0.444 ± 0.025
5AL	12.0 ± 0.6	12.7 ± 0.6	**12.3 ± 0.2**	14.3 ± 0.3	14.8 ± 0.6
6AL	NA	NA	NA	NA	NA
FRAL	1.16 ± 0.23	1.36 ± 0.11	**1.37 ± 0.21**	1.28 ± 0.13	1.16 ± 0.03
T6AL	6.30 ±1.50	7.47 ± 0.54	**6.94 ± 0.35**	2.58 ± 0.11	2.70 ± 0.16
7AL	19.9 ± 0.8	23.1 ± 0.7	**22.2 ± 1.2**	18.3 ± 1.5	19.7 ± 0.2
BZAL	4.53 ± 0.15	4.88 ± 0.27	**4.90 ± 0.18**	8.26 ± 0.31	7.83 ± 0.35
8AL	1.62 ± 0.19	1.61 ± 0.07	**1.32 ± 0.06**	1.87 ± 0.10	2.02 ± 0.16
T8AL	9.30 ± 2.10	13.2 ± 1.3	**14.1 ± 0.3**	18.0 ± 3.3	22.3 ± 0.9
TLAL	0.113 ± 0.016	0.128 ± 0.009	**0.126 ± 0.006**	0.094 ± 0.006	0.090 ± 0.004
9AL	16.0 ± 1.3	17.5 ± 1.8	**16.5 ± 0.6**	14.3 ± 1.3	14.8 ± 0.5
T9AL	6.97 ± 0.93	12.9 ± 0.8	**13.3 ± 0.9**	16.2 ± 2.2	21.0 ± 0.6
10AL	6.93 ± 0.80	6.90 ± 1.60	**5.77 ± 0.32**	2.48 ± 0.49	2.46 ± 0.51
Amount of IS added (µL)	35	38	**40**	42	45

Mean: Average of triplicate

b) Effect of amount of 0.1 M hydrochloric acid addition to sample

This method is only designed to add 330 µL of acid (0.1 M HCl) to the sample. The following table shows the variation of amount of acid to the sample. According to these data less than 20 µL differences from target volume (330 µL) in acid have not significantly changed the concentration of analytes.

Typical effects of amount of acid addition to sample

Analyte Code	Mean ± stdev (ng/mL)				
2AL	118 ± 34	160 ± 4	**172 ± 12**	196 ± 46	252 ± 11
ACAL	64.6 ± 8.5	61.8 ± 4.7	**67.9 ± 8.6**	76.9 ± 4.8	83.0 ± 11.0
3AL	43.5 ± 6.4	41.2 ± 0.5	**43.3 ± 1.2**	38.6 ± 4.0	41.7 ± 1.0
4AL	2.32 ± 0.17	2.19 ± 0.48	**2.22 ± 0.10**	2.89 ± 0.27	2.81 ± 0.35
I5AL	4.38 ± 0.41	4.39 ± 0.05	**4.57 ± 0.10**	4.25 ± 0.32	4.51 ± 0.21
CRAL	0.480 ± 0.100	0.487 ± 0.017	**0.533 ± 0.027**	0.621 ±0.100	0.659 ± 0.052
5AL	27.0 ± 4.2	24.7 ± 1.9	**23.6 ± 2.4**	18.8 ± 1.9	19.3 ± 0.7
6AL	28.2 ± 0.6	30.2 ± 3.0	**34.6 ± 2.7**	38.9 ± 2.9	38.4 ± 2.2
FRAL	1.52 ± 0.13	1.50 ± 0.26	**1.58 ± 0.08**	2.15 ± 0.58	2.17 ± 0.41
T6AL	7.20 ± 1.70	8.82 ± 0.20	**8.74 ± 0.88**	11.2 ± 2.8	11.5 ± 0.6
7AL	19.9 ± 1.5	18.7 ± 0.2	**20.6 ± 0.6**	19.4 ± 0.9	20.4 ± 0.5
BZAL	11.6 ± 1.6	11.3 ± 0.4	**11.0 ± 0.6**	9.50 ± 1.00	10.5 ± 1.0
8AL	2.57 ± 0.47	2.30 ± 0.22	**2.41 ± 0.13**	2.53 ± 0.21	2.56 ± 0.11
T8AL	47.1 ± 3.7	54.9 ± 6.3	**52.0 ± 3.5**	40.1 ± 3.9	45.9 ± 8.3
TLAL	0.036 ± 0.002	0.034 ± 0.004	**0.045 ± 0.013**	0.075 ± 0.014	0.045 ± 0.016
9AL	12.7 ± 1.8	14.2 ± 1.1	**14.8 ± 0.6**	13.3 ± 2.0	12.6 ± 5.1
T9AL	56.8 ± 3.2	65.4 ± 11.3	**66.0 ± 2.7**	73.5 ± 6.1	86.0 ± 17.0
10AL	0.580 ± 0.330	1.10 ± 0.27	**1.46 ± 0.27**	4.55 ± 0.52	4.49 ± 0.64
Amount of 0.1 M HCl added (µL)	310	320	330	340	350

Mean: Average of triplicate

c) Typical effect of agitation speed of sample during SPME extraction

In a normal method set up the agitation speed is 350 rpm. Small variation of speed (±50) have not significantly changed the concentration of analyte (see following Table)

Evaluation of agitation speed to the sample on the accuracy of the analyte Concentrations.

Analyte Code	Mean ± stdev (ng/mL)				
2AL	214 ± 18	202 ± 15	**205 ± 27**	257 ± 60	238 ± 15
ACAL	61.1 ± 2.6	58.1 ± 2.2	**61.0 ± 3.7**	80.3 ± 8.4	73.6 ± 8.6
3AL	34.5 ± 1.2	37.1 ± 2.5	**35.5 ± 0.9**	36.3 ± 4.3	37.1 ± 2.6
4AL	1.91 ± 0.11	1.98 ± 0.14	**1.99 ± 0.07**	3.63 ± 0.75	3.90 ± 0.53
I5AL	3.48 ± 0.14	3.61 ± 0.12	**3.63 ± 0.14**	3.63 ± 2.23	3.79 ± 0.13
CRAL	0.308 ± 0.039	0.340 ± 0.010	**0.330 ± 0.007**	0.348 ± 0.064	0.374 ± 0.003
5AL	15.8 ± 0.7	16.1 ± 0.7	**15.9 ± 0.3**	18.0 ± 1.0	17.8 ± 0.5
6AL	69.5 ± 0.8	69.4 ± 1.6	**69.9 ± 0.3**	67.1 ± 2.8	67.9 ± 2.1
FRAL	0.970 ± 0.221	1.13 ± 0.07	**1.13 ± 0.21**	1.47 ± 0.18	1.34 ± 0.11
T6AL	7.80 ± 2.50	10.1 ± 0.7	**9.68 ± 0.34**	10.7 ± 0.4	11.4 ± 0.9
7AL	14.4 ± 0.2	15.2 ± 0.9	**15.2 ± 0.7**	16.8 ± 1.7	17.5 ± 0.8
BZAL	5.54 ± 0.40	6.09 ± 0.17	**6.10 ± 0.12**	3.32 ± 0.26	3.83 ± 0.06
8AL	2.37 ± 0.08	2.54 ± 0.28	**2.34 ± 0.12**	1.43 ± 0.07	1.43± 0.02
T8AL	19.4 ± 0.5	23.1 ± 0.6	**25.3 ± 2.2**	18.0 ± 1.2	19.6 ± 2.6
TLAL	0.071 ± 0.009	0.082 ± 0.010	**0.079 ± 0.011**	0.097 ± 0.016	0.090± 0.011
9AL	13.1± 0.9	14.8 ± 0.8	**14.4 ± 0.5**	14.4 ± 1.5	16.4 ± 0.7
T9AL	29.7 ± 2.0	32.5 ± 0.6	**32.5 ± 4.9**	24.3 ± 1.4	27.1 ± 4.1
10AL	1.72 ± 0.93	1.40 ± 0.48	**2.06 ± 0.23**	1.74 ± 0.87	1.19 ± 0.03
Agitation speed (rpm)	300	325	350	375	400