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Analysis of underivatized polyamines by reversed phase liquid chromatography with electrospray tandem mass spectrometry

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Abstract

A reversed phase liquid chromatography–electrospray ionization-tandem mass spectrometric method (RP-LC–ESI-MS/MS) was developed to separate and detect polyamines with minimal sample pre-treatment and without any derivatization. Prior to MS/MS analysis, a complete chromatographic separation of polyamines was achieved by a linear gradient elution using heptafluorobutyric acid as a volatile ion-pair modifier, and signal suppression was prevented by post-column addition of 75% propionic acid in isopropanol to the column flow. The developed method was successfully applied to the identification of metabolites formed from N^1,N^{12}-diethylspermine in the reaction catalyzed by recombinant human polyamine oxidase and to the detection of eight different polyamines in a standard mixture.

Keywords: Polyamines; Polyamine analogues; HPLC/MS; Electrospray; Ion pairing

1. Introduction

The polyamines, spermine, spermidine and their diamine precursor, putrescine, are ubiquitous components in mammalian cells and have shown to be essential in the regulation of cell growth and differentiation [1]. Under physiological conditions, these aliphatic amines are positively charged, which allows electrostatic interactions with negatively charged cellular macromolecules, like DNA, RNA, proteins and phospholipids. It is known that the metabolism of polyamines, including their cellular uptake, biosynthesis and excretion, is tightly regulated, but their actual cellular functions and the specificity of their interactions are still not entirely clear.

N-Alkylated polyamine derivatives are widely used to deplete natural polyamine pools in mammalian cells. These compounds are actively transported into the cells but are incapable of fulfilling crucial cellular functions of the natural polyamines [2,3]. Some of the analogues, e.g. N^1,N^{11}-diethylnorspermine (DENSpm) and N^1,N^{12}-diethylspermine (DESpm), display antitumoral activity and are promising chemotherapeutic agents for the therapy of cancer and other hyperproliferative diseases, and hence their metabolism is of obvious interest [3,4].

FAD-dependent polyamine oxidase (PAO; EC 1.5.3.11) is one of the key enzymes in the catabolism of polyamines spermine and spermidine. The natural substrates for the enzyme are N^1-acetylspermidine, N^1-acetylspermine and N^1,N^{12}-diacetylspermine, but also some terminally *N*-alkylated polyamine analogues, e.g. DENSpm, DESpm and dibenzyl polyamine derivatives, serve as substrates for PAO [5–7]. DENSpm is the most widely studied *N*-alkyl polyamine analogue which has been shown to be metabolized by PAO. However, there are two metabolic pathways described in the literature, one suggesting that DENSpm is metabolized by de-ethylation by PAO, followed by step-wise spermidine/spermine-N^1-acetyltransferase (SSAT)/PAO mediated further degradation [5], and the other indicating that DENSpm and DESpm are

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catabolized like diacetylated polyamines by PAO [6]. Thus, the catabolic pathways of DENSpm and DESpm with PAO are not entirely confirmed so far.

Many analytical methods have been developed for polyamines, including thin-layer chromatography, ion exchange chromatography, gas chromatography (GC), high-performance liquid chromatography (HPLC), electrophoresis techniques (CE, CZE) and immunoassays (RIA, ELISA) [8–12]. Mass spectrometry (MS) has been used for detecting polyamines either in combination with HPLC or GC, or without prior chromatographic separation [10,13–16]. Techniques used for polyamine quantification are mainly based on chromatographic separations by using HPLC with pre- or post-column derivatization. Derivatization is needed to increase the sensitivity of the method when using traditional UV or fluorescence detection, since the polyamine backbone does not display native fluorescence and it absorbs at the same wavelength where interfering compounds are detectable. The main problems in derivatization are elongated analysis times and the possibility to have inaccurate results due to incomplete or unstable reaction with required functionalities, e.g. *N*-hydroxy compounds. Furthermore, an unselective labelling may lead to interfering by-products.

The analysis of minor compounds in complex matrices is usually difficult because of interferences by matrix components, even when extensive separation and clean up procedures are applied to the sample. One approach to eliminate these interferences is the use of HPLC coupled to tandem mass spectrometry (MS/MS) using triple quadrupole or ion trap mass spectrometers. As compared with conventional detection methods, MS/MS selectivity eliminates possible interfering peaks and improves both analytical sensitivity and specificity when analyzing low molecular weight compounds. Even the chromatographic separation of the compounds is not needed to be complete. Overlapping compounds, even having similar measured precursor ion, can be separated if they have diverse fragmentation pathways leading to different characteristic product ion.

Before this study only few methods exist where the derivatization procedure has not been used for polyamine analysis. These include an enzymic sensor array method detection, HPLC with conductometric, CZE with amperometric and CE with conductometric and CE with electrochemiluminescence detection [8,17–21]. Also GC of some polyamines without derivatization is possible, but derivatization with suitable reagents improves GC elution and resolution with considerable enhancement in the sensitivity of the detection system [9,11]. The online coupling of electrospray mass spectrometry (ESI-MS) with ion chromatography and RP-HPLC for analysis of underivatized polyamines has been explored by Feistner [22], but the coupling of HPLC and MS for metabolic profiling of the underivatized compounds proved to be unsuccessful due to compatibility problems between MS and studied separation methods. However, it was noted that the detection limits for the underivatized polyamines using ESI-MS/MS were quite high. Recently, two HPLC methods based to MS/MS detection have been published which employs the separation and detection of underivatized biogenic amines with good sensitivity from cheeses [15,16]. The first method uses ammonium acetate as a mobile phase additive and 150 mm × 2 mm Luna C18 column for separations of cadaverine, histamine, spermidine, spermine, tyramine and tryptamine [15]. The other method concerns the separation and analysis of histamine, 2-phenylethylamine and tyramine using trifluoroacetic acid (TFA) as a mobile phase additive [16]. Good separation was achieved for these three amines, but the method is not suitable for more polar polyamines which are not sufficiently retained on a reversed phase column using TFA containing mobile phases [22].

Direct and efficient HPLC separation method for underivatized polyamines have been developed based on octane sulfonic acid reversed phase separation and has been adapted by a number of laboratories [9]. However, octane sulfonic acid is non-volatile, and its use as an ion-pairing reagent is not suitable for direct online MS analysis of separated polyamines. Also volatile strong acids such as TFA or heptafluorobutyric acid (HFBA) have been used in general in RP-HPLC measurements as ion-pairing reagents to improve chromatographic performance, but they are known to cause signal suppression for basic compounds, when analyzed by ESI-MS [23]. Fortunately, this is mostly avoided by using post-column addition of a solution of 75% propionic acid (PrA) and 25% isopropanol (IPA) in a ratio 1:2 to the column flow [23].

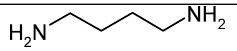
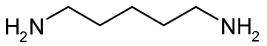
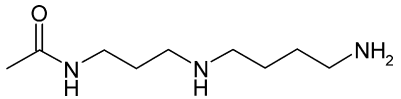
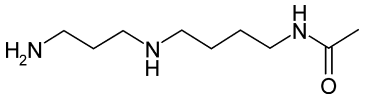
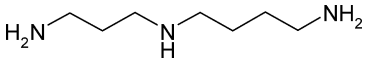
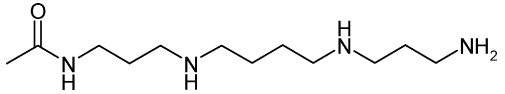
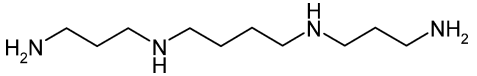
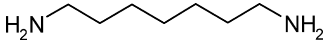
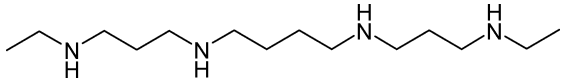
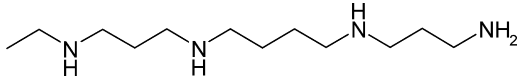
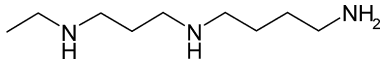
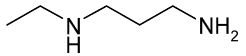
In the present work, we show that polyamines can be separated and analyzed by HPLC–MS/MS using volatile HFBA as an ion-pairing reagent. Five volatile acids were compared as ion-pairing reagents in chromatographic separation and the effects of these mobile phase additives to relative signal intensities of different polyamines were tested. In comparison with conventional methods of polyamine analysis, separation and analysis were carried out without derivatization and after simple sample preparation. Up to 10-fold signal intensity as compared with conventional strong acid containing mass spectrometric analysis was achieved by post-column addition of PrA in IPA [23]. The developed method was successfully applied to the identification of metabolites of DESpm as a substrate for recombinant human polyamine oxidase (hPAO). Metabolites formed in the reaction included *N*-ethylspermine, N^1-ethylspermidine, spermidine and N^1-ethyl-1,3-diaminopropane (EtDAP). EtDAP can be derived only from the endo-cleavage of DESpm. This is the first endo-cleavage reported for mammalian PAO.

2. Experimental

2.1. Instrumentation

LC separations, MS detection and analysis of the compounds were achieved with Agilent 6410 Triple Quad LC/MS equipped with Agilent 1200 Series Binary Pump SL pumping system and Agilent 1200 Autosampler. Data acquisition and analysis were performed using Agilent MassHunter Workstation software. The chromatographic separations were carried out using Phenomex Gemini reversed phase C18 column (3 μm, 30 mm × 2 mm, 110 Å). Ultrapure water was prepared using a Milli-Q Gradient system (Millipore, Milford, MA, USA).

Table 1
Structures and mass spectra properties of polyamines used in this study (N = number of theoretical plates)

	Structure	Formula	Calculated mass (amu)	Measured precursor ion $[M+H]^+$	Fragmentor voltage (V)	SRM product ion MS/MS $Q1>Q3$ (m/z)	Other product ions	Collision energy (eV)	Retention time for 0.1% HFBA	Without tee/with tee, but without post-column flow/with tee and post-column flow (N)
PUT		$C_4H_{12}N_2$	88.10	89.1	60	89.1 > 72.2		5	2.49	2031/1557/1645
CAD		$C_5H_{14}N_2$	102.12	103.1	60	103.1 > 86.1		5	2.97	
N^1AcSpd		$C_9H_{21}N_3O$	187.17	188.2	90	188.2 > 100.1	171.2, 72.2	15	4.52	11219/6512/7200
N^8AcSpd		$C_9H_{21}N_3O$	187.17	188.2	90	188.2 > 114.1	171.1, 72.2, 58.2	15	4.81	
Spd		$C_7H_{19}N_3$	145.16	146.2	90	146.2 > 72.2	112.2	15	5.97	20079/12608/16384
N^1AcSpm		$C_{12}H_{28}N_4O$	244.23	245.2	90	245.2 > 129.2	171.2, 112.2, 100.1	10	6.44	
Spm		$C_{10}H_{26}N_4$	202.22	203.2	90	203.2 > 129.2	112.2	10	7.19	42593/32108/31207
DAH		$C_7H_{18}N_2$	130.15	131.2	60	131.2 > 114.2	55.2	5	5.02	
DESpm		$C_{14}H_{34}N_4$	258.28	259.3	90	259.3 > 157.2	112.1, 86.1	10	7.53	
EtSpm		$C_{12}H_{30}N_4$	230.25	231.3	90	231.3 > 112.2		20	7.36	
EtSpd		$C_9H_{23}N_3$	173.19	174.2	90	174.2 > 72.2	112.2, 86.2	15	6.37	
EtDAP		$C_5H_{14}N_2$	102.12	103.1	90	103.1 > 86.2	58.3	5	3.62	

2.2. Reagents

2.2.1. Polyamine standards for LC/MS studies

Butane-1,4-diamine dihydrochloride (PUT), pentane-1,5-diamine dihydrochloride (CAD), and heptane-1,7-diamine (DAH) were from ICN Biomedicals Inc. N^1-(3-Amino-propyl)-butane-1,4-diamine trihydrochloride (Spd), *N*-[3-(4-amino-butylamino)-propyl]-acetamide dihydrochloride (N^1AcSpd), *N*-[4-(3-amino-propylamino)-butyl]-acetamide dihydrochloride (N^8AcSpd), *N*,*N*′-bis-(3-amino-propyl)-butane-1,4-diamine tetrahydrochloride (Spm) and *N*-{3-[4-(3-amino-propylamino)-butylamino]-propyl}-acetamide trihydrochloride (N^1AcSpm) were from Aldrich. *N*,*N*′-Bis-(3-ethylamino-propyl)-butane-1,4-diamine (DESpm) was synthesized essentially as described earlier [24]. The publication describing the efficient preparation methods for N^1-ethyl-propane-1,3-diamine dihydrochloride (EtDAP), N^1-(3-ethylamino-propyl)-butane-1,4-diamine trihydrochloride (EtSpd) and *N*-(3-amino-propyl)-*N*′-(3-ethyl-amino-propyl)-butane-1,4-diamine tetrahydrochloride (EtSpm) is in preparation.

Stock solutions of the standards were prepared by dissolving an appropriate amount in ultrapure water to yield concentration of 1 mM. All solutions were passed through 0.22 μm filter, stored at −20 °C and used after dilution to the required concentration.

2.2.2. Solvents used for LC separations and MS analysis

Ultra gradient HPLC-grade acetonitrile (ACN) was purchased from J.T. Baker. Trifluoroacetic acid (TFA, 99+%), pentafluoropropionic acid (PFPA, 97%) and heptafluorobutyric acid (HFBA, 99%) from Aldrich. Butyric acid (BA, >99.5%), acetic acid (AA, >99.8%), propionic acid (PrA, >99.5%) and isopropanol (IPA > 99.8%) from Fluka, and formic acid (FA, 99%) from Riedel-deHaën.

2.2.3. Chemicals for DESpm metabolic studies

Recombinant human polyamine oxidase (hPAO) was produced as described earlier [25]. All other reagents (formic acid, glycine, NaOH) were from Sigma.

2.3. Mass spectrometer conditions

Flow injection analysis (FIA) was used to optimize the fragmentor and source parameters. The drying gas temperature was varied between 300 and 350 °C, drying gas flow between 6 and 10 L/min and capillary voltage between 3500 and 4500 V. The optimized source parameters for MS analysis were as follows: drying gas temperature 300 °C and gas flow 8 L/min, nebulizer gas pressure 40 psi and capillary voltage 4000 V. Fragmentor voltage values were tested between 0 and 120 V for each polyamine to obtain the highest precursor ion abundance, and the optimized values are shown in Table 1. After optimization, positive-ion mass spectra for each polyamine were generated by FIA in full scan mode at the mass range *m*/*z* 50–400.

MS/MS experiments were based on collisionally activated dissociation occurring in the collision cell (quadrupole 2) of the triple quadrupoles, with a nitrogen collision gas. In order to determine the characteristic mass fragments for selected reaction monitoring (SRM) analysis, the product spectra of each polyamine was recorded in full scan mode by varying the offset voltage between 5 and 20 eV. The characteristic product ion was selected and the optimal collision energy value chosen to obtain the highest selected product ion abundance. Precursor ions, chosen product ions and collision energy values for all analytes used in the following SRM analysis are given in Table 1.

2.4. Relative signal intensity studies

The relative signal intensity for different solvent compositions was measured by comparing the relative peak area obtained from FIA of five polyamines (PUT, N^1AcSpd, Spm, DESpm and EtSpm). Five parallel injections were carried out and analyzed in the multiple reaction monitoring mode (SRM) using the chosen fragmentation and optimized fragmentor and collision energy values described in Table 1. Dwell time was adjusted to 200 ms, injection volume of the 100 μM sample was 1 μL and the delay between injections was 30 s. The sample was diluted with water. Solvent system consisted of 0.1% acid in water (solvent A) and 0.1% acid in ACN (solvent B) in a ratio 1:1. Acids tested were FA, AA, BA, TFA, PFPA and HFBA.

The influence of propionic acid to the relative signal intensity was carried out using the conditions mentioned above, where the solvent A was 0.1% (v/v) HFBA and 2% (v/v) PrA in water, and solvent B was 0.1% (v/v) HFBA and 2% (v/v) PrA in ACN.

2.5. Chromatographic conditions

Chromatographic separations were carried out by an analytical LC–MS/MS using a gradient solvent system at flow rate of 0.2 mL/min. A gradient of 0.1% (v/v) acid in water (solvent A) and 0.1% (v/v) acid in ACN (solvent B) was used. Gradient was increased from 0 to 50% B over 10 min. Reversed phase column was thermostated to 25 °C. MS/MS detection of the eluted analytes were carried out in the SRM mode using precursor ions, chosen product ions and optimized fragmentor and collision energy values as described in Table 1. Dwell time for all was adjusted to 50 ms. Two samples were used in the studies, one containing a mixture of eight polyamine standards (PUT, CAD, DAH, N^1AcSpd, N^8AcSpd, Spd, N^1AcSpm and Spm) and the other including a mixture of four *N*-alkylated polyamine analogues (DESpm, EtSpm, EtSpd and EtDAP). For retention time studies, two samples were diluted with water to the concentration of 100 μM and the injection volume of the sample was 1 μL. Acids studied in the solvent system were FA, AA, BA, TFA, PFPA and HFBA. Reliability was checked by at least two injections, except for FA, were only one injection was performed to each of the two polyamine mixtures.

The effect of propionic acid on the retention times was checked using the LC–MS/MS conditions mentioned above, where the solvent A was 0.1% (v/v) HFBA and 2% (v/v) PrA in water, and solvent B was 0.1% (v/v) HFBA and 2% (v/v) PrA in ACN. The injection volumes of the two samples were 1 μL, and the dilution to 100 μM was done with water.

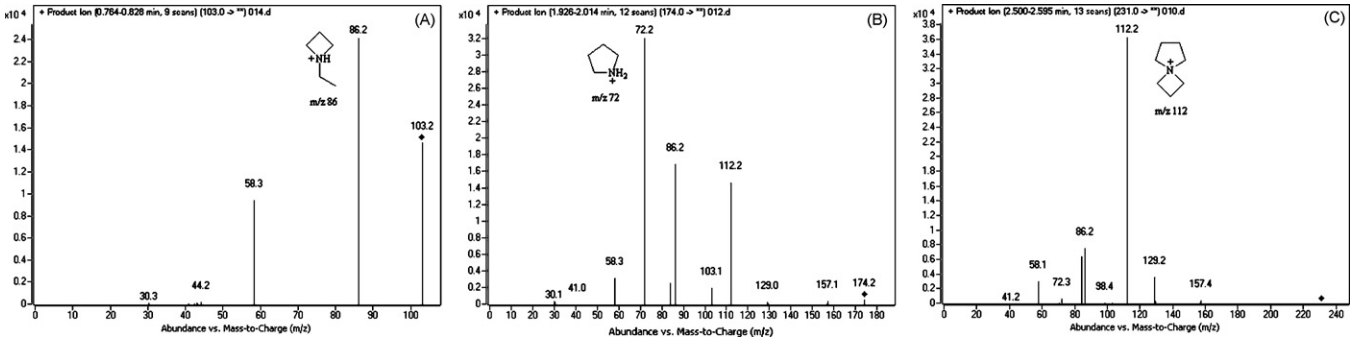


Fig. 1. Positive ion MS/MS product ion mass spectra and presumable product ions for three analytes. $[M+H]^+$ ion marked with diamond. EtDAP (A), EtSpd (B), and EtSpm (C).

The retention factor k for each solvent composition was calculated using the equation $k = (t_R - t_0)/t_0$, where t_R is the retention time and t_0 is the solvent front (0.42).

2.6. The effect of post-column addition of PrA to relative signal intensity and retention times

The post-column addition of a solution of 75% PrA and 25% IPA was performed through a peek mixing tee (VICI Jour Research AB, Sweden) at 0.1 mL/min into the column flow. Other LC–MS/MS conditions were as before, where the solvent A was 0.1% (v/v) HFBA in water and solvent B was 0.1% (v/v) HFBA in ACN.

Influence of the peek mixing tee and the post-column flow on the peak shape and retention times were studied as follows. Three different chromatographic separations were performed and each of them was repeated three times: one without the peek mixing tee, the other with the tee but without the post-column flow and the third with the tee and the post-column flow. The injection volume of the sample containing a mixture of eight polyamine standards (PUT, CAD, DAH, N^1AcSpd, N^8AcSpd, Spd, N^1AcSpm and Spm) was 1 μL in each run, and the dilution to 100 μM was made with water. Number of theoretical plates N, were then calculated for four of the analytes (PUT, Spd, Spm and N^1AcSpd) from the three chromatographic separations performed, using the equation $N = 5.54(t_R/W_{1/2})^2$.

The relative signal intensity was measured by comparing the relative peak areas obtained from the LC–MS/MS run with the tee but without the post-column flow, and the LC–MS/MS run with the tee and the post-column flow.

2.7. The final LC–MS/MS conditions for DESpm metabolic studies

A gradient solvent system at flow rate of 0.2 mL/min. A gradient of 0.1% (v/v) HFBA in water (solvent A) and 0.1% (v/v) HFBA in ACN. Gradient was increased from 0 to 50% B over 10 min. Column temperature was adjusted to 25 °C. Isocratic post-column addition of a solution of 75% PrA and 25% IPA was performed through a peek mixing tee at 0.1 mL/min to the column flow. MS/MS detection of the eluted analytes were carried out in the SRM mode using precursor ions, product ions and optimized fragmentor and collision energy values described in Table 1. Dwell time for all was adjusted to 50 ms.

LC–MS/MS conditions were tested prior to DESpm metabolic studies by two samples, one containing a mixture of eight polyamine standards (PUT, CAD, DAH, N^1AcSpd, N^8AcSpd, Spd, N^1AcSpm and Spm) and the other including four N-alkylated polyamine analogues (DESpm, EtSpm, EtSpd and EtDAP). Injection volume of the 100 μM sample was 10 μL and the sample was diluted with 0.1% HFBA in water. Reproducibility was checked by two injections.

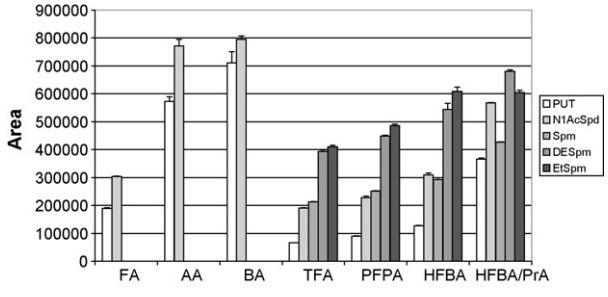


Fig. 2. Relative signal intensity for analytes in different solvent compositions. Five parallel FIA of five polyamines were carried out in the SRM mode using 0.1% acid in water and 0.1% acid in ACN in a ratio 1:1 as a solvent. Spm, DESpm and EtSpm were not able to detect properly using FA, AA or BA as an acid. The influence of the addition of 2% (v/v) PrA to the HFBA containing solvent system is shown in right. Error bars indicate the standard deviation.

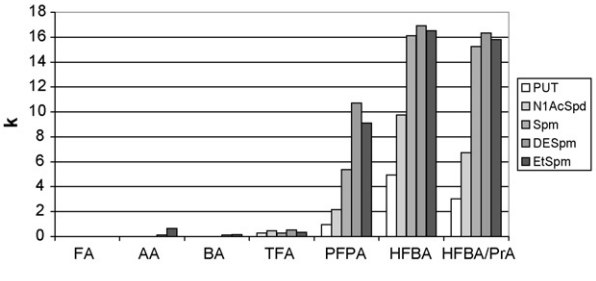


Fig. 3. The effect of solvent composition on the retention factor k. RP-LC–MS/MS separations were performed by linear gradient elution of 0–50% B in 10 min (solvent A: 0.1% (v/v) acid in water, solvent B: 0.1% (v/v) acid in ACN) at flow rate of 0.2 mL/min. Polyamines were not retained properly to the column using FA, AA, BA or TFA as an ion-pairing acid. The influence of the addition of 2% (v/v) PrA to the HFBA containing solvent system is shown in right.

2.8. Metabolic studies of DESpm

Enzymatic degradation of DESpm was performed with recombinant hPAO. Reactions were carried out in a total volume of 360 μL including 100 mM glycine–NaOH buffer pH 9.5, 1 mM DESpm and 1 μg hPAO. The reactions were allowed to proceed for the indicated time at +37 °C before the addition of 40 μL of 50% formic acid in water. First of the incubations was stopped immediately (incubating time 0 min), the second after 30 min incubation and the third after 60 min incubation. All solutions were passed through 0.22 μm filter and stored at −20 °C. Prior to LC–MS/MS analysis, samples were diluted to 1:10 with 0.1% HFBA in water. The final LC–MS/MS conditions described above were used to analyze the samples. Injection volume of the diluted sample was 10 μL. Reproducibility was checked by two injections.

3. Results and discussion

3.1. Mass spectrometry analysis

Structures, molecular masses, measured precursor ions and MS/MS data for the compounds studied are summarized in Table 1. Positive-ion mass spectra for each polyamine were generated under the conditions described in Section 2. All polyamines produced protonated $[M+H]^+$ molecule and no adduct formation was observed. When optimizing the MS conditions, it was found that diamines PUT, CAD and DAH give higher precursor ion abundance with lower fragmentor voltage values compared to rest of the analytes.

In order to determine the optimal conditions for SRM analysis, the positive ion product spectra of each polyamine was recorded as described in Section 2. The product ion spectra, which were obtained under the MS/MS conditions shown in Table 1, and presumable product ions formed for EtDAP, EtSpd and EtSpm as an example are shown in Fig. 1A–C. The MS/MS spectra of studied polyamines showed typical neutral loss of ammonia. The main fragment ions in the CAD and DAH MS/MS spectrum, at *m/z* 86 and 114, respectively, are probably similar type ring structures as described before for putrescine [22,26], but also linear product ion structures are as possible [15]. Diamines PUT and CAD had no other major fragment ions, but DAH had another fragment *m/z* 55. N^1AcSpd and N^8AcSpd had similar precursor ions, but MS/MS spectrum showed different behaviour in collision cell and allowed to choose divergent product ions, *m/z* 100 and 114, respectively, for SRM analysis.

3.2. The effect of different solvent compositions to relative signal intensity

The effect of different acid additives in the used solvent system to relative signal intensities of five selected polyamines (PUT, N^1AcSpd, Spm, DESpm and EtSpm) was studied as described in Section 2. Studied acid additives were FA, AA, BA, TFA, PFPA and HFBA (results are shown in Fig. 2). Common signal suppression for basic compounds was noticed when solvent system contained strong acids like TFA, PFPA and HFBA compared to weaker acids like AA and BA. On the other hand, it was found that Spm, DESpm and EtSpm could not be analyzed using solvent systems containing FA, AA or BA as an acid. These analytes were adsorbed on mass spectrometer tubing, and they came out as a broad peak later on. Possible explanation to this is the weak ion pair that forms between the sample and the weak acid anion, which enables the sample interaction with carbonyl groups present in the polyetheretherketone, material the tubing are made of (PEEK tubing from VICI Jour Research AB). It was also taken note that relative signal intensities varied between different analytes, which means that stable isotope analogues rather than structural homologues should be used in quantitative analysis.

Table 2
Retention times for studied polyamines under different solvent compositions

Compound	FA	AA	BA	TFA	PFPA	HFBA	HFBA + PrA
PUT	0.43	0.43	0.43	0.55	0.82	2.49	1.69
CAD	0.43	0.43	0.43	0.56	0.87	2.97	1.88
EtDAP	0.43	0.42	0.42	0.56	0.97	3.62	2.23
N^1AcSpd	0.43	0.43	0.43	0.62	1.33	4.52	3.24
N^8AcSpd	0.43	0.43	0.43	0.67	1.58	4.81	3.72
DAH	0.43	0.43	0.43	0.68	1.85	5.02	4.03
Spd	0.43	0.43*	0.44*	0.54	1.28	5.97	4.99
EtSpd	0.43	0.44*	0.44*	0.57	1.98	6.37	5.73
N^1AcSpm	0.43	0.44*	0.51*	0.63	2.89	6.44	5.93
Spm	0.44*	0.46*	0.46*	0.54	2.68	7.19	6.83
EtSpm	0.45*	0.70*	0.48*	0.57	4.25	7.36	7.06
DESpm	0.45*	0.48*	0.47*	0.64	4.92	7.53	7.28

A linear gradient elution of 0–50% B in 10 min was performed at flow rate 0.2 mL/min. Solvent A was 0.1% (v/v) acid in water and solvent B was 0.1% (v/v) acid in ACN. Acids tested were formic acid (FA), acetic acid (AA), butyric acid (BA), trifluoroacetic acid (TFA), pentafluoroacetic acid (PFPA) and heptafluoroacetic acid (HFBA). Polyamines are in the order they appeared with the HFBA containing solvent system. The influence of the addition of 2% (v/v) PrA to the HFBA containing solvent system is shown as HFBA + PrA. Severe tailing was observed for compounds marked with *.

3.3. Analytical chromatography

Chromatographic studies were performed using two samples, one containing a mixture of eight polyamines (PUT, CAD,

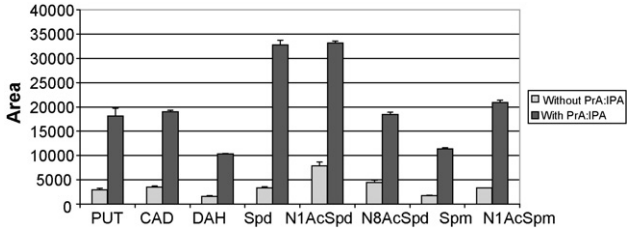


Fig. 4. The effect of the post-column addition of 75% PrA in IPA on relative signal intensity. RP-LC–MS/MS separation of eight polyamines was carried out by linear gradient elution of 0–50% B in 10 min (solvent A: 0.1% (v/v) HFBA in water, solvent B: 0.1% (v/v) HFBA in ACN) at flow rate of 0.2 mL/min. Injection volume of the 100 μM sample was 1 μL. Sample was diluted with water. Isocratic post-column addition of a solution of 75% PrA and 25% IPA in a ratio 1:2 was performed through a peek mixing tee at 0.1 mL/min into the column flow. Experiments were performed in triplicate and the error bars show the standard deviation.

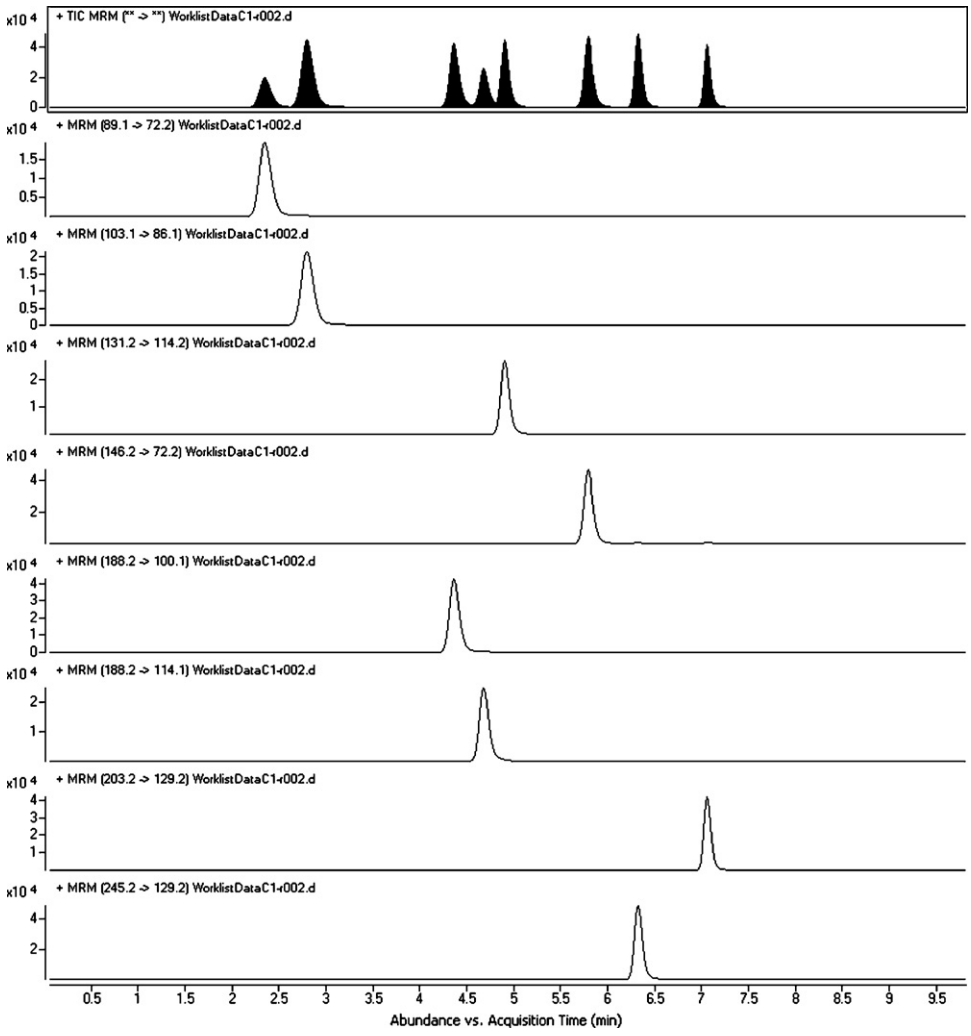


Fig. 5. Positive-ion RP-LC/MS/MS SRM chromatogram of mixture of eight polyamines, 100 μM each with injection volume of 10 μL. (1) Put, (2) Cad, (3) N^1AcSpd, (4) N^8AcSpd, (5) DAH, (6) Spd, (7) N^1AcSpm, and (8) Spm.

DAH, N^1AcSpd, N^8AcSpd, Spd, N^1AcSpm and Spm) and the other with four *N*-alkylated polyamine analogues (DESpm, EtSpm, EtSpd and EtDAP). The effect of different acids on retention times of these polyamines were investigated with FA, AA, BA, TFA, PFPA and HFBA as described in Section 2. Retention times for all polyamines in different solvent compositions are shown in Table 2 and the retention factor *k* for five selected analytes (PUT, N^1AcSpd, Spm, DESpm and EtSpm) in different solvent compositions are shown in Fig. 3. Results show that complete chromatographic separation of the eight polyamine standards (PUT, CAD, DAH, N^1AcSpd, N^8AcSpd, Spd, N^1AcSpm and Spm) and four alkylated polyamine analogues (DESpm, EtSpm, EtSpd and EtDAP) was achieved using HFBA as an acid. Also PFPA was able to retain polyamines in the column to some extent. When using PFPA as an ion-pairing agent instead of HFBA, it was noted that Spm and Spd eluted faster compared to other analytes (see Table 2). Other acids were not able to retain polyamines sufficiently to reversed phase material. Furthermore, weaker acids were not able to decrease the interaction of polyamines to columns free silanol groups, and severe peak tailing was observed to some of the polyamines. This interaction was greatly reduced using stronger acids.

The solvent used to sample dilution were also found to influence the chromatographic separations. When studying relative signal intensity and retention times, injection volume was 1 μL. When the injection volume was raised to 10 μL, samples had to be diluted with 0.1% HFBA instead of plain water prior to chromatography. With water, some of the compounds were not retained completely by the column, probably because of the insufficient replacement of the polyamine chloride ions with solvent acid ions. When 0.1% HFBA was used for dilution, all the compounds were retained.

3.4. Signal suppression and enhancement

Volatile strong acids used as ion-pairing reagents in reversed phase HPLC cause signal suppression for basic compounds, when analyzed by ESI-MS. This is also noted, when relative signal intensity measurements with different ion-pairing reagents are compared with each other (see Fig. 2.) This ion-pairing process masks the protonated sample cations from the ESI-MS

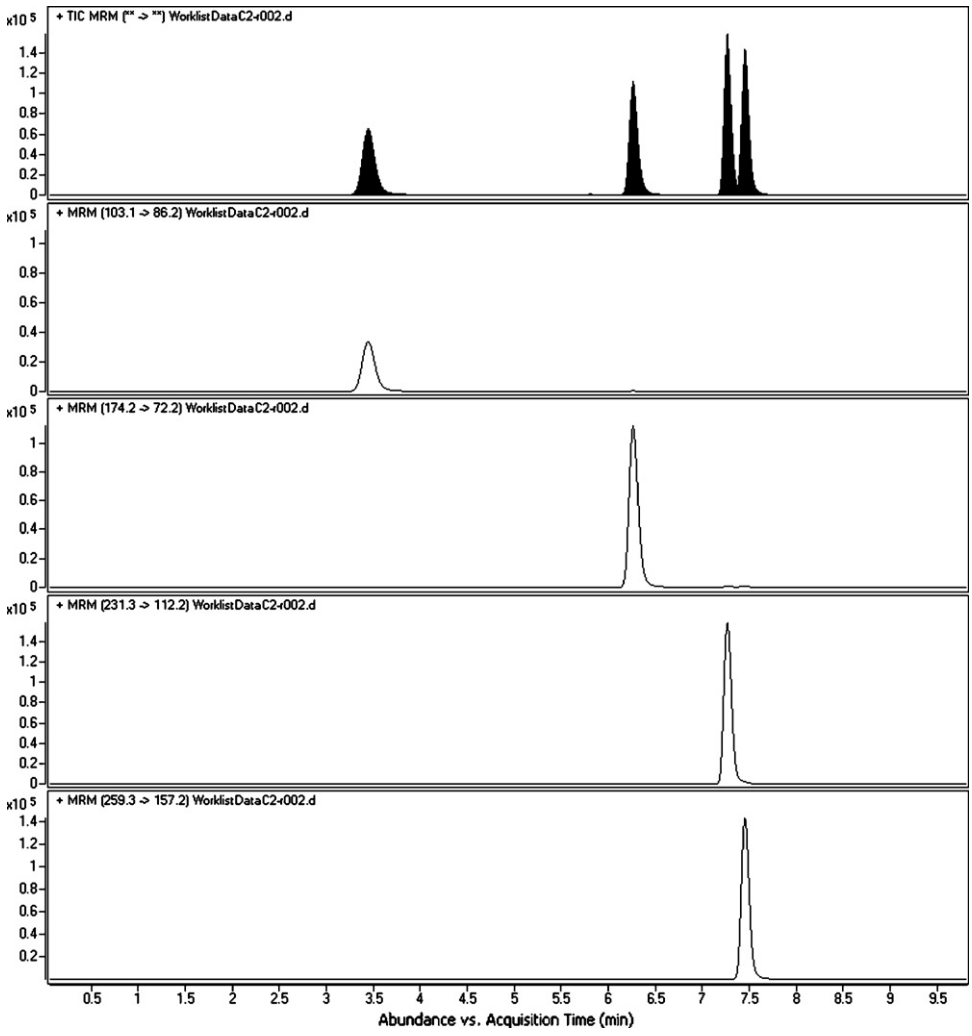


Fig. 6. Positive ion RP-LC/MS/MS SRM chromatogram of mixture of four *N*-alkylated polyamine analogues, 100 μM each with injection volume of 10 μL. (1) EtDAP, (2) EtSpd, (3) EtSpm, and (4) DESpm.

electric field by rendering them "neutral". Kuhlmann et al. [23] have proposed a practical method to enhance the signal for most basic analytes by employing post-column addition of a solution of 75% PrA and 25% IPA in a ratio 1:2 to the column flow. In this method, the acid anion, e.g. TFA, is removed by adding a high concentration of weak acid with lower volatility than TFA to the solution (e.g. PrA). The weak acid competes with the TFA anion for protons, and TFA, which is more volatile than PrA, will evaporate out of the droplet faster than the weak acid. As the droplet shrinks, the acid equilibrium is driven by mass action toward deprotonation of the weak acid. The weak ion pair that forms between the sample and the weak acid anion is ejected.

We also studied the influence of this method to the relative signal intensity (see Fig. 2). Results showed that addition of 2% PrA to the solvent system increased relative signal intensity slightly, up to ~3 times depending on the measured molecule. The influence of PrA addition on the retention times was on the contrary unfavourable, because it was noted that peaks started to unretain as compared with the result obtained without PrA (see Fig. 3).

Because of the undesirable effect on the retention times, isocratic post-column addition of a solution of 75% PrA in IPA was done through a peek mixing tee in a ratio 1:2 to the column flow as in [23]. Influence of the peek mixing tee and post-column flow to the peak shape and retention times was studied as described in Section 2. Number of theoretical plates *N*, were calculated for four of the analytes (PUT, Spd, Spm and N^1AcSpd) from the three chromatographic separations performed. These three values for each four analytes are shown in Table 1. The results show that the column efficiency was decreased because of the peek mixing tee volume. The post-column addition of 75% PrA in IPA had no significant additional effect on *N*, but increased the relative signal intensity 4–10 times depending on the analyte (see Fig. 4).

3.5. The final LC–MS/MS conditions

Two samples, one containing a mixture of eight polyamine standards (PUT, CAD, DAH, N^1AcSpd, N^8AcSpd, Spd, N^1AcSpm and Spm) and the other including four *N*-alkylated polyamine analogues (DESpm, EtSpm, EtSpd and EtDAP) were

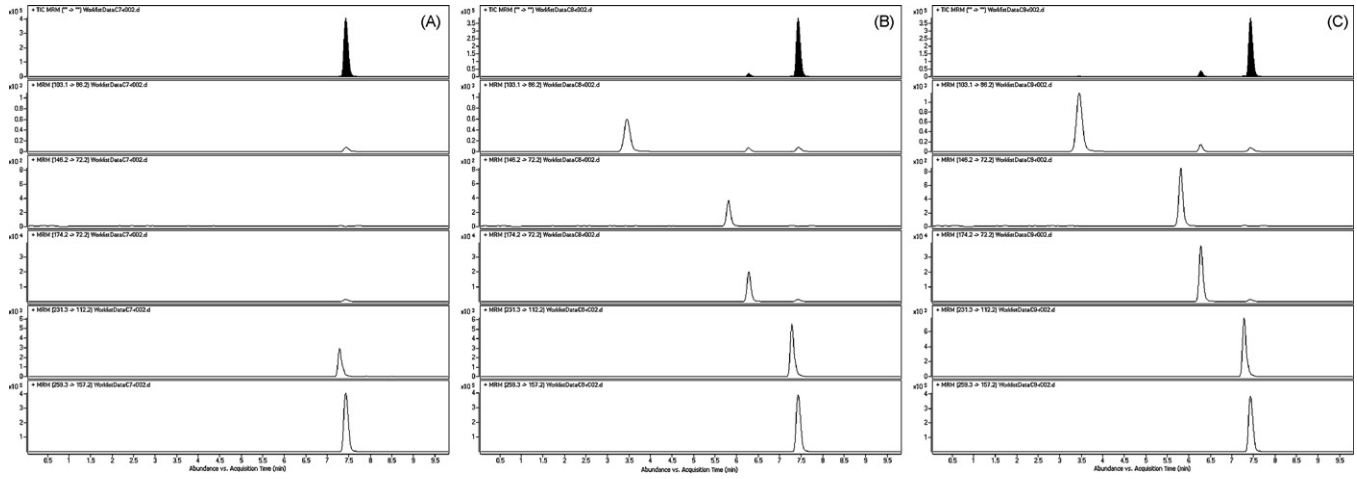


Fig. 7. Positive ion LC–MS/MS SRM chromatograms of three different incubation experiments carried out with DESpm as the substrate of hPAO ($a = 0$ min, $b = 30$ min and $c = 60$ min). Chromatographic separation and MS identification of DESpm metabolites was performed using the final LC–MS/MS conditions described in detail in Section 2.

tested using the developed LC–MS/MS method with HFBA as a volatile ion-pair and post-column addition of PrA to increase the signal intensity. Positive ion LC–MS/MS SRM chromatograms of the mixture of eight polyamine standards are shown in Fig. 5 and the mixture of four *N*-alkylated polyamine analogues in Fig. 6.

3.6. Metabolic studies of DESpm

Enzymatic degradation of DESpm was performed with recombinant hPAO and studies of DESpm metabolites were carried out using the final LC–MS/MS conditions described in detail in Section 2. Positive ion LC–MS/MS SRM chromatograms of three different incubation experiments are shown in Fig. 7 ($a = 0$ min, $b = 30$ min and $c = 60$ min). Metabolites formed in the catabolism of DESpm included EtSpm, EtSpd, Spd and EtDAP. Despite the DESpm used as an enzyme substrate in the study contained traces of EtSpm (see Fig. 7A), it is clearly seen in the chromatograms that the amount of EtSpm was increased after incubation with hPAO (see Fig. 7B and C). Because relative signal intensities are greatly dependent on the analyte measured, the determination of the quantitative amounts of metabolites requires labelled (e.g. deuterated) analogues as internal standards. In any case, the degradation products originated from DESpm by hPAO, which were found after MS analysis, clearly prove enzymatic de-ethylation of DESpm to EtSpm [5], and the appearance of EtSpd support the view that DESpm is degraded like diacetylated spermine by PAO [6]. Furthermore, we found both Spd and EtDAP. Spd may be derived from EtSpd [6], but interestingly EtDAP should be derived from endo-cleavage that has not been shown to occur with mammalian PAO. However, plant, bacterial and protozoan PAOs are known

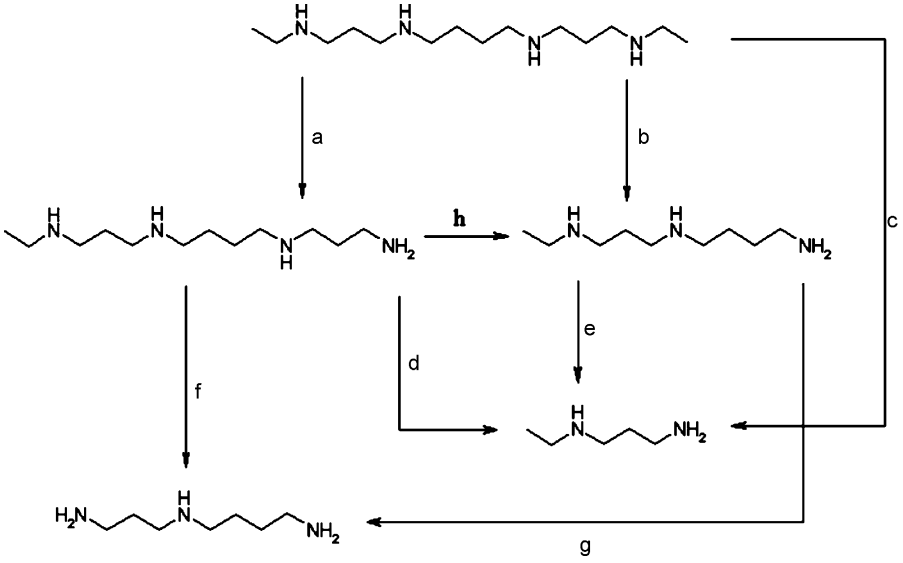


Fig. 8. Possible catabolic pathways of DESpm catalyzed by hPAO. (a) De-ethylation of DESpm to EtSpm [5], (b) endo-cleavage of DESpm to EtSpd, mimicking the catabolism of N^1,N^{12}-diacetylated spermine [6], (c–e) endo-cleavage of DESpm to EtDAP, (f) Spd derived from EtSpm [6], (g) de-ethylation of EtSpd to Spd, and (h) exo-cleavage of EtSpm to EtSpd.

to be responsible for the endo-cleavage (or terminal catabolism) of polyamines [27]. PUT was not detected from the metabolic studies of DESpm, suggesting that EtSpd is not recognized as a mimic of N^1AcSpd by hPAO. Schematic presentation of the possible catabolic pathways of DESpm is shown in Fig. 8. These results suggest that hPAO-mediated degradation is more complicated than shown before.

4. Conclusions

All polyamines in the study (PUT, CAD, DAH, N^1AcSpd, N^8AcSpd, Spd, N^1AcSpm, Spm and four *N*-alkylated polyamine analogues DESpm, EtSpm, EtSpd and EtDAP) were separated and detected without any derivatization using the developed LC–MS/MS method. Five volatile acids were tested as an ion-pairing reagent, of which HFBA proved to yield best chromatographic separation of the analytes with good symmetrical peak shapes. Even the closely related N^1AcSpd and N^8AcSpd were separated from each other. Some of the tested acids caused adsorption of underivatized polyamines to instrument capillaries during MS analysis and tailing of the peaks during LC gradient, but these were greatly reduced by using stronger acid like HFBA as ion-pairing additive. Common signal suppression in ESI-MS for basic compounds by strong acids was prevented using post-column addition of PrA to column flow.

The developed LC–MS/MS method described here can be recommended for use in qualitative polyamine analysis. Compared with conventional methods of polyamine analysis, the developed LC–MS/MS is easier to use and is substantially faster due to minimal sample pre-treatment and rapid chromatographic separation. Moreover, the method allows absolute identification of the products by highly sensitive MS and the possibility to eliminate interfering peaks arising from the matrix by selective MS/MS. The developed method was successfully applied to identify metabolites of DESpm as a substrate for recombinant hPAO. Metabolites formed in the hPAO reaction included EtSpm, EtSpd, Spd and EtDAP, suggesting that hPAO-mediated degradation is more complicated than believed earlier. Quantitative analysis, however, requires stable isotope analogues of the studied molecules as internal standards, and further studies are also needed to elucidate the exact origin of the formed Spd and EtDAP and the properties of EtSpm and EtSpd as the substrates of hPAO.

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