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BIOCHEMICAL ASSAY BY IMMOBILIZED ENZYMES AND A MASS SPECTROMETER

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Summary

By counting the volatile molecules produced by an immobilized-enzyme catalyzed reaction which is interfaced to a mass spectrometer via a semi-permeable membrane, a general approach to biochemical measurement and detection is obtained which offers the potential of high sensitivity, specificity and speed. In combination with molecule microscopy, this method should allow, for example, a mapping of suitable enzyme distributions in non-stained and non-fixed tissue slices.

Immobilized urease (urea amidohydrolase, EC 3.5.1.5) was used to assay urea using CO_2 as the volatile product, and alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) was used to assay NADH using ethanol as the volatile product.

Introduction

The use of gas chromatography-mass spectrometry for the detection and measurement of biochemicals is well established [1,2]. For comparison with the method presented here, important features of gas chromatography-mass spectrometry are: (1) existence of a time delay during the separation of sample molecules in the gas chromatograph, (2) thermal volatilization of sample molecules, and (3) direct introduction of sample molecules into the mass spectrometer following separation. As a consequence, gas chromatography-mass spectrometry is not readily adapted to real-time monitoring or measurements requiring a rapid time response. Furthermore, gas chromatography-mass spectrometry is generally limited to molecules with a vapor pressure greater than 10^{-6} mm Hg, and a molecular weight of less than 1200. Also, identification is obtained by comparison of an often complex mass spectrum (fragmentation pattern) with known patterns.

We present a new method which is based on the direct and rapid counting of volatile enzyme product molecules [3]. One or more selected enzymes, which are immobilized next to a semi-permeable membrane, catalytically convert a given substrate into products, of which at least one is reasonably volatile (e.g. CO_2), which then permeate the membrane and evaporate into the reduced pressure (vacuum) of a mass spectrometer. Since such volatile products are usually low molecular weight molecules which do not fragment significantly compared to the substrate [1,2], the mass spectrometer can often be operated at only one mass peak. Substrate identification is thus obtained via the enzymes' specificity. This allows the use of a relatively inexpensive mass spectrometer, and optimization for sensitivity. In the configurations currently used (Figs. 1 and 2a), the steady-state, mass spectrometer count rate at the volatile product molecular weight is proportional to the enzyme-catalyzed reaction rate. Over a wide range of concentration, this rate is proportional to substrate concentration.

Methods and Results

The current volatile enzyme-product apparatus [4] (Fig. 1) has been used for rapid measurements of urea and NADH. A buffered, aqueous solution was circulated past a layer of enzyme immobilized next to a synthetic semipermeable membrane, the other side of which was exposed to the vacuum of the mass spectrometer. Small concentrated amounts of substrate were injected into the circulating solution. When substrate molecules reached the immobilized enzyme layer, a rapid increase in the mass spectrometer count rate at the volatile product mass peak occurred. Since the enzyme reaction is localized near the semi-permeable membrane, a large fraction, f_R (about 0.25 for the urease configuration and 0.75 for the alcohol dehydrogenase configuration) of the volatile product permeates the membrane and evaporates. Similar fractional recoveries of volatile products have been obtained in electrochemical mass spectrometry [5].

Initially we used urease (urea amidogydrolase, EC 3.5.1.5), which catalyzes the reaction:

$$\text{urea} + H_2O \xrightarrow[(EC\ 3.5.1.5)]{\text{Urease}} 2NH_3 + CO_2$$

While both products are permeable and volatile, we monitored the CO_2 count rate, since a large vacuum background at the molecular weight of NH_3 was present due to OH fragments produced from H_2O, which also permeates the membrane into the mass spectrometer. We began with blank runs in which there was no immobilized enzyme and in which the concentration of urea in the circulating buffer was increased stepwise from zero by injecting small, concentrated quantities of urea. No change in the CO_2 count rate was observed. Solubilized urease was then injected into the circulating solution (Fig. 2b). After a delivery time delay and following a transient due to: (1) diffusion of substrate across the unstirred boundary layer and product diffusion through the polyester backing and membrane, and (2) mixing of the injected material while circu-

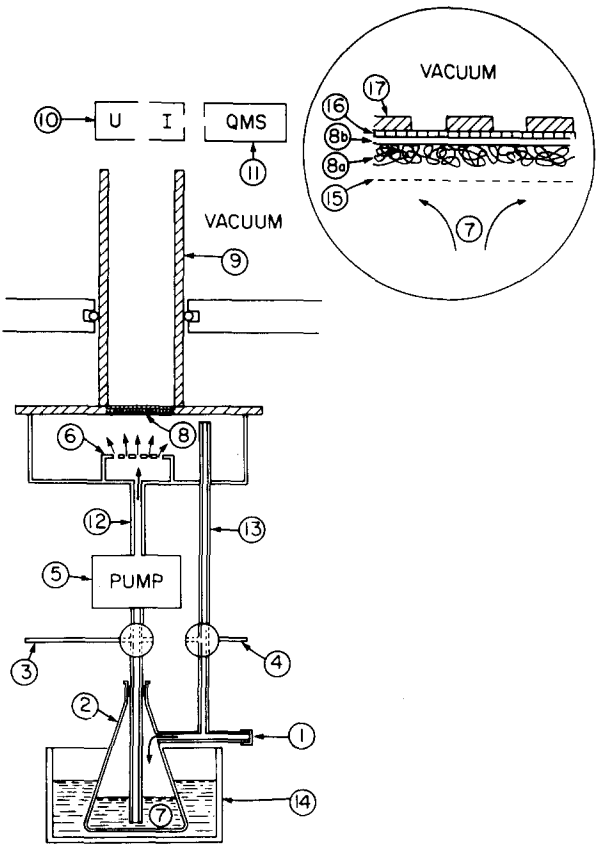

Fig. 1. Schematic representation of the apparatus, which consists of a circulating sample solution, immobilized enzyme, semi-permeable membrane (see insert) and mass spectrometer. 1 is a sample injection port, 2 is a stirred flask for mixing, 3 and 4 are valved inlets and outlets respectively for partial degassing by N_2 bubbling, 5 is a peristaltic pump, 6 is a perforated disc providing additional mixing and more uniform delivery of sample 7 to the enzyme layer and the dimethyl silicone membrane 8. A heated transfer tube 9 guides the permeable and volatile molecules into the universal ionizer 10 of the quadrupole mass spectrometer (QMS) 11. Tygon tubing was used for section 12 and 13, where CO_2 absorption effects may be responsible for our inability to greatly degas the sample. 14 is a constant temperature bath. The total circulating sample solution volume was about 150 ml, and the delivery time from sample injection to the first response was 10—20 s depending on pump speed. The insert is an enlarged schematic of the enzyme layer and membrane region shown for the immobilized urease experiment. Starting from the sample 7 side, a simple, one-dimensional, unstirred boundary layer 15 of thickness $x \simeq 10^{-2}$ cm is assumed. Then there is the approx. $3 \cdot 10^{-2}$ cm thick polyester backing 8a on which the urease is immobilized. The dimethyl silicone membrane itself 8b of $2.5 \cdot 10^{-3}$ cm thickness is directly supported by a single piece of 10 μm thick Nuclepore filter 16, followed by a perforated stainless steel disc 17 containing several holes for the main mechnical support. Finally there is the greatly reduced pressure or vacuum of the mass spectrometer.

lating (causing the damped oscillation component), the steady state CO_2 count rate increased linearly in time. This is as expected for a CO_2-producing reaction occurring homogeneously in a sample volume which is large relative to membrane area and permeability. The steady slow rise in count rate for the solubilized urease reaction is proportional to the accumulation of CO_2 in the circulating solution, and the count rate is thus a measure of the time integral of the reaction rate.

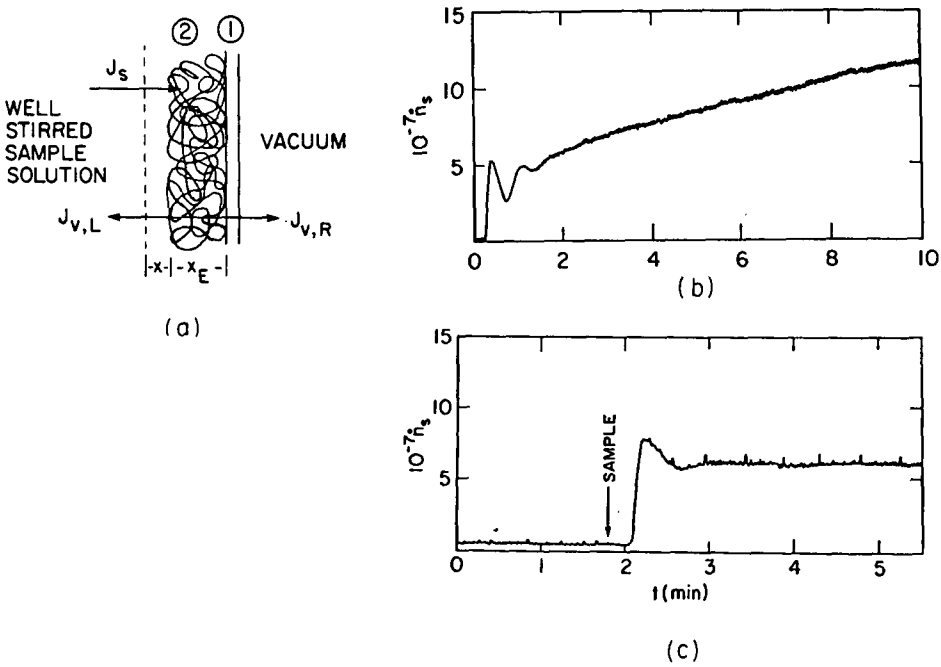

Fig. 2. (a) Idealized configuration of the enzyme-layer and semi-permeable dimethyl silicone membrane used in our experiments. 1 is the semi-permeable membrane. 2 is the enzyme layer of thickness $x_E \simeq 3 \cdot 10^{-2}$ cm, containing sufficient enzyme activity that the reaction rate is limited by the diffusion of substrate to the enzyme layer at low substrate concentrations, C_S. $x \simeq 10^{-2}$ cm is the thickness of a simple, one-dimensional, unstirred boundary layer. In the steady state, J_S is the substrate flux density reaching the enzyme layer, while $J_{V,R}$ and $J_{V,L}$ are the right and left-going volatile product flux densities. For values of C_S less than the apparent K_m, the steady state reaction rate is diffusion limited, is equal to J_S and is proportional to C_S. Thus, since $J_{V,R} = f_R J_S$, the count rate, $\dot{n}_s$, of the mass spectrometer is $\epsilon_T A_M f_R J_S$, where ϵ_T is the total counting efficiency ($2 \cdot 10^{-7}$ in our prototype apparatus) and $A_M = 1.2$ cm^2 is the membrane area. (b) Time response of the apparatus following injection of a large amount of solubilized urease into the circulating sample solution containing substrate urea. $\dot{n}_s$ is the mass spectrometer input count rate (ions $\cdot$ s^{-1}) at mass 44 (CO_2). No immobilized enzyme was present. Solubilized enzyme was injected at $t = 0$ and, following a delivery time, a transient due to incomplete mixing was observed which damped into a linear ramp. (c) Time response of the apparatus to urea injections with urease immobilized onto the fiber backing of the membrane. Where shown, 1.0 ml of 10^{-1} M urea was injected into the circulating sample, giving a concentration change of $6.7 \cdot 10^{-4}$ M. Following a delivery time, a transient response in the mass spectrometer count rate was again observed, but in this case the steady state count rate, $\dot{n}_s$, was constant in time. The initial peak is associated with the mixing of injected urea and gives rise to a damped oscillation, the period of which is equal to the circulation time. The short periodic spikes are due to an intermittent vacuum pump problem (observed separately).

In a subsequent experiment, urease was immobilized by cross linking onto the polyester-fiber backing of the membrane. When small amounts of urea were then injected to increase stepwise the sample substrate concentration, the nature of the time response was quite different from that exhibited in Fig. 2b. Following a similar transient ($\tau_{63\%} = 6$ s for the first component) the steady state count rate exhibited a constant value (Fig. 2c). The change in steady state count rate, $\Delta\dot{n}_s$, was noted after each urea concentration change, ΔC_s. A response curve was determined (Fig. 3) by plotting the cumulative $\Delta\dot{n}_s$ as a function of urea concentration. The observed sensitivity was $9 \cdot 10^{10}$ cps $\cdot$ M^{-1}, as compared with a mathematical model's prediction of $4 \cdot 10^{10}$ cps $\cdot$ M^{-1}.

We have also obtained results in which alcohol dehydrogenase (alcohol:

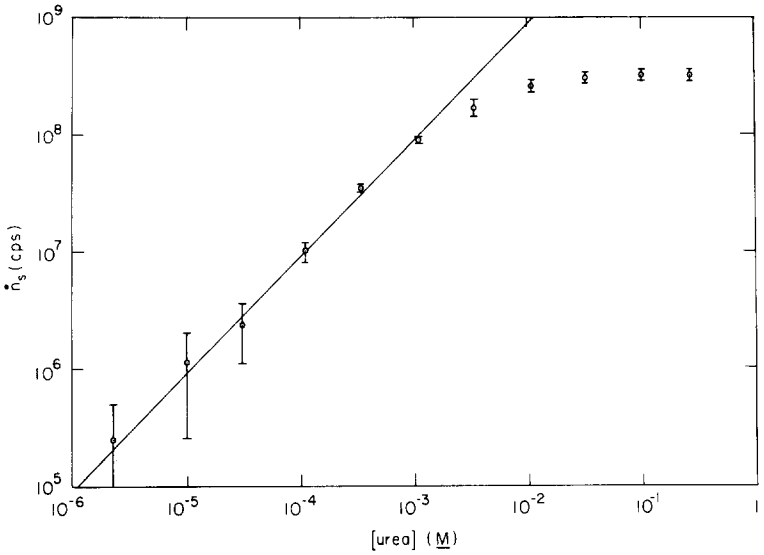

Fig. 3. Response curve of the prototype volatile enzyme product apparatus to a single sequence of eleven stepwise increases in urea concentration (Fig. 2c). The ordinate gives the cumulative change in the steady state count rate, $\dot{n}_s$, of CO_2 ions at the input of the multiplier of the mass spectrometer, while the abscissa gives the cumulative urea concentration in molarity. The response curve is consistent with a simple model in which the steady state count rate is proportional to the apparent reaction velocity of the immobilized enzyme reaction. In the simple and highly non-optimal prototype apparatus, a detectable response (signal-to-noise = 1) was seen at [urea] = $2.3 \cdot 10^{-6}$ M. The response increased linearly with concentration to [urea] $\simeq 10^{-3}$ M, thereafter leveling off into saturation as is expected for an enzyme-catalyzed reaction. The straight line fit to the low concentration region was constrained to a first power law, and fits the data well. The sensitivity in this region is $\Delta\dot{n}_s/\Delta C_s = 9 \cdot 10^{10}$ cps $\cdot$ M^{-1}, which compares favorably with the mathematical model's prediction of $4 \cdot 10^{10}$ cps $\cdot$ M^{-1}. The error bars are estimates based on the short term noise observed in the total count rate.

NAD^+ oxidoreductase, EC 1.1.1.1) is the immobilized enzyme and ethanol is the detected volatile. Although some fragmentation does occur with ethanol, it was found sufficient to monitor the major mass peak of 46. By supplying acetaldehyde at a concentration approx. 7 times the K_m for the solubilized reaction, we used the reaction

$$\text{acetaldehyde} + \text{NADH} \xrightarrow[\text{(EC 1.1.1.1)}]{\text{Alcohol dehydrogenase}} \text{ethanol} + \text{NAD}$$

to measure NADH, a molecule frequently used in colorimetric assays with coupled enzyme reactions. Alcohol dehydrogenase was immobilized by entrapment between two membranes (Fig. 4, inset). Because of the thick diffusional layer between the dialyzer and silicone membranes and the low permeability of the dialyzer membrane, the alcohol dehydrogenase reaction was more diffusionally limited. Thus, the response time, as illustrated in Fig. 4, following NADH injections was considerably longer ($\tau_{63\%}$ = 80 s) and the sensitivity in the linear portion of the response curve (Fig. 5) was considerably smaller, $1 \cdot 10^9$ cps $\cdot$ M^{-1}. This is also in fair agreement with the simple model [3], which predicts $0.3 \cdot 10^9$ cps $\cdot$ M^{-1}.

Since many relatively simple molecules permeate suitable membranes and are

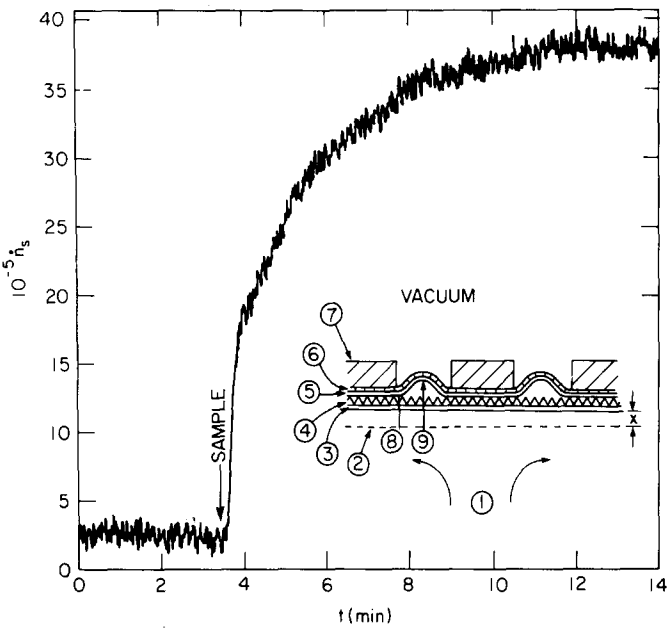

Fig. 4. Response of the apparatus to an injection of 0.2 ml of 10^{-1} M NADH with alcohol dehydroganse immobilized by entrapment as shown in the insert. The NADH concentration change was $1.3 \cdot 10^{-4}$ M. Ethanol (measured at $M = 46$) is the detected volatile product. Following a 12-s delivery time, a slow, somewhat complicated transient response was observed. $\dot{n}_s$ is the count rate (ions/s) at the input of the multiplier of the mass spectrometer. The NADH used contained an ethanol contamination which is not subtracted here, but for which a steady state correction is made in the response curve of Fig. 5. The insert shows a schematic representation of the membrane-enzyme region. 1 is the circulating sample stream, 2 is an assumed unstirred boundary layer, 3 is a dialyzer membrane, 4 is a nylon mesh of thickness $5 \cdot 10^{-3}$ cm which serves as a spacer, and 5 is an unbacked MEM-213 membrane. 6 is a single piece of 1-μM pore size, 10-μM thick Nuclepore filter, and 7 is a supporting stainless steel perforated disc. Due to bulging of the MEM-213 membrane and Nucleopore filter, the layer of entraped enzyme (8 and 9) varies in thickness, a configuration which is believed responsible for the somewhat complicated time response (with both fast and slow components).

volatile, a large number of enzymes and substrates [6] appear suitable for the volatile enzyme product method. Examples are: acetylcholinesterase (EC 3.1.1.7) with acetic acid as the volatile, catalase (EC 1.11.1.6) with O_2 as the volatile, cysteine desulphydrase (EC 4.4.1.1) with H_2S as the volatile, and sarcosine oxidase (EC 1.5.3.1) with HCHO as the volatile. The use of coupled reactions greatly increases the possibilities.

Many applications involving rapid, sensitive biochemical measurements of substrates, cofactors, inhibitors and enzymes are possible. In the latter case, the sample to be assayed for a particular enzymatic activity is passed through a narrow channel bounded by the membrane and vacuum of the mass spectrometer on one side, and a dialyzer membrane on the other. The dialyzer prevents the escape of enzyme, while admitting the required substrates and cofactors. In this way, sample volumes could be kept small and uncontaminated.

The sensitivity should be adequate to perform metabolic experiments with single cells, provided the background counting rate can be reduced by proper vacuum and sample degassing techniques. Metabolic fluxes (CO_2 and lactate) from single cells could be measured with a time constant of about 10 s, and the

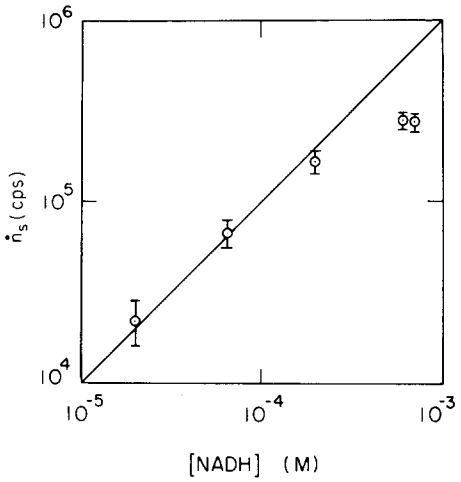

Fig. 5. Response curve for the measurement of NADH using alcohol dehydrogenase for a single sequence of increasing NADH concentrations, with ethanol as the detected volatile product. Here $\dot{n}_s$ is the cumulative change in the mass 46 count rate as a function of NADH concentration, corrected for ethanol contamination, which accounted for 15% of the steady state change shown in Fig. 4. After injecting ethanol to determine directly the total permeability from sample solution to vacuum, a correction was made by subtracting the count rate calculated for the injection of contaminant ethanol. The response curve exhibits saturation, as expected for an enzyme catalyzed reaction. The cost of NADH prohibited going to significantly higher NADH concentrations and fewer data exist for the alcohol dehydrogenase experiments. Nevertheless with thinner dimethyl silicone membranes [8], thinner enzyme layers, and degassing of the sample to reduce background, it appears feasible to extend significantly the performance of this enzyme system. The experimental sensitivity in the linear portion of the response is $1 \cdot 10^9$ cps $\cdot$ M^{-1}, which is in agreement with the estimate $0.3 \cdot 10^9$ cps $\cdot$ M^{-1} given by the mathematical model.

change in such fluxes due to a variety of stimuli could be monitored. This technique could also be combined with molecule microscopy [7] to provide a means for spatially mapping enzyme activity with a resolution of about 1 μm in, for example, thin tissue slices. Neither stain nor fixative need be used. A very thin (300 Å) synthetic membrane [8], mechanically supported by a Nuclepore filter with 300 Å pores, would separate the sample from the vacuum system of a molecule microscope in order to prevent dehydration. When selected substrates and cofactors are provided, the spatial distribution of enzyme activity should give rise to a corresponding spatial variation in the flux of volatile product, which should permeate the membrane without significant spreading.

Experimental details

The response of the quadrupole mass spectrometer (Extranuclear Corp., Pittsburgh, Pa.) was observed for three types of experiments:

(1) Fig. 2b. Solubilized urease (Sigma Chemical Co., St. Louis, Mo: Type VI) was injected into 150 ml circulating solution of 0.1 M Tris buffer, pH 7.2 at $30 \pm 1°C$ which contained $5 \cdot 10^{-2}$ M urea. A MEM-213 dimethyl silicone membrane (General Electric Co., Membrane Products, Schnectady, N.Y.) of thickness 0.001 inch with a polyester fiber backing on the side exposed to the aqueous solution and without immobilized urease was used. 4.1 Sigma units of

urease were injected (1 Sigma unit = 1 mg urea nitrogen evolved per 5 min).

(II) Fig. 2c. Prior to increasing stepwise the urea concentration of the circulating solution, urease was immobilized onto the fiber backing of the membrane described in I by physisorption followed by crosslinking with glutaraldehyde [9,10]. The buffer and temperature used for I were also employed for II.

(III) Fig. 4. Prior to increasing stepwise the NADH (Sigma, Grade III β-NADH) concentration of the circulating solution, alcohol dehydrogenase (Sigma, Product No. A-1762) was immobilized by entrapment [11] between a Technicon Type C dialyzer membrane and a MEM-213 dimethyl silicone membrane of thickness 0.001 inch and without a fiber backing (see Fig. 4 for details of construction of the membrane-enzyme-membrane sandwich). 0.1 M phosphate buffer, pH 7.2 at 30 ± 1°C with $5.5 \cdot 10^{-3}$ M acetaldehyde was used. The injected NADH contained ethanol contaminant as determined by the manufacturer in the amount 0.5 molecules ethanol per molecule NADH. After the experimental run represented by Fig. 5 was completed, ethanol was injected on the aqueous side and the membrane-enzyme-membrane sandwich permeability to ethanol was determined to be $2 \cdot 10^{-6}$ cm $\cdot$ s^{-1}, whereas the MEM-213 permeability to ethanol was $8.9 \cdot 10^{-6}$ cm $\cdot$ s^{-1}. Based on these calibrations, the change in steady state count rate shown in Fig. 4 is found to have a 15% contribution from the ethanol contaminant.

For all the experiments the pressure in the mass spectrometer vacuum system due to all species was about 10^{-5} mm Hg. The total counting efficiency for individual molecules which permeate the membrane is $\epsilon_T = 2 \cdot 10^{-7}$, while in future designs $\epsilon_T \geqslant 10^{-4}$ is likely to be obtained.

Acknowledgements

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