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Determination of hair polyamines as *N*-ethoxycarbonyl-*N*-pentafluoropropionyl derivatives by gas chromatography–mass spectrometry

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Abstract

An efficient method is described for the simultaneous determination of hair polyamines, such as 1,3-diaminopropane, putrescine, cadaverine, spermidine and spermine, by gas chromatography–mass spectrometry (GC–MS) with the selected ion-monitoring (SIM) mode. The method is based on the extractive two-phase ethoxycarbonyl (EOC) reaction of amino functions in aqueous solutions combined with subsequent pentafluoropropionyl (PFP) derivatization of the remaining active hydrogen atoms for the direct analysis by GC–SIM-MS. The detection limits for SIM of the polyamines as *N*-EOC-*N*-PFP derivatives ranged from 1 to 10 ng/g hair, while their recovery rates varied in the range of 76.42–93.38%. This method demonstrated a good overall accuracy (% bias) and precision (% C.V.) as 3.32–11.05% and 5.88–14.71%, respectively. When applied to 0.6 *M* HCl extracts of hair samples from 11 healthy men and 19 healthy women, all five polyamines were positively detected at the concentrations of 8.82–871.87 ng/g. Both in median and mean concentrations, the most abundant hair polyamine was spermidine, followed by spermine, putrescine, 1,3-diaminopropane and cadaverine in the male group, while the order of 1,3-diaminopropane and cadaverine was reversed in the female group. The levels of polyamines, except for cadaverine, in hair specimens studied were found to be higher in men than in women.

Keywords: Derivitisation, GC; Hair; Polyamines

1. Introduction

Among the structurally diverse biogenic amines, naturally occurring diamines and polyamines, such as 1,3-diaminopropane, putrescine, cadaverine, spermidine and spermine, are known to be closely related with cell growth in rat brain during development. They are also important in tissues with cellular proliferation and in human gliomas [1,2]. Rapid tumor growth has been associated with markedly altered polyamine biosynthesis and accumulation [3] and an increase in concentrations of urinary polyamines might provide a diagnostic tool to evaluate malignant tumor activity [4–12]. In recent years, the altered levels of brain polyamines were found to be associated with Alzheimer's disease [13,14]. The accurate detection and identification of all the poly-

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amines simultaneously in a single analysis are thus becoming more important for the study of their biochemical roles.

When high-resolution capillary gas chromatography (GC) in combination with mass spectrometry (MS) is employed for the polyamine analysis, it is a prerequisite to block active hydrogen atoms in amino groups prior to analysis. Among the various derivatization methods developed for this purpose, perfluoroacylation [11,12,15–20] and alkylsilylation [21,22] are performed after multiple sample-purification steps that are normally very tedious and time consuming. In our recent works [11,12], solid-phase extraction using Sep-Pak column and 0.1 *M* HCl in methanol (as an eluent) after solvent-washing was found to be efficient for the recovery of polyamines and *N*-heptafluorobutyrylated polyamines. However, the heptafluorobutyl (HFB) derivatization required the complete evaporation of the methanol aqueous eluate to dryness, which was a very cumbersome step in view of the overall analysis time.

As a different approach, the direct-derivatization of polyamines in aqueous solutions to make them extractable by volatile organic solvents was more preferred. It is also mainly alkoxycarbonylation (AOC) in alkaline aqueous solution with methyl-, ethyl- and isobutyl chloroformate as reagents has been used in the literature [23–27]. With extractive two-phase AOC procedures [26,27], derivatization and extraction of amines in aqueous solutions are achieved in the nearly same time. In our previous amine-profiling and screening analysis [27], extractive two-phase AOC with isobutyl chloroformate (isoBCF) present in the dichloromethane was found to be efficient in the recovery of 57 diverse biogenic amines. However, the isobutoxycarbonylation (isoBOC) procedure was little suitable for polyamines than the other amines, especially spermine, because the multi-isoBOC groups introduced made the derivatives less volatile and the remaining polar active hydrogen atoms in amino groups cause peak tailing. Accordingly, it was desirable to improve the volatility and GC properties of polyamines. This might be accomplished by performing AOC with less bulky methyl- or ethyl chloroformate, with subsequent perfluoroacylation or alkylsilylation of the remaining active hydrogen atoms. However, attempts were rarely made to adopt this method for the polyamine analysis.

Among the biological specimens screened for polyamine assays, noninvasive urine was most extensively investigated [4,5,10–12,19,28]. However, the inherent problems of the urine specimen, such as the fluctuation of its composition during the day and the hygienic practice during its collection and handling, prompted us to search for another type of noninvasive samples. Recently, the value of hair as the noninvasive biosample has been recognized in the biomedical field [29–32]. It is well-established that hair reflects long-term chronic nutriture, while the blood and urine reflects acute status since hair grows approximately at 1 cm per month [33]. Hair provides biochemical information on the cumulative effects of exogenous and endogenous environments over a wide period of time. According to recent reports [29,30], X-ray scattering patterns of hair samples are characteristic of breast cancer. In our previous works [31,32], hair was found to serve well in yielding valuable physiological information on the endogenous steroids. To date, no attempts have been made to measure polyamine levels of hair.

The present study was undertaken to combine extractive two-phase AOC with subsequent perfluoroacylation of the remaining hydrogen atoms for the investigation of their biochemical roles in human hair. This was done in order to conduct for the polyamine analysis of hair specimens from 11 healthy men and 19 healthy women by GC–MS with the selected ion-monitoring (SIM) mode. In this study, ethyl chloroformate and pentafluoropropionyl anhydride were employed as the reagents for AOC and perfluoroacylation, respectively. Structures of the derivatives that are new to the literature were confirmed by mass spectral patterns.

2. Experimental

2.1. Hair specimens

Tip parts cut from scalp hair strands were collected from 11 healthy men (age 28 to 53) and 19 healthy women (age 24 to 57). Hair samples were washed sequentially with ethanol, water and acetone according to Baumgartner's report [34]. After they were dried (60°C), the hair samples were cut into short lengths of about 1–2 mm and aliquots of 100

mg weighed into glass test tubes. Prior to analysis, the hair samples were kept at room temperature.

2.2. Chemicals

The six polyamine standards, such as 1,3-diaminopropane, putrescine (1,4-diaminobutane), cadaverine (1,5-diaminopentane), spermidine (*N*-[3-aminopropyl]-1,4-diaminobutane), spermine (*N,N*-bis[3-aminopropyl]1,4-diaminobutane) and 1,6-diaminohexane (used as an internal standard) were obtained as free base forms from Sigma (St. Louis, MO, USA). Ethyl chloroformate (ECF) and pentafluoropropionyl anhydride (PFPA) were purchased from Acros Organics (Geel, Belgium). Other chemicals were of the guaranteed reagent grade and diethyl ether as an extraction solvent was distilled from calcium hydride powder just before use.

2.3. Preparation of standard solutions

Each stock solution of six polyamines was prepared at the concentrations of 0.1 mg/ml in a 0.1 *M* HCl solution. These solutions were used to prepare working solutions of various concentrations (0.001, 0.1 and 10.0 μg/ml) in a 0.1 *M* HCl solution and kept below 4°C. The internal standard (I.S.) working solution was prepared by diluting a 1,6-diaminohexane stock solution at 1.0 μg/ml in a 0.1 *M* HCl solution.

2.4. N-Ethoxycarbonylation and N-pentafluoropropionylation

Aliquots of the polyamine working solutions were diluted to the desired concentrations (5–1000 ng/ml) with 0.6 *M* HCl and added with I.S. (1 ppm×20 μl). *N*-ethoxycarbonylation was performed by shaking 1.0 ml of the diluted polyamine solutions with diethyl ether (1.0 ml) containing ECF (50 μl) after adjusting to pH 11–12 with 2 *M* NaOH solution (0.9 ml) for the reaction of basic amino functions [27,35]. After it was shaken for 20 min with subsequent centrifugation (5 min at 2500 *g*), the ether layer was separated by placing it in a dry ice–acetone bath. The aqueous phase was re-extracted with ether (2 ml) and the combined ether extracts were evaporated to dryness under a stream of nitrogen. For the subsequent pentafluoropropionyl (PFP) derivatization, PFPA (20 μl) and ethyl acetate (100 μl) were added to the dried *N*-EOC residues. The mixture was placed in a heating block (50°C) for 30 min and then taken to dryness under a stream of nitrogen. The resulting *N*-EOC-*N*-PFP residue was dried in a vacuum desiccator over P_2O_5/KOH for at least 30 min prior to instrumental analysis. The residue was reconstituted in ethyl acetate (40 μl) for the direct GC–MS analysis.

2.5. Validation of the analytical method

Standards solutions containing five polyamines at varying concentrations (0.01–1.0 ng/ml) were subjected to EOC reaction with a subsequent PFP reaction, as described above, to determine detection limits. Calibration samples were prepared using 1 ml of 0.6 *M* HCl solution spiked with increasing amounts (5–1000 ng/ml) of polyamine standards and fixed amount of I.S. (1 ppm×20 μl) in the same manner in triplicate. Likewise, samples for intra- and inter-day assays and for recovery tests were prepared individually in triplicate at 20 and 50 ng/ml using 1 ml of 0.6 *M* HCl solution. The sample used was a quality control hair formed by identical proportions of hair samples from the 24 individuals. These samples were analyzed in duplicate for five polyamines studied in a day and they were measured 8 days over a 2-month period without particular care for the reproducibility of the present method in the same manner as described for hair.

2.6. Sample preparation

To 100 mg of hair samples an I.S. (1 ppm×20 μl) and 1 ml of a 0.6 *M* HCl solution were added. The mixture was kept at 40°C overnight (~16 h), then washed with *n*-pentane. The aqueous layer was adjusted to pH 11–12 with a 2 *M* NaOH solution and then subjected to an EOC reaction, followed by a PFP reaction as described above.

2.7. Instrumental conditions

GC–MS analyses both in scan and SIM modes were performed with a Hewlett-Packard HP Model 6890 Plus gas chromatograph interfaced to an HP model 5973 MSD (Hewlett-Packard, Avondale, PA, USA). The electron energy was 70 eV and the ion

source temperature was 230°C. Samples were injected into a DB-5 (SE-54 bonded phase) fused-silica capillary column (J&W Scientific, Folsom, CA, USA; the dimensions 30 m×0.25-mm I.D., 0.25-μm film thickness) in the split-injection mode (5:1) at 260°C and the oven temperature was initially 140°C and raised to 210°C (2 min) at 8°C/min, then to a final temperature of 320°C (3.75 min) at 20°C/min. Helium, as a carrier gas, was set to a column head pressure of 88 kPa (column flow: 1.0 ml/min at 140°C).

2.8. Data acquisition

In the scanning mode, the mass range was 50–800 u at a rate of 0.42 scans/s. In the SIM mode, three characteristic ions for each polyamine were used for peak-identification, while one ion underlined was selected for quantification, as listed in Table 1. Each peak in the hair samples was identified by matching the area ratios of three ions with those of polyamine standards. The underlined quantitation ions correspond to $[M-119]^+$ ions for 1,3-diaminopropane, putrescine and 1,6-diaminohexane, to an $[M-308]^+$ ion for cadaverine and to $[M-73]^+$ ions for spermidine and spermine.

The start time for SIM was programmed from 5.0 to 20.0 min to set up two groups of 12 ions (for 1,3-diaminopropane, putrescine, cadaverine and 1,6-diaminohexane) and six ions (for spermidine and spermine) to be monitored. A dwell time of 80 ms was chosen for the first group and 120 ms for the second group. The relative voltage of electron multiplier was set to 400 V higher than that in the scanning mode for each ion monitored.

2.9. Calculation

The detection limit for each polyamine was calculated based on the weight giving a signal three times

Table 1
GC–SIM-MS and validation data for the analysis of polyamines as their *N*-EOC-*N*-PFP derivatives[a]

Polyamine	MW	Retention time (min)	Start time (min)	Ions selected (*m*/*z*)	LOD[b] (ng/g hair)
GC–SIM-MS data					
1,3-Diaminopropane	510	6.10	5.0	391, 202, 176	1
Putrescine	524	7.40		405, 333, 216	5
Cadaverine	538	8.70		419, 329, 230	10
1,6-Diaminohexane	552	10.00		433, 361, 244	NA[c]
Spermidine	653	14.90	12.0	608, 580, 534	5
Spermine	782	18.20		782, 709, 548	10

	Calibration range (ng/ml)	Linearity (*r*)[d]	Recovery (%)[e]
Validation data			
1,3-Diaminopropane	5–200	0.984	88.64
Putrescine	5–200	0.979	93.38
Cadaverine	5–200	0.999	81.05
1,6-Diaminohexane	NA	NA	NA
Spermidine	10–1000	0.997	86.70
Spermine	10–1000	0.999	76.42

[a] Analyzed on a DB-5 (30 m×0.25-mm I.D., 0.25-μm film thickness) fused-silica capillary column initially at 140°C and raised to 210°C (2 min) at 8°C/min, then to a final temperature of 320°C (3.75 min) at 20°C/min, in SIM mode at three ions for each polyamine for peak identification and one ion underlined for quantification at dwell time of 80 ms for the first group and 120 ms for the second group and relative voltage of electron multiplier set to 400 V higher than that in the scanning mode for each ion monitored.

[b] Limit of detection.

[c] Not applicable, used as an internal standard.

[d] Linearity was described with linear correlation coefficients for calibration curves.

[e] Data are expressed as mean values.

the peak-to-peak noise of the background signal. A least-squares regression analysis was performed on the measured peak area ratios against the increasing weight ratios of polyamines to I.S. in order to test the linearity of SIM responses and to plot calibration curves for the quantitative measurement of polyamines. The values of unknown concentrations in the hair were determined from the regression lines of the calibration curves.

3. Results and discussion

3.1. GC–MS properties of polyamines as N-EOC and N-EOC–N-PFP derivatives

Under the present extractive two-phase reaction with ECF in the aqueous solution using diethyl ether as the organic solvent phase, all amino groups of polyamines studied were ethoxycarbonylated into their *N*-EOC derivatives, which were instantly transferred to a volatile ether layer, thus shortening the overall sample-preparation time. When the recovered *N*-EOC derivatives, without subsequent perfluoroacylation, were analyzed, all diamines displayed single peaks with a little tailing, while spermidine and spermine were not detected as appreciable peaks (Fig. 1A). This indicates that the remaining active hydrogen atoms of the two polyamines must be blocked prior to GC analysis. When the subsequent perfluoroaclylation with PFPA was performed, separation of all six polyamines as *N*-EOC-*N*-PFP derivatives was achieved with excellent peak shapes, higher responses and shorter analysis time compared to *N*-EOC derivatives for the same amount (Fig. 1B). The faster elution (1–2 min) due to the increase in volatility of *N*-EOC-*N*-PFP derivatives completed the separation within 20 min and the enhancement of responses by factors of 7–19 reduced the detection limits at the same rates.

Mass spectral patterns of six polyamines as their *N*-EOC-*N*-PFP derivatives are displayed in Fig. 2. The molecular ion peaks were either unobservable or very weak due to the preferential cleavage of labile N–C bonds in the carbamate groups. The base peak ions at m/z 202 for 1,3-diaminopropane and m/z 216 for putrescine and the second most abundant ion at m/z 230 for cadaverine correspond to $[M-308]^+$ ions formed by the consecutive losses of $N(CO_2C_2H_5)(COC_2F_5)$ and $HCO_2C_2H_5$ from molecular ions. The intensity of $[M-308]^+$ ions were abruptly decreased from diaminohexane. The formation of base peak ions at m/z 176 for cadaverine and diaminohexane, at m/z 204 for spermidine and at m/z 377 for spermine, as well as for an intense ion at m/z 116 and $[M-73]^+$, $[M-119]^+$ and $[M-234]^+$ ions, are demonstrated in the fragmentation pattern of spermine (Fig. 3). Ions at m/z 116 and 377, which were present in spermidine and spermine only, correspond to $[CH_2N(EOC)CH_3]^+$ and $[(COC_2F_5)(CO_2C_2H_5)NCH_2CH_2CH_2N(CO_2C_2H_5)CH_2]^+$, respectively. The $[M-119]^+$ ions were prominent in all polyamines except for cadaverine and spermine, but $[M-73]^+$ ions were intense in spermidine and spermine only, probably due to the presence of more EOC groups. The intense ion at m/z 176 observed in all polyamines corresponds to $HN(COC_2F_5)CH_2$. The base peak ion at m/z 204 for spermidine, but minor for putrescine and cadaverine, corresponds to $HN(COC_2F_5)CH_2CH_2CH_2$. The generation of these two ions may be explained by the cleavage of a C–C bond with a proton transfer within the EOC-*N*-$(CH_2)_3$-*N*-PFP ion structure (Fig. 3). Minor ions, such as $[M-191]^+$, $[M-263]^+$ and $[M-276]^+$ions, were derived by the losses of $HCO_2C_2H_5$ from $[M-191]^+$ ions with a proton transfer, CH_2CH_3 from $[M-234]^+$ ions and $CH_2CH_2CH_2$, from $[M-234]^+$ ions, respectively.

3.2. Optimal GC–SIM-MS conditions and method validation

Either $[M-73]^+$ or $[M-119]^+$ high-mass ions were intense enough for the quantification of all polyamines, except for cadaverine, where the second most abundant ion at m/z 230 was selected (Fig. 2). With two more additional ions chosen for each polyamine in the SIM acquisition mode, accurate peak identification was achieved (Table 1). SIM activation started from 5.0 min for the first group of 12 ions of early eluting diamines and then from 12 min for the second group of six ions of late-eluting spermidine and spermine. In the preliminary experiments, dwell times of 80 ms for the first group and 120 ms for the second group were found to yield the

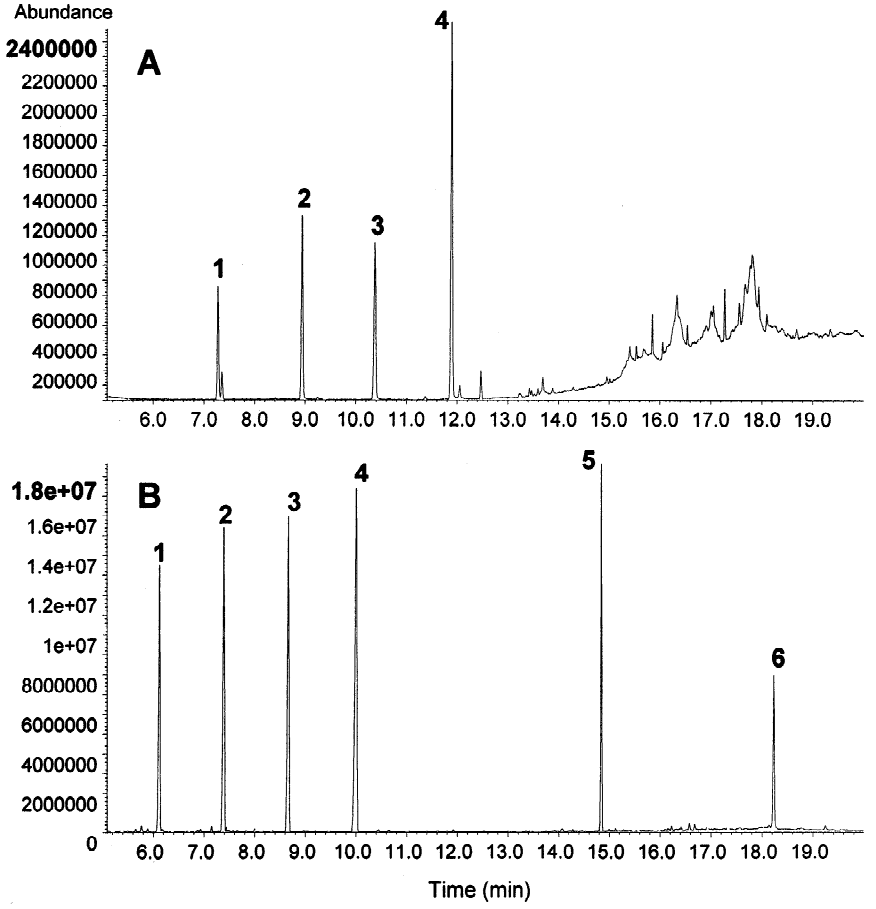

Fig. 1. Total ion chromatograms of six polyamines (2 ng each) as their (A) *N*-EOC and (B) *N*-EOC-*N*-PFP derivatives separated on DB-5 (30×0.25-mm I.D., 0.25-μm film thickness) fused-silica capillary column. The oven temperature was programmed: from 140°C to 210°C (2 min) at 8°C/min and then to 320°C (3.75 min) at 20°C/min. Peaks: 1=1,3-diaminopropane; 2=putrescine; 3=cadaverine; 4=1,6-diaminohexane; 5=spermidine; 6=spermine.

highest ion abundance for each polyamine. Under the present SIM conditions, the detection limits were in the range of 1–10 ng/g hair.

The whole procedure of extractive two-phase EOC reaction with subsequent PFP derivatization and GC–SIM-MS analysis was tested for its validity (Table 1). Linear responses to 1,3-diaminopropane, putrescine and cadaverine were obtained in the range of 5–200 ng/ml and to spermidine and spermine in the range of 10–1000 ng/ml with correlation coefficients varying from 0.979 to 0.999. The accuracy (% bias) for recovery tests varied from 3.3 to 11.0%, while recoveries ranged from 76.42 to 93.38% with precision (% C.V.) varying from 5.9 to 14.7% for three different runs. A quality control (QC) sample prepared from 100 mg of hair samples of the 24 individuals was routinely analyzed with every batch of hair. The polyamine levels, measured eight times over a 2-month period without particular care, gave the following mean values (ng/g): 1,3–diaminopropane, 17.16±1.27; putrescine, 78.41±5.93; cadaverine, 21.05±2.31; spermidine, 270.38±29.88; spermine, 205.11±18.44. Therefore, the overall efficiency and precision of the three combined steps appear to be sufficient for the simultaneous measurement of polyamines in unknown hair samples.

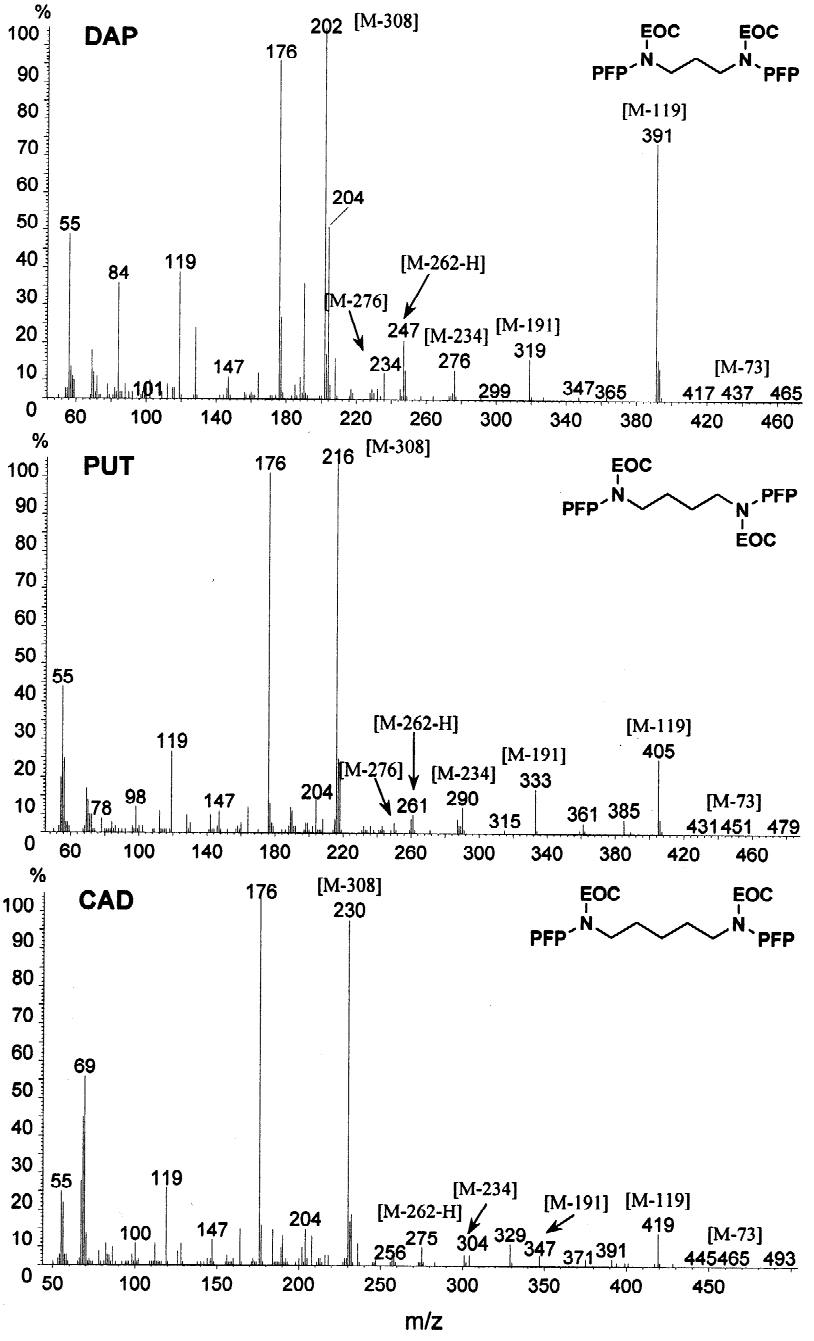

Fig. 2. Electron-impact mass spectra of six polyamines as their *N*-EOC-*N*-PFP derivatives obtained in the scanning mode at a rate of 0.42 scans/s with a mass range of *m*/*z* 50–800. DAP=1,3-diaminopropane; PUT=putrescine; CAD=cadaverine; DAH=1,6-diaminohexane; SPD=spermidine; SPM=spermine.

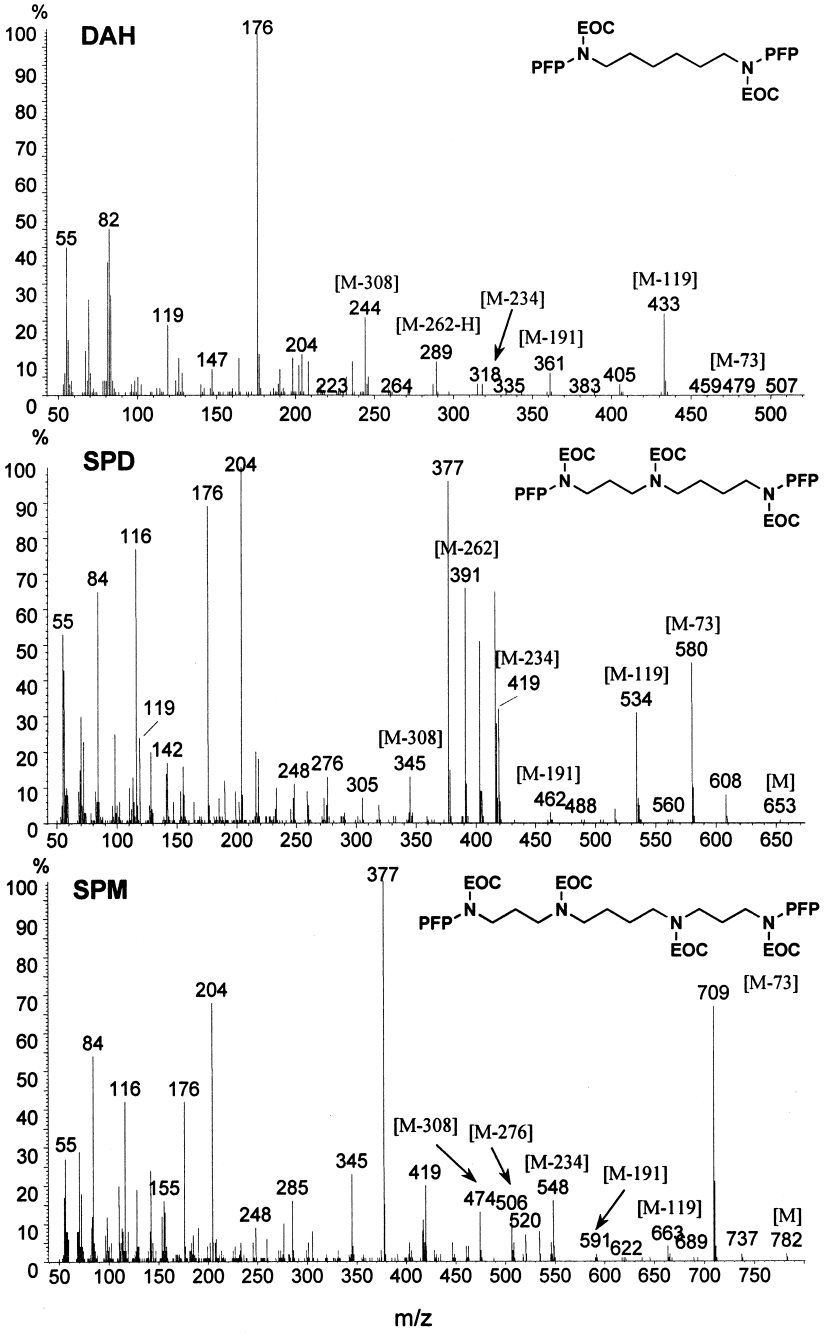

Fig. 2. (*continued*).

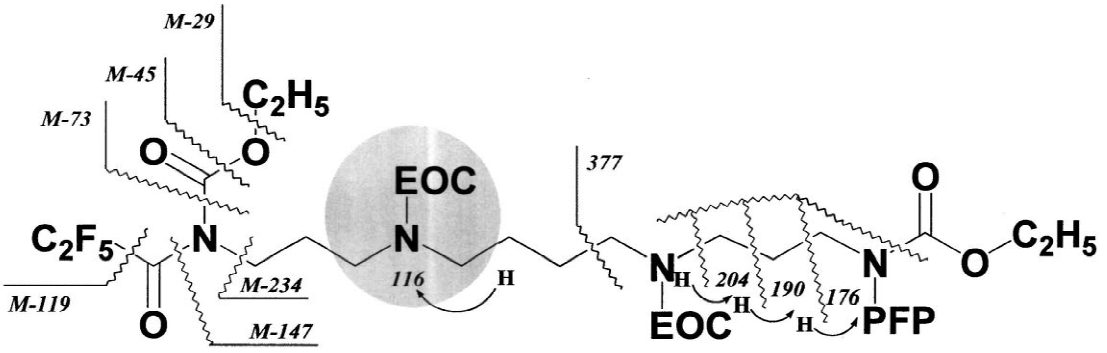

Fig. 3. Fragmentation pattern of spermine as *N*-EOC-*N*-PFP derivative.

3.3. Quantification of polyamines in human hair

After washing sequentially with ethanol, water and acetone, hair samples collected from 11 healthy men and 19 healthy women were treated with a 0.6 *M* HCl solution under mild conditions to extract polyamines from hair matrices. When the present three–step method was applied to the aqueous extracts of hair, an excellent separation of five polyamines was achieved with no significantly interfering background peaks, as demonstrated in a typical selected-ion–current chromatogram (Fig. 4).

From aliquots of 100 mg of all hair samples studied, five polyamines were positively detected and their concentrations varied from 8.82 to 871.87 ng/g (Table 2). Large variations in the levels of polyamines from subject to subject were observed. Both in median and mean amounts, the most abundant polyamine was spermidine, followed by spermine, putrescine, 1,3-diaminopropane and cadaverine in the male group, while the order of 1,3-diaminopropane and cadaverine was reversed in the female group. The levels of hair polyamines, except for cadaverine, turned out to be higher in men than in women.

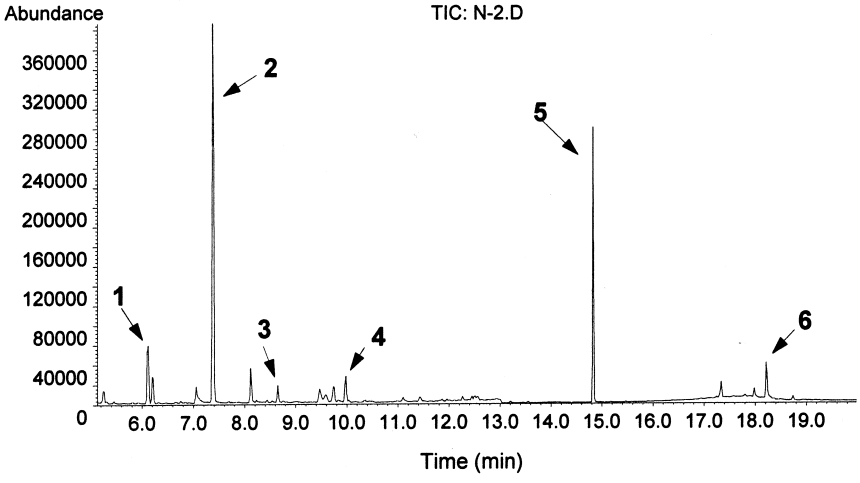

Fig. 4. Selected-ion current chromatogram of hair polyamines as their *N*-EOC-*N*-PFP derivatives from a male subject obtained in the SIM acquisition mode by monitoring three ions for each amine. The start time was programmed from 5.0 for the first group of 12 ions and then from 12.0 min for the second group of six ions with a dwell time of 80 ms for the first group and 120 ms for the second group. GC conditions are described in Fig. 1. Peaks: 1=1,3-diaminopropane; 2=putrescine; 3=cadaverine; 4=1,6-diaminohexane (I.S.); 5=spermidine; 6=spermine.

Table 2
Polyamine levels (ng/g) in hair samples from 11 men and 19 women[a]

Polyamine	Male hair (n=11) Mean±SD[b] (Median, Range)	Female hair (n=19) Mean±SD[b] (Median, Range)
1,3-Diaminopropane	27.38±2.86 (25.77, 21.48–30.68)	15.76±5.15 (14.81, 8.82–24.71)
Putrescine	100.15±16.83 (103.77, 71.69–124.99)	44.42±11.14 (41.03, 19.09–87.09)
Cadaverine	23.25±6.97 (15.58, 11.47–42.71)	27.84±13.47 (24.74, 10.26–73.40)
Spermidine	446.38±111.10 (340.63, 309.05–689.46)	188.09±71.92 (177.88, 96.37–476.77)
Spermine	359.74±144.78 (137.06, 70.28–871.87)	112.44±57.00 (99.99, 21.34–259.01)

[a] Acidic extracts of hair (100 mg) collected from 11 healthy men (aged 28–53) and 19 healthy women (aged 24–57) was subjected to extractive two-phase EOC reaction with subsequent PFP derivatization for the GC–SIM-MS analysis. GC–SIM-MS conditions are described in Table 1.
[b] Standard deviation.

4. Conclusion

A major advantage of the present method is the selective and rapid recovery of polyamines from an aqueous solution by an extractive two-phase EOC reaction using volatile diethyl ether as the organic phase. Moreover, the subsequent PFP derivatization of the remaining active hydrogen in the amino groups enhanced the GC–SIM-MS properties. It enhanced these properties by lowering the detection limits down to 1–10 ng/g hair and allowing simultaneous measurement of hair polyamines with excellent peak shapes, higher responses and shorter analysis time. An extension of the present method for the study of biochemical roles of polyamines in cancer and other pathological cases is underway.

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