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# Pseudouridine detection improvement by derivatization with methyl vinyl sulfone and capillary HPLC–mass spectrometry

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**Abstract**

A method is presented for improved detection of pseudouridine in nucleoside mixtures based on the specific derivatization with methyl vinyl sulfone followed by analysis by capillary HPLC–mass spectrometry. Reaction conditions were optimized in order to obtain the best yield and specificity. The method was successfully applied to different nucleoside mixtures.


*Keywords:* Pseudouridine; Modified nucleosides; RNA

## 1. Introduction

Currently, in RNA more than hundred modifications have been identified at the nucleotide level [1]. These so-called posttranscriptional modifications are introduced by enzymes. The first modified nucleoside detected in cellular RNA was pseudouridine (Ψ) [2,3]. It is also the most abundant modification with about 95 occurrences per mammalian ribosome. Ψ is synthesized by pseudouridine synthases at the polyribonucleotide level. These enzymes converts the *N*-glycosidic (C1′–N1) into a C-glycosidic (C1′–C5) bond by rotation of the nucleobase [4]. Because of this arrangement N1 and N3 are both available for hydrogen bonding, allowing pseudouridine to increase base stacking and restrict mobility of the backbone 5′ to the site of the pseudouridine [4,5].

Over the last decennium, mass spectrometry has taken an important place in the detection and mapping of posttranscriptionally modified nucleosides [6]. However, Mass spectrometry of pseudouridine is not straightforward because: (1) Ψ has the same elemental composition and hence is isobaric with uridine, which is present in excess in RNA from natural sources, (2) Ψ does not release its base and (3) Ψ is poorly retained on reversed phase columns [7] and elutes in the solvent front making it sometimes difficult to spot. Several methods have been proposed for the specific sequence placement of pseudouridine. A method based on the reaction of Ψ with *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium) ethylcarbodiimide *p*-tosylate (CMC) was described by Bakin and Ofengand [8], and later applied with matrix assisted laser desorption ionization (MALDI) mass spectrometry as the detection method [9]. More recently, a different approach was used based on the Michael type addition of acrylonitrile in weakly alkaline conditions followed by MALDI mass spectrometry [10]. Because the addition proceeds through a deprotonated nucleobase, the acidity of the base protons determines largely the reactivity of Ψ and its unmodified counterpart, uridine. Both these methods allow specific sequence placement of Ψ in the RNA strand, but so far no data is provided about any increase in detection sensitivity of Ψ in nucleoside mixtures. We report a novel derivatization reagent for Ψ, methyl vinyl sulfone (MVS). The derivatization reaction is also a Michael type addition and the reaction product has a higher electrospray ionization efficiency compared to non-derivatized Ψ, allowing lower detection limits. The high specificity (more than 80% compared to U) makes this reagent very useful for sensitive and specific detection of Ψ in presence of high amounts of U.

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## 2. Experimental

### 2.1. Chemicals

All nucleosides were analytical grade: C and G were obtained from Janssen Chimica (Beerse, Belgium), A and Ψ from Sigma (St. Louis, MO, USA) and U as well as acetonitrile HPLC grade from Acros (New Jersey, USA).

MVS was purchased from Sigma and contained 0.02% *p-tert*-butylphenol as stabilizing agent. For the reactions a 1‰ (v/v) solution of MVS in water was used. A, C, U and Ψ nucleosides were dissolved in water (Milli-Q plus, Millipore, Billerica, MA, USA) at a concentration of 0.8 mM, G was dissolved at a concentration of 0.17 mM.

The nucleoside mixture (Supelco, Bellefonte, PA, USA) contained 12 nucleosides (C, G, I, $m^1A$, $m^3C$, $m^5C$, Cm, $m^7G$, Ψ, T, $s^2C$ and U, for abbreviations see [1]). All nucleosides were present at a concentration of 10–100 μg/ml.

The *E. coli* tRNA (Sigma, St. Louis, MO, USA) was precipitated with ammonium acetate/ethanol prior to use in order to remove salt excess. Four percent sodium acetate buffers were prepared by dissolving sodium acetate (Acros New Jersey, USA) to the desired concentration and adjusting the pH by addition of acetic acid (Chem-Lab, Zedelgem, Belgium). Absolute ethanol was from Fisher scientific (Loughborough, Leicestershire, UK).

### 2.2. Methyl vinyl sulfone derivatization of single nucleosides

In a 1.5 ml eppendorf tube 5 μl nucleoside (for A, C, U or Ψ) or 20 μl nucleoside (for G) solution, 5 μl ethanol and 5 μl buffer were mixed. Finally, MVS was added, the solution was vortexed and placed in a heating block at constant temperature. Reaction time, temperature, pH and amount MVS were varied and optimized.

### 2.3. Methyl vinyl sulfone derivatization of the nucleoside mixture

Five microliters of the mixture of 12 nucleosides (4 nmol of reacting species: Ψ, T, $m^5C$, I) were mixed with 5 μl ethanol and 5 μl 4% sodium acetate buffer pH 7, and allowed to react with 2 μl methyl vinyl sulfone 1‰ (23 nmol) at 70 °C. After 3 h, the mixture was dried, redissolved in 10 μl water and analyzed by capillary HPLC–MS. Dilutions were made to determine the detection limit with and without derivatization.

### 2.4. Methyl vinyl sulfone derivatization of Ψ in an E. coli tRNA hydrolysate

Thirty micrograms (1 nmol) of *E. coli* tRNA was hydrolyzed to nucleosides following the protocol published by Crain and McCloskey [11]. Five microliters 4% sodium acetate buffer, 5 μl ethanol and 21 μl MVS 1‰ (240 nmol) were added. The solution was mixed and kept at 70 °C for 3 h. The reaction mixture was then dried, redissolved in 5 μl water and analyzed by capillary HPLC–MS.

### 2.5. Capillary HPLC–MS

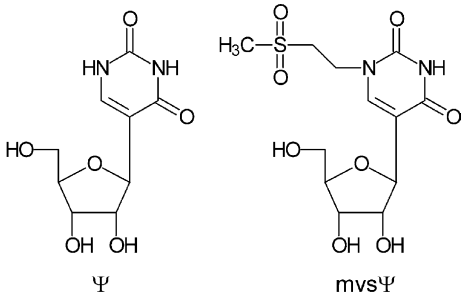
Off-line HPLC analysis was done on a Ultimate capillary HPLC (Dionex, Sunnyvale, CA, USA). The instrument was interfaced to a Famos carousel microautosampler. Injection volume was 0.1 μl. A PepMap reversed phase C18 column 150 mm × 0.3 mm, 3 μm particles, (Dionex, Sunnyvale, CA, USA) was used at room temperature (20 °C ± 2 °C). The column was eluted at a flow rate of 5 μl/min using an ammonium formiate (50 mM, pH 7)/acetonitrile (80% in ammonium-formiate 50 mM, pH 7) gradient. A two step gradient was used: 1–48% acetonitrile in 7 min, followed by 48–80% acetonitrile in 1.5 min. Diode array absorbance data were acquired at 260 nm. The area under the curve (AUC), calculated by the Chromeleon v6.50 software, was used to evaluate the different reaction conditions.

On-line HPLC–MS analysis was performed on a capillary liquid chromatograph (CapLC, Waters Milford, MA, USA) coupled with an orthogonal acceleration/quadrupole time-of flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK). Both instruments were controlled by the MassLynx 3.4 software (Micromass, Manchester, UK). 0.5 μl sample was injected onto an Atlantis dC18 150 mm × 0.32 mm column packed with 3 μm particles (Waters, Milford, USA) and kept at 30 °C. The mobile phase was ammonium formiate (25 mM, pH 7) as solvent A and 100% methanol as solvent B. Flow rate was 5 μl/min and the gradient was set to a concave curve (curve 7) from 0% B to 30% B in 30 min followed by an increase of 5% B/min. The electrospray source was directly coupled to the outlet of the chromatograph. Spectra were acquired in continuum mode from *m*/*z* 105 to 700 at 2 s intervals. The resolution was 9000 (full peak width at half height). Capillary voltage was set to 3000 V, cone voltage 35 V and the collision cell was kept at 10 V.

### 2.6. General Ψ derivatization procedure

Our results (see Section 3) allow the formulation of a general derivatization procedure for increasing the detection sensitivity of pseudouridine in nucleoside mixtures.

The sample should be dissolved to approximately 0.1–1 nmol/μl of Ψ or other reacting species (e.g. inosine, $m^5C$, ribosylthymine) and an equal amount (v/v) of both 4% sodium acetate pH 7 and ethanol should be added. For this concentration, 6–7 equivalents MVS are sufficient to produce a maximum reaction yield after 3 h at 70 °C. After reaction, the mixture should be dried and redissolved in a small amount of water followed by LC–MS. Because amino groups also react with MVS, higher amounts of the reagent should be used in samples containing, e.g. $NH_3$, triethylamine. In the




| ion | m/z | |
|---|---|---|
| | Ψ | mvsΨ |
| $[M+H]^+$ | 245 | 351 |
| $[M+Na]^+$ | 267 | 373 |
| $[M+H-2H_2O]^+$ | 209 | 315 |

Fig. 1. Structure and signature ions for pseudouridine (Ψ) and methyl vinyl sulfone derivatized pseudouridine (mvsΨ). $[M+H-2H_2O]^+$ is a fragment formed in the electrospray source after loss of two $H_2O$ molecules from the sugar residue of the Ψ nucleoside.

case of the tRNA sample, a 15- to 20-fold higher amount of MVS reagent was sufficient.

## 3. Results and discussion

Reaction of methyl vinyl sulfone with pseudouridine yields mainly the N1 substituted Ψ nucleoside (Fig. 1). For a discussion of the comparison of the N1 and N3 reactivity, see reference [10]. There will always be a small amount of uridine, if present, that will coreact with MVS. By adapting our reaction conditions, we could limit this amount to maximum 10%. Although derivatized Ψ (mvsΨ) and U (mvsU) have the same mass, they differ sufficiently in retention time to allow distinction between these 2 species. Other nucleosides also react with MVS, but they differ in mass and retention time with mvsΨ. Only when their concentration is expected to be substantial or when excess amounts of U (e.g. in RNA from natural sources) are present, higher amounts of MVS are necessary to obtain the maximum yield of mvsΨ.

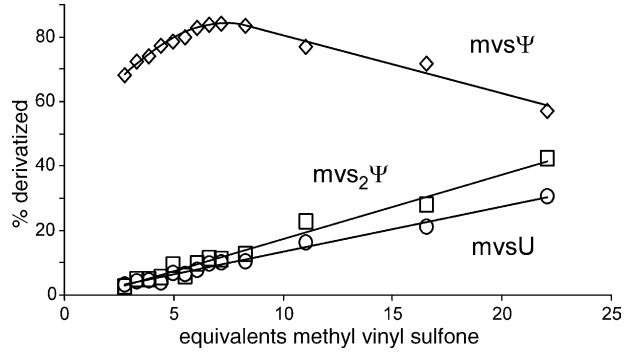



Fig. 2. Influence of methyl vinyl sulfone concentration on the derivatization of pseudouridine and uridine at pH 7.0, 70 °C and 3 h of reaction time. (◊) mvsΨ; (◯) mvsU and (□) $mvs_2Ψ$.

### 3.1. Methyl vinyl sulfone derivatization of single nucleosides

In order to optimize the derivatization procedure, reaction conditions were varied and the reaction mixtures were analyzed by off-line capillary HPLC. In a first series of experiments, the reactivity of the four common ribonucleosides and Ψ with MVS were evaluated. Under all conditions tested C, G or A were not derivatized (data not shown). The yield profile as a function of added MVS is given in Fig. 2. The yield is dependent of the MVS/nucleoside ratio, a maximum of 85% is obtained at 5–7 equivalents MVS. After addition of higher amounts of MVS, the mvsΨ proportion decreases due to the appearance of disubstituted Ψ ($mvs_2Ψ$). At these higher amounts of reagent also higher levels of mvsU are noticed. At the maximum in the mvsΨ proportion plot, 10% U is modified.

The relative sensitivity of Ψ detection can be derived from Fig. 3. A partial converted pseudouridine solution was analyzed by HPLC–MS. The UV trace shows that mvsΨ elutes later than the unreacted Ψ and gives an unmodified/modified Ψ ratio of 2:1, but in the extracted ion chromatograms, this ratio is about 1:1.2. The electrospray ionization efficiency of mvsΨ is apparently higher than for the unmodified nucleoside. The same figure also shows that mvsΨ is better retained on the Atlantis dC18 column shifting the reten-

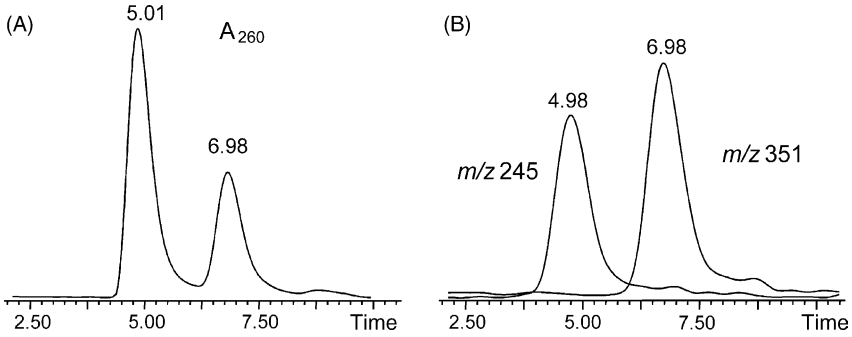



Fig. 3. HPLC–MS analysis of an incomplete reaction mixture of pseudouridine with methyl vinyl sulfone. (Panel A) UV trace at 260 nm. (Panel B) overlaid extracted chromatograms for $[M+H]^+$ of pseudouridine (*m/z* 245) and methyl vinyl sulfone derivatized pseudouridine (*m/z* 351).

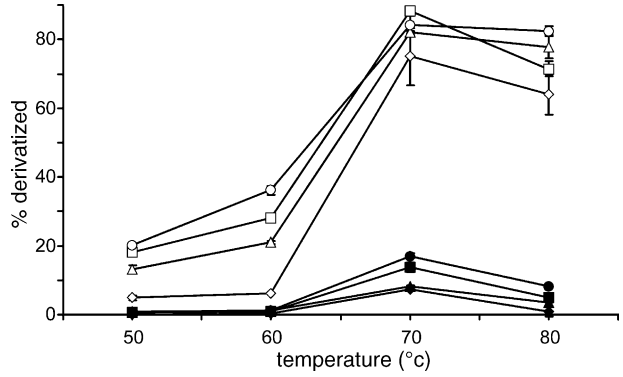



Fig. 4. Derivatization of pseudouridine with methyl vinyl sulfone in the presence of uridine at different temperatures and pH. Error bars represent the standard deviation ($n = 3$). (◊) pH 6.0; (△) pH 7.0; (□) pH 8.0 and (◯) pH 8.6. Open and closed symbols represent pseudouridine and uridine products respectively.

tion time by 2 min, on the PepMap column this is even 4 min.

The effect of temperature and pH on the derivatization is shown in Fig. 4. At lower temperatures, the derivatization yield of Ψ is not sufficient and depends on the pH. At 70 °C the derivatization yield reaches its maximum and is independent from the pH. Because at pH 7 lower mvsU amounts are detected, this pH was chosen in combination with a temperature of 70 °C for the tests on the nucleoside mixtures. Our tests showed a reaction time of 3 h to be sufficient at this high temperature (2–15 h was tested, data not shown).

### 3.2. Methyl vinyl sulfone derivatization of nucleoside mixtures

The mixture of 12 nucleosides was subjected to a MVS treatment and the reaction mixture was analyzed by HPLC–MS. In Fig. 5A reconstructed ion chromatograms (RICs) of $[M + Na]^+$ and $[M + H{-}2H_2O]^+$ (see Fig. 1 for signature ions) of a 1/60 dilution of this derivatized nucleosides mixture (thus containing 1.7 pmol/μl Ψ and 850 fmol Ψ injected) are compared to the corresponding ions in the same dilution of a non-derivatized mixture. The signal to noise ratios of both signals of the derivatized mixture are higher than the ones of the non-derivatized mixture. If the sample is further diluted, it was no longer possible to identify Ψ in the non-derivatized mixture, while reliable identification is still possible in the derivatized sample. Fig. 5B shows the mass

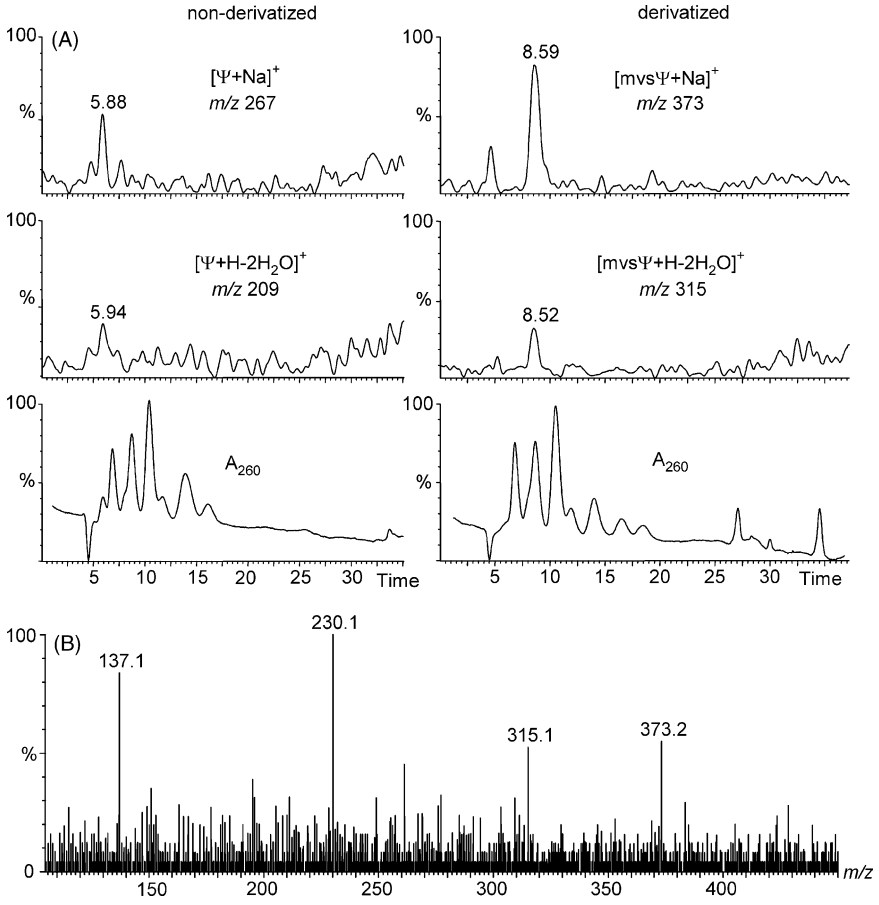



Fig. 5. HPLC–MS of a mixture of 12 nucleosides. (Panel A) $[M + Na]^+$, $[M + H{-}2H_2O]^+$ and UV at 260 nm traces for 850 fmol Ψ in a nucleoside mixture and 850 fmol mvsΨ in a derivatized nucleoside mixture. (Panel B) mass spectrum obtained after dilution and injection of 320 fmol mvsΨ in a derivatized nucleoside mixture.

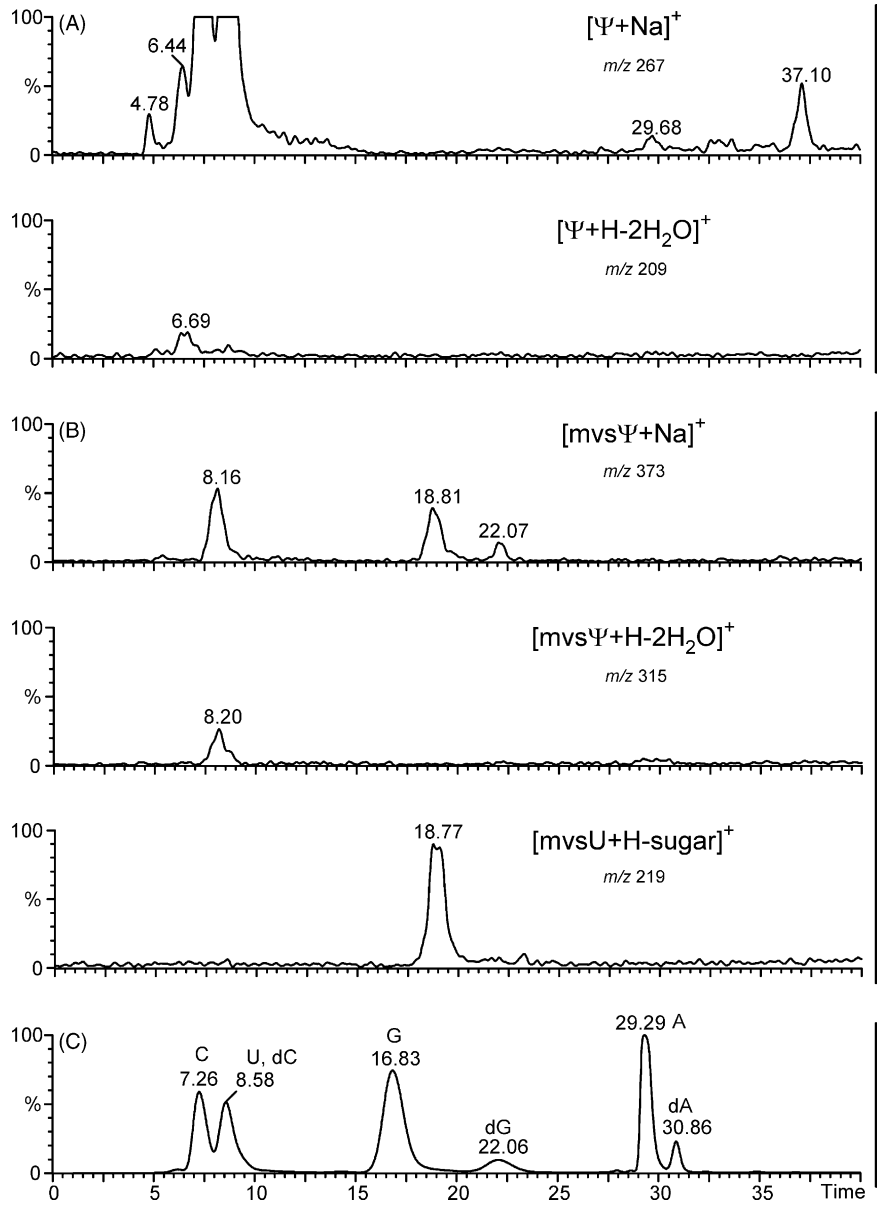



Fig. 6. HPLC–MS of the nucleosides of *E. coli* tRNA. (Panel A) RIC for $[M + Na]^+$ and $[M + H{-}2H_2O]^+$ of Ψ in an unmodified sample. (Panel B) RIC for $[M + Na]^+$ and $[M + H{-}2H_2O]^+$ of Ψ after derivatization with MVS. (Panel C) UV trace of the unmodified sample at 260 nm.

spectrum obtained from 320 fmol mvsΨ. The traces for the protonated molecular ion for Ψ and mvsΨ are not plotted in Fig. 5A because of the interference of coeluting C and U. The nucleoside mixture contains inosine, $m^5C$ and T, which also reacted with MVS (data not shown). Because the retention time and masses of these nucleosides differ sufficiently with pseudouridine this causes no problems.

In order to verify the applicability of our procedure, a tRNA mixture from *E. coli* was hydrolyzed to nucleosides and derivatized with MVS. The mixture was evaluated with capillary HPLC–MS (Fig. 6). Without derivatization no reliable identification of Ψ was possible, because of the weakness of the signal in the RIC for *m/z* 209 ($[M + H{-}2H_2O]^+$) and several peaks at the retention time of interest (6–9 min.) in the RIC for *m/z* 267 ($[M + Na]^+$). In contrast, identification of Ψ is straightforward after derivatization because of the good correspondence between RICs for *m/z* 373 ($[M + Na]^+$) and *m/z* 315 ($[M + H{-}2H_2O]^+$) of mvsΨ. The intensity of these signals is higher than the signals of the unmodified counterparts, thus allowing lower detection limits.

Because during enzymatic degradation of the tRNA to single nucleosides, ammonium acetate and ammonium bicarbonate buffers were used [11], higher amounts of MVS are necessary in this case (see Section 2.6). In addition, the excess of U (compared to Ψ) present in the mixture also shows peaks in the chromatogram, but these can easily be assigned to mvsU because of the peak in the trace at *m/z* 219 corresponding to the derivatized uracil base.

## 4. Conclusion

An easy derivatization method using methyl vinyl sulfone which leads to improved detection sensitivity of pseudouridine by capillary HPLC–MS has been developed and applied to nucleoside mixtures. Experimental parameters were optimized in order to maximize specificity and sensitivity. The method is useful for identification of pseudouridine in excess of other nucleosides as in samples originating from enzymatic digestion of natural ribosomal or transfer RNA.

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