QUANTITATIVE ANALYSIS OF VOLATILE ALDEHYDES IN TOBACCO, TOBACCO PRODUCTS, FIBRE-BASED MATRICES AND TOBACCO DERIVED PRODUCTS WITH (b) (4)

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Purpose

To quantitatively determine the amount of the volatile aldehydes formaldehyde, acetaldehyde, acrolein and crotonaldehyde in tobacco, tobacco products, fibre-based matrices and tobacco derived products (also called Purified Products) with (b) (4)

Applies to

APS

General information

Principle of the method

The volatile aldehydes are relatively small molecules (30-70 Da, **Figure 1**) that have undergone derivatisation (b) (4). Following weighing in of the samples, extraction and derivatisation are performed in the same step, (b) (4) in **Figure 1e**. Extraction of aldehydes in the sample as well as derivatisation (b) (4) Calculations are performed in (b) (4). The capacity per instrument and person is (b) (4) single samples/week.

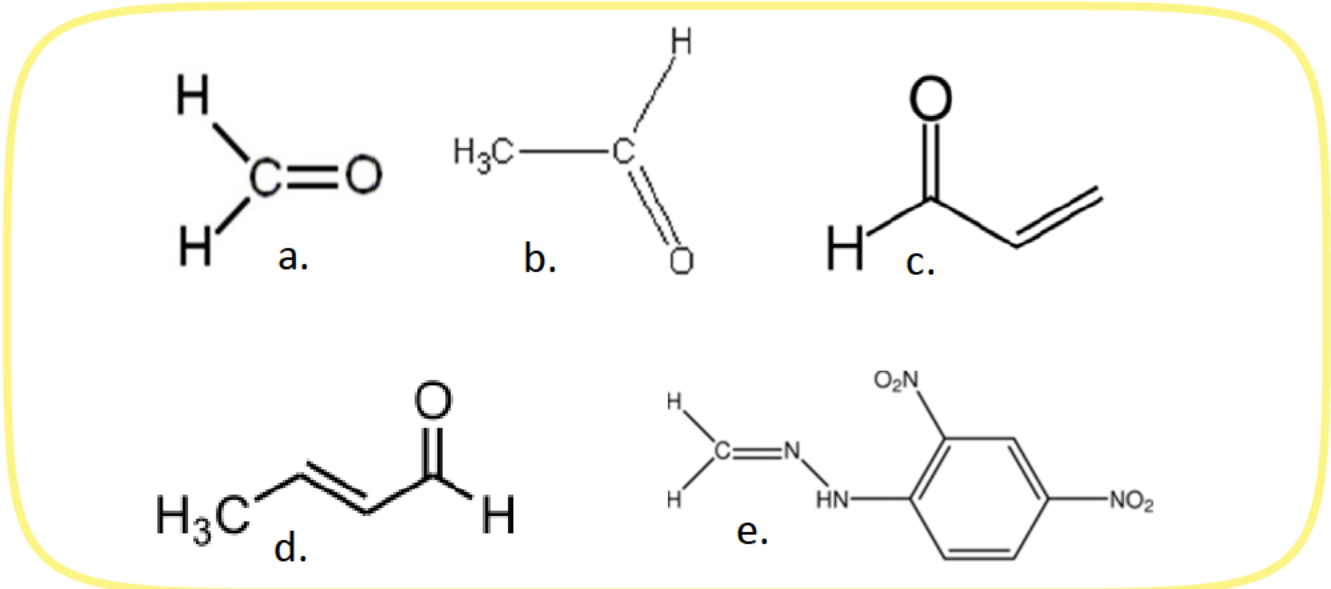


Figure 1. The chemical structural formula for formaldehyde (a), acetaldehyde (b), acrolein (c), crotonaldehyde (d) and DNF derivative of formaldehyde (e).

This method (APS) is based on the Coresta Recommended Method (b) (4), but differs in some aspects, see **Table 0**.

Table 0. The differences between CRM (b) (4) the APS method.

CRM (b) (4)	APS method
(b) (4)	

Note: All reference documents and additional information stated “available upon request” are in Swedish. They are available upon request but need to be translated into English first.

Method scope, measurement range and measurement uncertainty

Scope

The method is used for quantitative analysis of the volatile aldehydes formaldehyde, acetaldehyde, acrolein and crotonaldehyde in tobacco, tobacco products, fibre-based matrices and tobacco derived products, such as Zyn.

Method’s calibration range

Method’s measurement range

(b) (4)

Measurement uncertainty

(b) (4)

(b) (4)

(b) (4) This is in all likelihood due to the fact that the extracts from the various analysis timepoints have stood for varying amounts of time after shaking for one hour, as the shaking was not coordinated during the validation period. In order to mitigate the measurement uncertainty contribution, it is important to remove the isohexane phase from the sample extract within 30 minutes after the end of the extraction.
(b) (4)

In addition, it is vital to make additions of standard solutions, (b) (4) and (b) (4) without any interruption, and to be extra careful when adding standard and internal standard solutions.

Literature references

(b) (4)

Internal reference documents (available upon request)

(b) (4)

Risk assessment and safety instructions

Summarised risk assessment

A complete risk assessment of the method is available in (b) (4) When preparing the stock solutions of (b) (4) weighing in should be done in a fume cabinet or at a weighing point with a point extractor.
Wear nitrile gloves, protective goggles and a lab coat to prevent skin exposure when working with (b) (4).

(b) (4)

Substances hazardous to the environment

(b) (4)

Flammable substances

(b) (4)

Mixtures containing flammable solvents are usually also classified as flammable. Internal procedures for handling flammable substances and mixtures must be followed.

Hazard and precautionary statements

Safety data sheet for substances and solvents used in the method are in (b) (4) (b) (4). Classification below according to the International Agency for Research on Cancer (IARC).

Formaldehyde,	IARC Group 1; known carcinogenic to humans, (b) (4)
Acetaldehyde,	IARC Group 2B; possibly carcinogenic to humans, (b) (4) (b) (4)
Acrolein	Deadly by inhalation, (b) (4)
Crotonaldehyde (trans)	Deadly by inhalation, (b) (4)
(b) (4)	

Equipment

Apparatus

Two different (b) (4) systems are used for the analysis. See the relevant appendix for the system's individual settings.

Appendix 1. (b) (4)

Appendix 2.

Other equipment and laboratory utensils

(b) (4)

Chemicals, reagents and solvents

(b) (4)

(b) (4)

Check samples

(b) (4)

Preparation of standards and internal standards

(b) (4)

parafilm around the lid.
Hazard symbol: *Not subject to labelling*

Internal standard stock solution (b) (4)
Around 1000 mg of (b) (4) is weighed on an analytical scale in the fume cabinet in the same way as the non-deuterated in a 100 ml volumetric flask and diluted to the mark with MQ water. Shelf life of 6 months in a refrigerator with parafilm around the stopper.
Hazard symbol: *Systemic Health Hazards GHS08 (Danger)*

Internal standard stock solution (b) (4)
Around 1000 mg of (b) (4) is weighed on an analytical scale in the fume cabinet in the same way as the non-deuterated in a 100 ml volumetric flask and diluted to the mark with MQ water. Shelf life of 6 months in a refrigerator with parafilm around the stopper.
Hazard symbol: *Systemic Health Hazards GHS08 (Warning)*

Internal standard solution for spiking (IS): (b) (4)
(b) (4)
Pipette 5000 µL of (b) (4) and 1000 µL of (b) (4) internal standard stock solution into a 100 ml volumetric flask. Dilute to the mark with MQ water. Pour into a 100 ml flask. Shelf life of 30 days in a refrigerator with parafilm around the lid.
Hazard symbol: *Systemic Health Hazards GHS08 (Danger)*

Calibration standards
Calibration standards are prepared at every analysis timepoint in the same way as the samples, but (b) (4). Six calibration standards are prepared following the addition of Mix Standards 1 and 2 and IS as set out in **Table 3**. *NOTE: Spiking is done in 40 ml extraction solution, not in empty vessels*. Prepared calibration standards are stored in refrigerated auto injectors or refrigerators where they have a shelf life that is as long as the prepared samples, see "Sample stability" below.
Hazard symbol: *Not subject to labelling*

Table 3. (b) (4)

(b) (4)

Each calibration standard contains the corresponding 10 ppm of each internal standard.

Comparison of old and new standards

As the shelf life for stock standards and mix standards is set to 30 days, no comparisons of the new and old standards will be made. On the other hand, be observant if the check sample has a deviating value.

If calculations of concentrations are done manually, these are made as below.

Calculate the exact concentration for (b) (4) stock solutions

(b) (4)

Calculate the exact concentration for (b) (4) standard solution

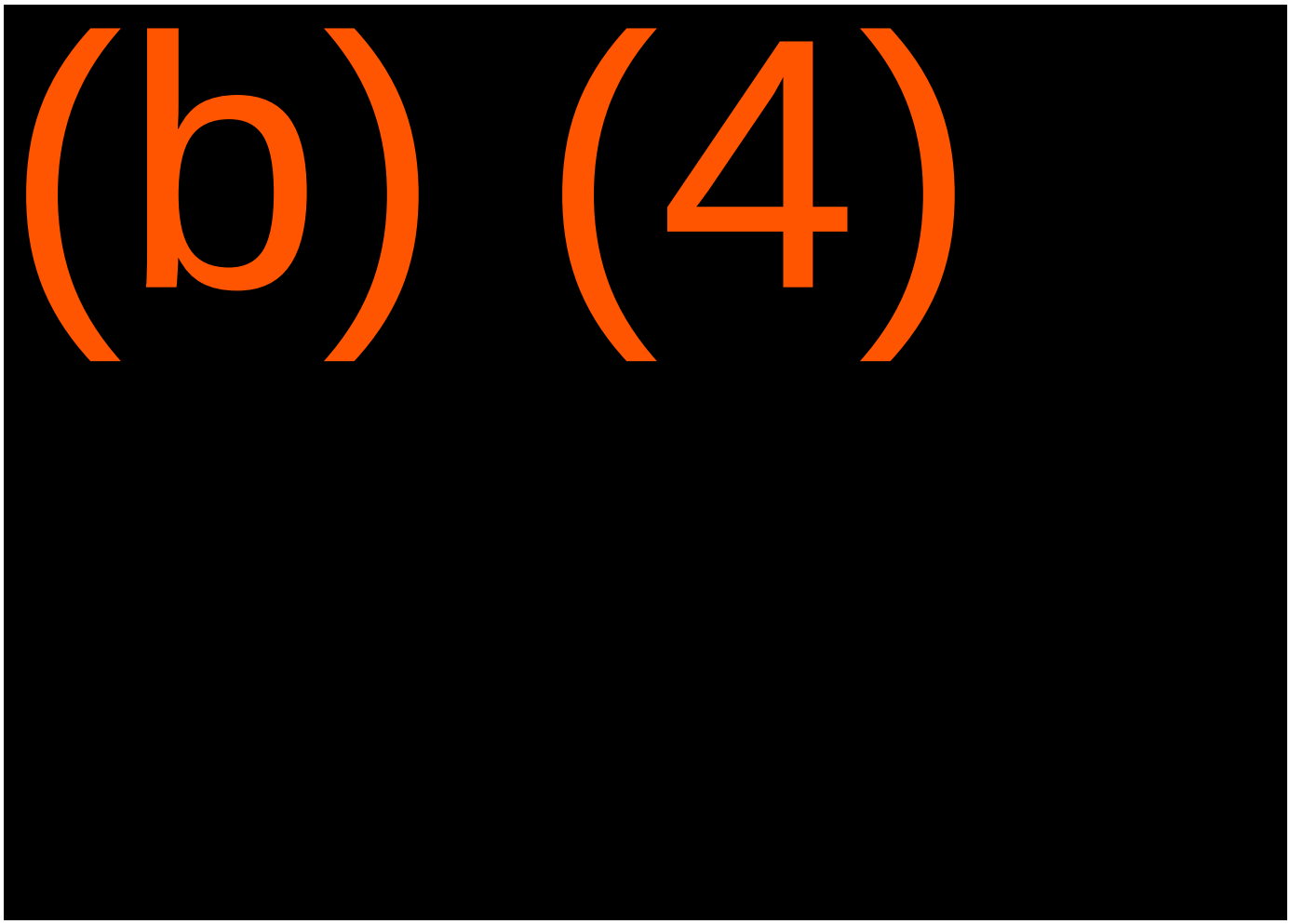
(b) (4)

Calculate the exact concentration of (b) (4) calibration standard solutions

(b) (4)

Preparation of other solutions

(b) (4)



Sample handling

Sample storage and preparation

Samples are stored and prepared in accordance with (b) (4) (available upon request).

Sample amount

The minimum required sample size for duplicate analysis and reanalysis is [redacted] g. The minimum amount of sample for performing a simple analysis is [redacted] g.

Analysis

Calibration and verification of apparatus

Before the analysis, the performance of the system should be evaluated by visually checking the chromatogram from the injection of Cal 2. Start by injecting four Cal 2 to equilibrate the system.

A standard curve is generated in each sequence by injecting all of the calibration solutions first and last in the sequence. The check samples and the samples that are to be analysed, are injected in between as follows:

- Cal 2 for equilibrium (and for checking signal/noise)
- Cal 1-6
- (blank)
- Check samples 1-2
- (blank)
- Samples
- (blank)
- Check samples 1-2
- (blank)
- Cal 1-6

Sample stability

The prepared calibration standards and samples must be analysed together within 3 days and must be refrigerated from the time that they are prepared.

Analytical procedure

Preferably use frozen samples. Allow them to thaw at least 30 minutes before weighing in. (b) (4) Place Mix standards 1 and 2 and Mix

(b) (4)

(b) (4)

Special instructions

The samples can be weighed out the day prior to extraction if they are stored in sealed Erlenmeyer flasks in the refrigerator. Prepare all the solutions and equipment to ensure that points 4 to 8 are performed as smoothly as possible without any interruptions. After shaking, the extracts must be left for 30 minutes before point 10 is completed.

Documentation

Write on the work list when and who weighed out the samples, prepared them and evaluated the instrument run, as well as any comments on the analysis or control chart.
The preparation date for the internal standard solution used for spiking of samples and the date when the calibration standard was prepared is noted.
(b) (4)

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Data

Collection and storage of data

(b) (4)

Calculations

(b) (4)

(b) (4)

Quality assurance

Control chart

For the internal quality control; in each run, there are two check samples which are injected before and after the unknown samples in the sequence. Check samples and the evaluation of results in the control chart are handled as multi-analysis in line with (b) (4)

he mean value is shown in the X-chart and the percentage difference between the values in the R-chart.

If the analysis results can not be approved in compliance with the criteria above, the following measures should be taken:

- Check that the analysis conditions are correct, and remedy any non-conformity, such as changing to new mobile phases or new analytical column.

Comments regarding the problems are entered in the control chart and in the event of extended comments, refer to the work list.

Standard curve criteria

For each quatification, the adjustment of the calibration standards to suit the standard curve and the accuracy of the curve are checked. (b) (4)

As the calibration standards are injected both at the beginning and end of each run, two calibration points are obtained for each level in the standard curve. Three points at different levels or two points at the same level plus one point on another level may be excluded in order to allow the remaining calibration points to be approved in line with the criteria. If the standard curve does not meet the set requirements, the samples and new standards need to be re-prepared for re-analysis.

(b) (4)

A response greater than the highest calibration standard

Samples with responses greater than the highest calibration standard are prepared with around half the sample, for a new analysis. The reporting is solely based on the re-analysis. The sample that is injected after a sample with a high concentration can be injected if there is any suspicion of carry-over. In this case, this must be done before the sample and the associated calibration standards are too old. Otherwise, a new preparation must be made.

Reporting of analysis results

(b) (4)

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Revision history

(b) (4)

(b) (4)

Person responsible

Director APS

Validation

Validation report

(b) (4)

Table 4. (b) (4)

(b) (4)

Selectivity

(b) (4)

(b) (4)

Cross talk

Cross talk has not been experimentally investigated as it is unlikely that it will occur when it divides by 2 atomic mass units or more between the parent ions in the MRM transitions.

Carry-over

(b) (4)

Repeatability

Repeatability was determined by analysing the same matrix six times in constant conditions on a total of nine different matrices, see matrices and concentrations in **Table 4**.

(b) (4)

Table 5. (b) (4)

(b) (4)

See the validation sheet for calculations and a more detailed presentation of the results.

Precision within the laboratory

(b) (4)

Table 6. (b) (4)

(b) (4)

Accuracy (trueness)

(b) (4)

Table 7 (b) (4)

Table 7. (b) (4)

(b) (4)

See the validation sheet for calculations of accuracy for the spiked samples

Bias from accuracy

The estimated concentration error in the method to the true (spiked) concentration in % (bias) is calculated as the square root of the sum of squares of accuracy -100 from the Accuracy and the uncertainty in the addition of the amount of analyte. Bias from accuracy is used for calculating measurement uncertainty. **Table 8** shows the bias for the different analytes.

Table 8. Bias % from accuracy for each aldehyde.

Analyte	Bias %
(b) (4)	

Limit of detection (LOD) and limit of quantification (LOQ)

(b) (4) "LOQ Reporting limit" is the definitively determined LOQ as based on S/N in the sample matrices as well as those in the lowest-used standard levels. (b) (4) See the validation sheet for calculations.

Table 9. (b) (4)

Analyte	LOD standard (ppm)	LOQ standard (ppm)	**LOQ Reporting limit (ppm)**
(b) (4)			

Linearity

(b) (4)

(b) (4)

Table 10 shows the correlation coefficients and the span of the accuracy (b) (4) **Table 11** shows the standard curve measurement uncertainty contribution to the total measurement uncertainty when one and then two standard curves are used in the quantitation process.

Table 10. (b) (4)

Analyte	Correlation coefficient	Accuracy standard points
(b) (4)		

Table 11. (b) (4)

Analyte	Measurement uncertainty **one** standard curve	Measurement uncertainty **two** standard curves
(b) (4)		

The validation sheet shows a graph of the regression line and the analytical data together with the slope and y-intercept of the line, along with the percentage accuracy at each calibration level.

Matrix effect

Typically, in the method validation of LC-MS methods, the matrix effect in MS is investigated together with the extraction yield and overall process efficiency. In this validation, only the matrix effect has been investigated as the analysis contains a derivatisation step, and it is not possible to separate the extraction yield from the derivatisation yield.

The matrix effect has been determined b (b) (4) This was performed in duplicate samples at levels 1 ppm and 10 ppm, resulting in a total of 16 trials. The peak areas for (b) (4) (b) (4), which gives the matrix power in MS. The matrix effect is presented in **Table 12**. (b) (4)

(b) (4)

Table 12. (b) (4)

Analyte	Matrix effect %
(b) (4)	

See the validation sheets for a more detailed presentation of the results.

Robustness

The robustness of the method is estimated from the variation of the check sample over time. This is not used here as the method is new and therefore there is an inadequate volume of check sample data. Sample and standard stability are presented under the robustness point. The stability of sample extracts and standard solutions have proven to be critical for the robustness of the method, and have therefore been studied in more detail.

Stability of the sample extract

(b) (4) in **Table 13**. The results demonstrate that the sample extract is not stable in the two-phase system and therefore (b) (4)

Table 13. (b) (4)

(b) (4)	

The stability of (b) (4) is presented in **Table 14**. (b) (4)

(b) (4)

Table 14. (b) (4)

(b) (4)

Standard stability

The stability of (b) (4) was examined in standard solutions (b) (4) (b) (4). The results for (b) (4) are presented in **Table 15**. The shelf life is good in the refrigerator 30 days for (b) (4) (b) (4). However, it is good enough to meet the requirements for this multi-method.

Table 15. (b) (4)

(b) (4)

Measurement range

The measurement range of the method is presented in **Table 16**. If any sample exceeds the concentration for the strongest standard, a smaller amount of sample must be weighed out and the sample is re-prepared and re-analysed.

Table 16. Measurement range for each aldehyde.

Analyte	When reporting (ppm)
(b) (4)	

Measurement uncertainty

The relative measurement uncertainty for all tested matrices (coverage factor 2) are presented in **Table 17** for single and duplicate samples.

Table 17. (b) (4)

(b) (4)

The measurement uncertainty contribution for (b) (4) In order to reduce the contribution to measurement uncertainty, it is important to remove (b) (4) the sample extract within 30 minutes of the end of the extraction.
The measurement uncertainty for (b) (4) It is likely due to that (b) (4) is used as internal standards for these two, instead of their own isotope labelled internal standards. (b) (4)

In addition, it is important to make the additions of standard solutions, internal standard, DNF and (b) (4) without any interruption, and to be extra careful when adding standard and internal standard solutions.

Additional validation for (b) (4)

Limit of detection (LOD) and Limit of quantification (LOQ) for (b) (4)

LOD/LOQ was investigated by studying repeatability and accuracy for Cal 2, which is the lowest spiked level in the calibration standards and the reporting limit of the method and practical LOQ. (b) (4)

The FDA Guideline for Industry-Bioanalytical Method Validation, states that repeatability and accuracy must be < 20% for the lowest calibration standard. **Table 18** shows RSD % for repeatability and the span for % bias accuracy for Cal 2.

Table 18. (b) (4)

Analyte	RSD (%)	% Bias from Cal 2
Formaldehyde	11.6	-10 – 17
Acetaldehyde	4.4	-3.0 – 6.3
Acrolein	5.1	-5.7 – 5.8
Crotonaldehyde	7.2	-7.1 – 10.6

To determine S/N in samples with matrix is not an issue for form- and acetaldehyde as they are always present at higher levels. S/N for crotonaldehyde and acrolein has not been investigated in a matrix as the concentrations almost exclusively fall below the reporting limit (Cal 2).

See the validation sheet for calculations and a more detailed presentation of the results.

Linearity for Agilent 6495

Linearity has been investigated by analysing six (form- and acetaldehyde) or five (acrolein and crotonaldehyde) calibration solutions in three series with concentrations of 0 to 60 ppm for form- and acetaldehyde, and 0.1-12 ppm for acrolein and crotonaldehyde. Linear and square curve fitting using the least squares method has been examined as well as unweighed and weighted curves. Square curve fitting weighted by $1/y^2$ produced the best correlation coefficients and best accuracy. **Table 19** shows correlation coefficients and the span for the accuracy of the calibration points for each aldehyde. **Table 20** shows the standard curve measurement uncertainty contribution to the total measurement uncertainty when one and then two standard curves are used in the quantitation process.

Table 19. The correlation coefficients and the span of accuracy in the calibration points for each aldehyde.

Analyte	Correlation coefficient	Accuracy standard points
Formaldehyde	0.9994	-10 – 7.1
Acetaldehyde	0.9997	-4.8 – 4.0
Acrolein	0.9988	-5.4 – 8.0
Crotonaldehyde	0.9975	-7.5 – 13

Table 20. (b) (4)

Analyte	Measurement uncertainty **one** standard curve (%)	Measurement uncertainty **two** standard curves (%)
(b) (4)		

See the validation sheet for calculations and a more detailed presentation of the results.

The cross validation analysis of the same samples on (b) (4)
Three different batches, with samples of (b) (4) were analysed using both (b) (4) The systematic percentage difference of the mean value ("Bias") was evaluated between the instruments. Samples, check samples and standards were included in the comparison, **Table 21.** (b) (4) For the instruments to be assessed equivalent, the mean value should be less than 10%. (b) (4)

Table 21. Evaluation of systematic difference (Bias) between Instruments*.

Analyte	Mean value difference (%)	Mean value RSD (%)
(b) (4)		

(b) (4)

The conclusion is that (b) (4) **provide equivalent results for all** (b) (4) .

Conclusion

19/03/2018: (b) (4)

Appendices

1. (b) (4)
2.

Appendix 1. (b) (4)

(b) (4)

UPLC parameters

Eluent solution (mobile phase)
Eluent A: 10 mM ammonium acetate, pH 4.7 ± 0.1 with acetic acid
Eluent B: Acetonitrile (LC-MS grade)

(b) (4)

Table 22. Time programming of UPLC pumps.

Time (min)	Flow (ml/min)	% Eluent A	% Eluent B	Gradient type
(b) (4)				

(b) (4)

(b) (4)

Mobile phase switch

The switching of mobile phase to waste at the beginning and end of the gradient is made with a switch valve built-into the MS. (b) (4) :

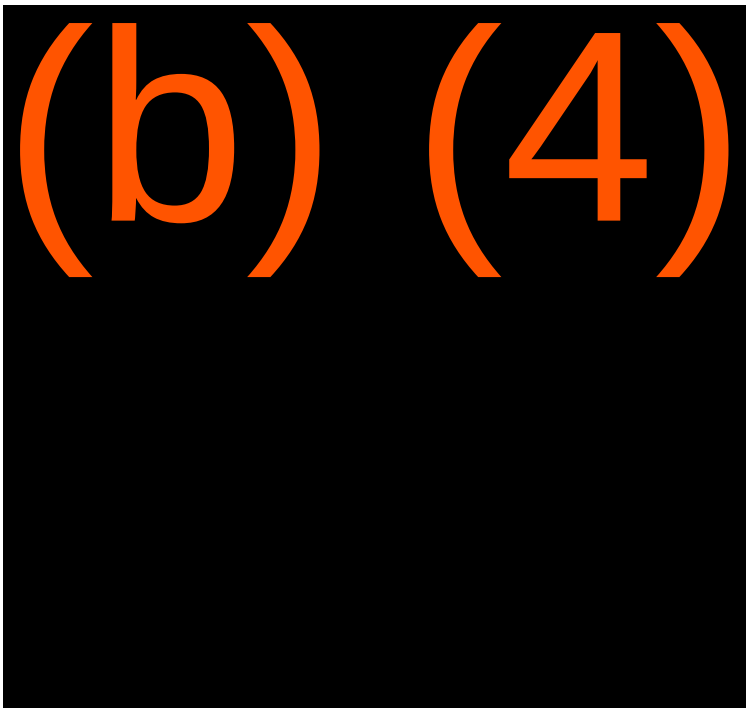
Initial flow state: Waste

Event	Time(sec)	Name	Action

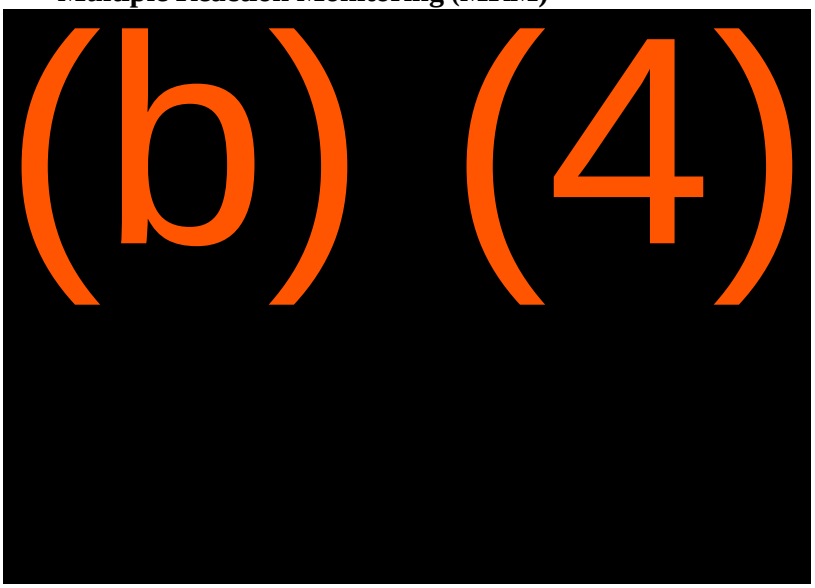
(b) (4)

(b) (4)-parameters

(b) (4)



Multiple Reaction Monitoring (MRM)



Tables 23 and 24 summarise the substance-specific parameters for the quantification and confirmation of the (b) (4) .

(b) (4)

Appendix 2. (b) (4)

(b) (4)

UPLC parameters

Eluent solution (mobile phase)
Eluent A: 10 mM ammonium acetate, pH 4.7 ± 0.1 with acetic acid
Eluent B: Acetonitrile (LC-MS grade).

The UPLC pumps are programmed in (b) (4) according to **Table 25**.

Table 25. (b) (4)

Time (min)	A (%)	B (%)	Flow (mL/min)	Max. Pressure Limit (bar)
(b) (4)				

Multisampler

(b) (4)

(b) (4)

Multisampler Pretreatment

(b) (4)

Binary Pump

(b) (4)

Column Comp.

(b) (4)

MS/MS-parameters (QQQ)

(b) (4)

Time segments:

#	Start Time	Scan Type	Div Valve	Delta EMV(+)	Delta EMV (-)	Stored
(b) (4)						

The substance-specific acquisition parameters for the (b) (4) are summarised in **Table 26**.

Table 26. (b) (4)

Comp. name	ISTD ?	Precursor Ion (m/z)	MS1 Res	Product Ions (m/z)	MS2 Res	Dwell Time msec	Fragmentor	Coll. Energy	Cell Acc. Vol.	Polarity
(b) (4)										

Source parameters

(b) (4)

Table 27 summarises the substance-specific parameters for the quantification and confirmation of the (b) (4) .

Table 27. (b) (4)

(b) (4)										

(b) (4)

Chromatogram

Examples of chromatograms and typical retention times and the peak shape of (b) (4) (b) (4) can be viewed in **Figure 3**.

Swedish Match

Quality and Environmental Management System

Document Title
Quantitative Analysis of volatile aldehydes with (b) (4)

Part of Process
Contract Analysis APS

Document Type
Method Description

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(b) (4)