Megazyme

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FORMALDEHYDE

ASSAY PROCEDURE

K-FRHYD 05/20

(*50 Manual Assays per Kit) or
(500 Auto-Analyser Assays per Kit) or
(500 Microplate Assays per Kit)

The number of tests per kit can be doubled if all volumes are halved

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INTRODUCTION:

Formaldehyde is a chemical that is used abundantly in a large number of industries and found in many consumer products. At room temperature, it is a colourless, flammable gas and highly reactive. Formaldehyde is found in the environment, it is released through biomass combustion (e.g. forest fires) or biomass decomposition. Other combustion processes (power plants, incineration, etc.) also represent sources of formaldehyde emissions in the atmosphere. However, formaldehyde is also extensively produced industrially worldwide for use in the manufacture of resins, as a disinfectant and fixative, or as a preservative in consumer products. Background levels of formaldehyde in food products are variable and range from values below 1 mg/kg in milk to over 200 mg/kg in some fish species.[1] Due to its toxicity and its widespread use, exposure to formaldehyde is a significant consideration for human health.

The Formaldehyde Assay Kit (**K-FRHYD**) is a simple, reliable and accurate method for the measurement and analysis of formaldehyde in a variety of sample types including environmental samples and food.

PRINCIPLE:

Formaldehyde is quantitatively oxidised to formic acid in the presence of formaldehyde dehydrogenase (FADH) and nicotinamide-adenine dinucleotide (NAD^+)

$$\text{Formaldehyde} + NAD^+ + H_2O \xrightarrow{\text{(FADH)}} \text{Formic acid} + NADH + H^+$$

The amount of NADH formed in this reaction is stoichiometric with the amount of formaldehyde. It is the NADH that is measured by the increase in absorbance at 340 nm.

ACCURACY:

Standard errors of less than 2% are achieved routinely. Repeatability data is shown in Table 1 (page 9).

SENSITIVITY, LINEARITY AND PRECISION:

The smallest differentiating absorbance for the assay (Limit of Detection) is 0.003 absorbance units, this corresponds to 0.033 μg of formaldehyde per test or 0.016 μg/mL of formaldehyde in a sample treated as per the standard procedure, and using the maximum sample volume of 2.0 mL in the test. The Limit of Quantification is 0.11 μg of formaldehyde per test or 0.054 μg/mL of formaldehyde in a sample treated as per the standard procedure, which is derived from an absorbance difference of 0.09 and the maximum sample volume of 2.0 mL in the assay. The assay is linear over the range of 0.1 to 14 μg

of formaldehyde per assay, this is shown in Figure 1 (page 10).

INTERFERENCE:

Under the assay conditions described, formaldehyde is quantitatively converted. The presence of acetaldehyde (at a concentration higher than that of formaldehyde) leads to a sample-dependent creep reaction that can be accounted for by extrapolation back to the time of addition of FADH (see notes on creep measurement on page 5). Some overestimation will still be observed if the acetaldehyde present in the test exceeds 10 μg.

Glutaraldehyde has been shown to interfere only at concentrations greatly exceeding the formaldehyde concentration. Good recoveries were achieved in tests containing 100 μg glutaraldehyde, which corresponds to 0.1% glutaraldehyde in a sample treated as per the standard procedure and using 0.1 mL per test.

No interference was observed in tests containing methanol and 2-propanol, while high levels of ethanol (the equivalent of 100% ethanol in a sample treated as per the standard procedure and using 0.1 mL per test) resulted in a sample-dependent creep reaction. This can again be accounted for by use of the linear extrapolation calculator and excellent recoveries are achieved. Very low levels of formaldehyde are measurable in a high ethanol matrix.

If the conversion of formaldehyde has been completed within the time specified in the assay (approx. 10 min), it can be generally concluded that no interference has occurred. Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to. For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 50 assays in manual format (or 500 assays in auto-analyser format or 500 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (12 mL, pH 9.0) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: NAD^+.
Freeze dried powder.
Stable for > 5 years below -10°C.

Bottle 3: Formaldehyde dehydrogenase solution (2.5 mL) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years below -10°C.

Bottle 4: Paraformaldehyde control powder (~ 3 g).
Stable for > 2 years; store sealed at 4°C.

PREPARATION OF REAGENT SOLUTIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 12 mL of distilled water.
Stable for > 1 year at 4°C or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

3. Use the contents of bottle 3 as supplied.
Stable for > 2 years below -10°C.

4. Accurately weigh 300 mg of the paraformaldehyde control powder into a 13 mL polypropylene tube and dissolve it in ~ 5 mL of 0.1 M potassium pyrophosphate buffer pH 9.0 (Dilution buffer B). Quantitatively transfer the solution into a 500 mL volumetric flask followed by ~ 300 mL of 0.1M potassium pyrophosphate buffer pH 9.0. Incubate the sample in a water bath set at 60°C for 10 min until sample is fully dissolved. Cool the solution to room temperature and adjust the volume to 500 mL with dilution buffer. Further dilute this preparation 10-fold by addition of 0.5 mL to 4.5 mL of 0.1 M potassium pyrophosphate buffer pH 9.0.

NOTE: The formaldehyde control powder is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of formaldehyde is determined directly from the extinction coefficient of NADH.

Paraformaldehyde is toxic by inhalation and moderately toxic by skin contact. All due care should be taken when using this component.

PREPARATION OF ADDITIONAL BUFFERS (not supplied):

(A) Concentrated Potassium Pyrophosphate Buffer, 1 M, pH 9.0

Dissolve 330.3 g of tetrapotassium pyrophosphate (anhydrous; Sigma Cat. No. 322431) in ~ 800 mL of distilled water. Adjust the pH to 9.0 using 8 M HCl. Adjust volume to 1 L. Add 0.2 g sodium azide and dissolve by stirring.

(B) Dilution Buffer (Potassium Pyrophosphate 100 mM, pH 9.0)

Add 100 mL of concentrated Potassium pyrophosphate buffer (A) to 800 mL of distilled water. Adjust the pH to 9.0 with 1 M HCl or 1 M NaOH and adjust the volume to 1 L. Stable for > 1 year at 4°C.

> **NOTE:** If diluted buffer is prepared without adding sodium azide as a preservative, then it should be stored at 4°C and used within a week..

EQUIPMENT (RECOMMENDED):

1. Disposable 13 mL polypropylene tubes, e.g. Sarstedt cat. no. 60.541.685 PP.
2. Volumetric flask (500 mL).
3. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
4. Micro-pipettors, e.g. Gilson Pipetman® (100 µL and 200 µL).
5. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of Buffer and NAD^+ solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
6. Top-pan balance.
7. Analytical balance.
8. Spectrophotometer set at 340 nm, e.g. MegaQuant Wave (Megazyme cat. no. **D-MQWAVE**).
9. Vortex mixer.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm

Cuvette: 1 cm light path (glass or plastic)
Note: It is essential to stopper the cuvettes

Temperature: ~ 25°C

Final volume: 2.55 mL

Sample solution: 0.1-14.0 µg of formaldehyde per cuvette (in 0.10-2.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C) sample solution solution 1 (buffer) solution 2 (NAD^+)	2.10 mL - 0.20 mL 0.20 mL	2.00 mL 0.10 mL 0.20 mL 0.20 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:		
solution 3 (FADH)	0.05 mL	0.05 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 15 min). If the reaction has not stopped after 15 min, continue to read the absorbances at 2 min intervals**.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of solution 3 (using the *MegaCalc*™).

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{formaldehyde}$.

The value of $\Delta A_{formaldehyde}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of formaldehyde can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{formaldehyde} \quad [g/L]$$

where:

V	=	final volume [mL]
MW	=	molecular weight of formaldehyde [g/mol]
ε	=	extinction coefficient of NADH at 340 nm
	=	6300 [$l \times mol^{-1} \times cm^{-1}$]
d	=	light path [cm]
v	=	sample volume [mL]

It follows for formaldehyde:

$$c = \frac{2.55 \times 30.03}{6300 \times 1.0 \times 0.1} \times \Delta A_{formaldehyde} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of formaldehyde:

$$\frac{c_{formaldehyde}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme web site (www.megazyme.com).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for formaldehyde can be performed using either a single point standard or a full calibration curve.

2. For each batch of samples that is applied to the determination of formaldehyde, either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
distilled water solution 1 (buffer) solution 2 (NAD^+)	48.68 mL 5.50 mL 5.50 mL (after adding 12 mL of H_2O to bottle 2)
Total volume	59.68 mL

Preparation of R2:

Component	Volume
distilled water solution 3 (FADH)	6.00 mL 1.35 mL
Total volume	7.35 mL

EXAMPLE METHOD:

R1: 0.200 mL
Sample: ~ 0.01 mL
R2: 0.025 mL

Reaction time: ~ 15 min at 25°C
Wavelength: 340 nm
Prepared reagent stability: > 2 days when refrigerated
Calculation: endpoint
Reaction direction: increase
Linearity: up to 0.14 g/L of formaldehyde using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:
1. The Microplate Assay Procedure for formaldehyde can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of formaldehyde either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.255 mL
Linearity: 0.01-1.4 µg of formaldehyde per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
solution 2 (NAD^+)	0.020 mL	0.020 mL	0.020 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
solution 3 (FADH)	0.005 mL	0.005 mL	0.005 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 15 min). If the reaction has not stopped after 15 min, continue to read the absorbances at 2 min intervals**.			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of solution 3.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of formaldehyde present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.1 and 14 µg. The sample solution must therefore be diluted sufficiently to yield a formaldehyde concentration between 0.001 and 0.14 g/L.

Dilution Table

Estimated concentration of formaldehyde (g/L)	Dilution with water	Dilution factor (F)
< 0.14	No dilution required	1
0.14-1.40	1 + 9	10
1.40-14.0	1 + 99	100
14.0-140	1 + 999	1000
> 140	1 + 9999	10000

If the value of $\Delta A_{formaldehyde}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

2. Sample handling.

(a) Formaldehyde is extremely volatile, so all samples and assay controls must be stored in tightly closed containers.

(b) Solutions containing formaldehyde should always be dispensed into the buffer or aqueous solutions to minimise loss through evaporation.

(c) Formaldehyde is readily oxidised by atmospheric oxygen. Consequently, it is essential to analyse samples as soon as possible after preparation. Control solutions should be used on the day of preparation.

(d) The preferred assay control material is paraformaldehyde powder as it is less volatile than formalin and is not as readily oxidised or polymerised.

APPENDIX:

A. Linear range of assay

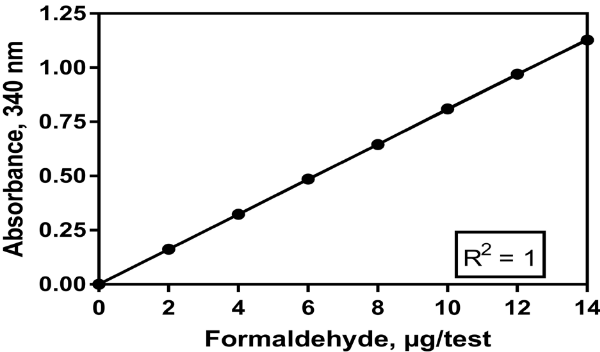

Figure 1. Linearity of the formaldehyde assay.

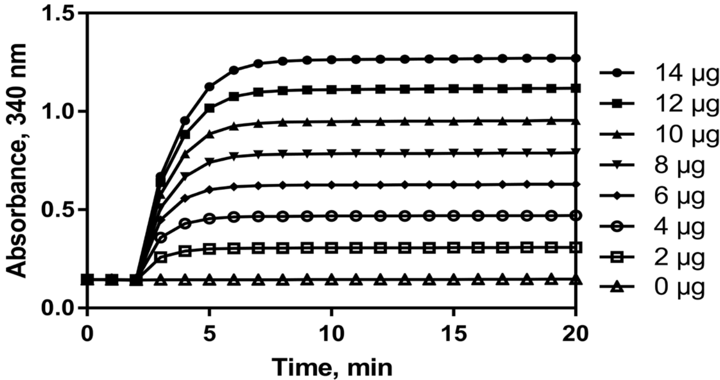

Figure 2. Increase in absorbance at 340 nm on incubation of 0-14 µg of formaldehyde per test.

µg formaldehyde/ test	n	Mean, ΔA	Standard Deviation	% CV
2	12	0.162	0.0030	1.83
4	12	0.323	0.0034	1.05
6	12	0.486	0.0038	0.78
8	12	0.645	0.0119	1.85
10	12	0.810	0.0110	1.36
12	12	0.971	0.0116	1.20
14	12	1.128	0.0129	1.15

Table 1. Intermediate precision values obtained using a range of formaldehyde standards in the test.

REFERENCES:

1. European Food Safety Authority. (2014). Endogenous formaldehyde turnover in humans compared with exogenous contribution from food sources. *EFSA Journal,* **12,** 3550-3561.

NOTES:

Bray Business Park, Bray,
Co. Wicklow,
A98 YV29,
IRELAND.

Telephone: (353.1) 286 1220
Facsimile: (353.1) 286 1264
Internet: www.megazyme.com
E-Mail: info@megazyme.com

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