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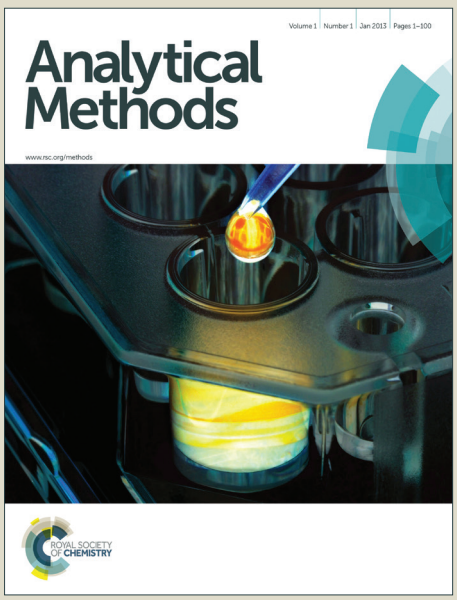

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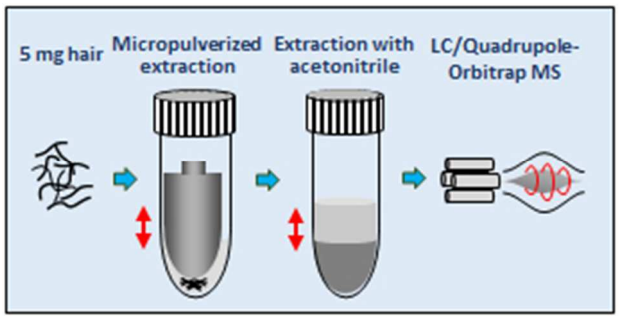

Determination of sedative-hypnotics in human hair by micropulverized extraction and liquid chromatography/quadrupole-Orbitrap mass spectrometry.

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Abstract

A method was developed for quantification of 13 major sedative-hypnotics in hair by liquid chromatography/high-resolution accurate mass spectrometry. A hair sample (5 mg) was micropulverized with 3.0 mol L^{-1} aqueous ammonium phosphate (pH 8.4). Liquid–liquid extraction was carried out twice with acetonitrile, the organic layer was concentrated and then reconstituted with 50 μL of 0.1 % formic acid in 10 % acetonitrile, and then filtered. The filtrate (10 μL) was analyzed with a Q Exactive™ hybrid mass spectrometer coupled with a liquid chromatograph. Using multiplexed selected ion monitoring mode, the quadrupole mass filter of the Q Exactive™ successfully eliminated large interferences, which enabled detection of the small peaks of the analytes. The lower limits of quantification (LLOQ) were 1 pg mg^{-1} for brotizolam, diazepam, *N*-desmethyldiazepam, estazolam, flunitrazepam, nitrazepam, triazolam, ramelteon and zolpidem, and 4 pg mg^{-1} for alprazolam, *N*-desmethylfludiazepam, etizolam and zopiclone. Accuracy and precision of the repeated analyses for all the analytes met FDA guidelines at three concentrations (LLOQ, 100 pg mg^{-1} and 2 ng mg^{-1}). The developed method was applied to hair samples from four patients with sleep disorders. Daily uses of brotizolam (8.8 pg mg^{-1}), ethyl loflazepate

(443 pg mg^{-1} as desmethylfludiazepam), flunitrazepam (46.0 pg mg^{-1}) and zolpidem (10.2 ng mg^{-1}) were detected for Donor A. Occasional uses of etizolam (below the LLOQ) and zolpidem (60.0 pg mg^{-1}) were detected for Donor B. Occasional use of etizolam (4.9 pg mg^{-1}) was detected for Donor C. The concentration of zopiclone in the gray hair of Donor D was >10 ng mg^{-1} in black hair and 187 pg mg^{-1} in white hair.

Introduction

Sedative-hypnotics are commonly abused in drug-facilitated sexual assaults (DFSA). In these cases, hair is sometimes the only suitable sample matrix for drug detection because most drugs will have become metabolized and eliminated from the body before samples are taken. The United Nations Office on Drugs and Crime (UNODC) proposed segmental hair analysis of sedative-hypnotics in their guidelines for the forensic analysis of DFSA in 2011.[1] Although high sensitivity is required for the detection of low-dose benzodiazepines, improvements achieved for electrospray ionization mass spectrometry over the past decade have encouraged the development of analytical methods for sedative-hypnotics in hair.

LC/MS/MS analysis of sedative-hypnotics in hair has been reported by several groups. The first method with acceptable sensitivity was presented by Villain et al.[2] Sixteen sedative/hypnotics were extracted overnight using concentrated phosphate buffer (pH 8.4) and detected with a triple quadrupole mass spectrometer. The calibration range of their method was 0.5–200 pg mg^{-1} using 20 mg of hair. They reported that administration of 10 mg of zolpidem (ZOL) and 6 mg of bromazepam to human volunteers resulted in drug accumulation in hair at 1.8 and 4.7 pg mg^{-1}, respectively. Afterwards, Xiang et al. also proved a segmental hair analysis using the same extraction

medium.[3] Estazolam (EST) was detected at less than 0.5 to 0.61 pg mg^{-1} after administration of 1 mg of EST to human volunteers. Considering these reports, a picogram per milligram level of sensitivity is generally required to detect a single dose of benzodiazepines. When it comes to segmental analysis, the ability to successfully detect drugs in small samples is strongly preferred. We reported that the mass of a 2 cm segment of hair ranged from 0.092 to 0.186 mg.[4] Accordingly, up to 435 pieces of hair would be required for 2 cm segmental analysis in duplicate if 20 mg of hair was used for the analysis. This estimation is roughly compatible with an earlier study that required four sample lots of 100 hairs each.[5] Collection of 400 hairs makes this method invasive, and the sample size should be reduced.

To date, many studies have analyzed sedative-hypnotics in hair. Most of these methods have not achieved acceptable sensitivity for segmental analysis, but they can be used for non-segmental, chronic or overdose cases.[6-13]

In earlier reports, sedative-hypnotics in hair were extracted by solid–liquid extraction with concentrated phosphate buffer [2,3,7] or methanol.[9,10] However, the extraction efficiencies of the drugs from hair have not been evaluated in detail. Therefore, we determined the relative extraction efficiencies of seven psychoactive drugs from an incurred hair sample.[14] Concentrated phosphate buffer (3.0 mol L^{-1}, pH 8.4) provided good extraction of the drugs, whereas methanol did not. Taking these results into consideration, we established a new sample preparation method using micropulverized extraction to shorten the extraction time, and it was successfully applied to the analysis of ZOL in hair with a liquid chromatograph-triple quadrupole mass spectrometer.[14] The method actualized rapid analysis of ZOL in hair with the required sensitivity (LLOQ, 1 pg mg^{-1}) and the sample size requirement was acceptable (10 mg). Although the triple

quadrupole mass spectrometer is thought to be the most suitable for quantification because of its wide linear range, additional structural information about the target molecules is required to improve specificity especially for forensic evidence.

High-resolution accurate mass spectrometry (HRAMS), especially using time-of-flight (TOF) or Orbitrap analyzers, has become widespread in analytical toxicology. TOF and Orbitrap are compatible with atmospheric pressure ionization such as electrospray ionization, and the instrument size and price are suitable for analytical laboratories.[15] The advantage of Orbitrap over TOF is its higher resolution power, which is important for identification. However, TOF is superior to Orbitrap in terms of acquisition speed, which is important for unknown screening.[15] As for toxicological hair analysis, the Orbitrap analyzer has been already applied to the determination of 28 benzodiazepines and metabolites in hair.[7] They employed a sample preparation similar to the method of Villain[2] and an acceptable LLOQ (1–10 pg mg^{-1}) was achieved with an LTQ Orbitrap mass spectrometer, although the sample size requirement (50 mg) was too large to carry out segmental analysis.

In this study, twelve sedative-hypnotics, including benzodiazepines, zopiclone and ramelteon, were analyzed for the first time using our previously established method. Following micropulverized extraction, a quadrupole-Orbitrap hybrid mass spectrometer was used for the first time to identify the analytes. The sample size requirement was reduced to 5 mg. The developed method was validated and verified by application to hair samples from patients with sleep disorders.

Experimental

Materials

Negative control hair samples were obtained from six volunteers (one male and five female). Hair samples were obtained from three male Japanese patients (Donor A, 56 years old, black hair; Donor B, 40 years old, black hair; Donor C, 42 years old, black hair) and one female Japanese patient (Donor D, 65 years old, gray hair), who had been prescribed and had orally ingested sedative-hypnotics for the treatment of sleep disorders. These hair samples and control hair samples were obtained by cutting the hair of the volunteers after obtaining informed consent. The samples were collected at different times and stored in a desiccator at room temperature before analysis. This study was approved by the ethics committee of the National Research Institute of Police Science (Japan). The hair samples were washed with 0.1 % sodium dodecyl sulfate for 3 min, then washed with water and methanol for 3 min each using a sonicator, and dried in air. The washed hair samples were cut into small pieces (2–3 mm) with scissors.

Authentic standards of alprazolam (ALP), brotizolam, diazepam (DIA), EST, etizolam (ETI), flunitrazepam (FLUN), nitrazepam, *N*-desmethylfludiazepam (DMF, a metabolite of ethyl loflazepate) and triazolam (TRI) were purchased from Wako Pure Chemical Industries (Osaka, Japan). ZOL was purchased from Sigma-Aldrich (St. Louis, MO). ALP-d_5, *N*-desmethyldiazepam (DMD), DIA-d_5, DMD-d_5, DMF-d_4, EST-d_5, FLUN-d_7, nitrazepam-d_5, ramelteon, TRI-d_4, ZOL-d_6, zopiclone (ZOP) and ZOP-d_4 were purchased from Cerilliant (Round Rock, TX).

Water (LC/MS grade) acetonitrile (LC/MS grade), ammonium phosphate, ammonium formate (1 mol L^{-1}), and formic acid (LC/MS grade) were purchased from Wako Pure Chemical Industries. Formic acid in water (LC/MS grade, 0.1 %, v/v) was purchased from Thermo Fisher Scientific (Waltham, MA).

Sample preparation

A washed hair sample (5 mg, not pulverized) was precisely weighed into a screw-cap polypropylene tube (2 mL volume, product number 1392-200, Fukae Kasei, Kobe, Japan). A stainless steel cylinder (product number 0064593-000, Taitec, Koshigaya, Japan), 10 μL of an aqueous internal standard mixture (200 ng mL^{-1} each of the free bases of the 10 deuterium analogues detailed above) and 200 μL of 3.0 mol L^{-1} (45 % w/v) ammonium phosphate (PB, pH 8.4 adjusted with aqueous ammonia) were added. The tube was capped tightly and cooled on a cool rack (IsoFreeze Flipper, product number 5610-40, Scientific Specialties Incorporated, Lodi, CA) for 3 min, and then the tube was shaken vertically for 2.5 min at 42 Hz with a pulverizer (μT-12, Taitec). The cooling and pulverizing cycle was repeated three more times. The stainless steel cylinder was transferred to a Safe-Lock polypropylene tube (2 mL volume, Eppendorf, Hamburg, Germany) and washed with 100 μL of PB using a vortex mixer. The washing liquid was placed back into the screw-cap tube, and 200 μL of acetonitrile was added. The tube was capped and shaken for 3 min at 53 Hz with the pulverizer (μT-12). After centrifugation (20,000 ×*g*, 3 min), the acetonitrile layer was transferred to a 2 mL Safe-Lock tube. This liquid–liquid extraction was repeated again, and the acetonitrile fractions were combined and evaporated to dryness under a nitrogen gas stream at 50 °C. The residue was reconstituted with 50 μL of water–acetonitrile (9:1, v/v) containing 0.1 % formic acid, and the solution was filtered with a 0.2 μm polytetrafluoroethylene microporous membrane (product number CIPT02, Ciro Manufacturing, Boca Raton, FL, USA). Ten milliliters of the standard solutions (0.50, 2.0, 10, 50, 200, 1000 and 5000 ng mL^{-1}) in water–acetonitrile (9:1, v/v) containing 0.1 % formic acid were added before pulverizing when preparing calibration and

positive control samples.

LC/HRAMS measurements

LC/HRAMS was performed using an Ultimate 3000 liquid chromatograph and a Q Exactive™ mass spectrometer (Thermo Fisher Scientific). A Cadenza CD-C18 reverse-phase column (Imtakt, Kyoto, Japan, 150 × 3 mm, 3 μm) was used for separation at 40 °C. The mobile phase, delivered at a flow rate of 0.3 mL min^{-1}, was 0.1 % formic acid in water (A)–acetonitrile (B). The proportion of solvent B in the mobile phase was changed over a linear gradient as follows: 0–2 min 10 % B, 2–10 min 10–35 % B, 10–25 min 35 % B, 25–30 min 35–90 % B, 30–35 min 90 % B, and 35–45 min 10 % B. The sample injection volume was 10 μL. The post-column flow was connected to the mass spectrometer from 8 to 35 min after injection.

Heated electrospray ionization in positive ion mode was used to produce protonated molecules of the analytes. The experimental parameters were optimized as follows: spray voltage 3.5 kV, vaporizer temperature 300 °C, capillary temperature 350 °C, sheath gas 40 (arbitrary unit, a.u.), auxiliary gas 10 (a.u.), sweep gas 0 (a.u.) and S-lens RF level 40. Eight multiplexed timed selected ion monitoring (SIM) was used for isolation and accumulation of ions with quadrupole and C-trap at a width of *m/z* 2 as described in Table S1. The Orbitrap mass analyzer was used for detection at a resolving power of 140,000 (nominal value at *m/z* 200). The acquisition range was *m/z* 150–400. External mass calibration was carried out daily as recommended by the manufacturer. Data analyses were carried out with Qual Browser software (Thermo Fisher Scientific).

Calibration curves

Calibration curves were constructed by plotting eight concentrations (1.0, 4.0, 20, 100, 400, 2000, 5000 and 10,000 pg mg^{-1}) in duplicate at a mass tolerance of 10 ppm. The calibration curve equations were calculated with Quan Browser software (Thermo Fisher Scientific). The corresponding internal standards are described in Table S1.

Validation study

Control samples used for evaluation of the accuracy and precision at low (1 and 4 pg mg^{-1}), moderate (100 pg mg^{-1}) and high (2,000 pg mg^{-1}) concentrations were prepared by spiking 10 μL of the appropriate standard solutions to a blank hair sample before micropulverized extraction. Six blank hair samples were analyzed for the evaluation of false positives.

Review of modified sample preparation

To evaluate the effects of cooling prior to pulverization and collection of the homogenate on the stainless steel cylinder after pulverization, the hair specimen of Donor B was analyzed repeatedly with and without these modifications (n = 4 each). The absolute peak area of ETI was compared.

Results and discussion

Sample preparation

In toxicological hair analysis, extraction efficiency directly affects quantitative values, because the standards that are used to construct calibration curves are always prepared by spiking the analytes just before extraction, and complete extraction from unknown samples is the premise of the analysis. Despite the importance of extraction efficiency, it

is virtually impossible to determine absolute extraction efficiency because the true concentration of the analyte in incurred or fortified hair samples is unknown. Considering these circumstances, the sample preparation method that we developed previously for the efficient extraction of 7 psychoactive drugs (7-aminoflunitrazepam, amitriptyline, nortriptyline, DMF, FLUN, mianserin, and ZOL) was used for this study with the following minor modification.[14] When pulverizing, the tube had become hot because of friction inside the tube. This increased the internal air pressure and caused leakage of the suspension. Therefore, in the present study, the pulverization process was completed in four intervals and a cooling period was used before each pulverization. In addition, the homogenate on the stainless steel cylinder after pulverization was recovered to maximize the total yield. These modifications increased the absolute peak area of ETI by 20±12 % (n = 4). Although the improvement in the total recovery was not significant, the cooling process is recommended to avoid accidental leakage of the homogenate during pulverization.

Trace analysis with Q Exactive™

A broad intra-scan dynamic range is required to detect small peaks in the presence of a large matrix peak when acquiring mass spectrometry data in scan mode. The nominal intra-scan dynamic range of the Q Exactive™ is 5000:1, and we have demonstrated that it cannot be used for trace analysis in some situations (Fig. S1). Compounds A and B were detected as large interferences and they were overlapped with the peaks of TRI, ETI and ALP under the chromatographic conditions. Without the hair matrix, ions for analytes equivalent to 1 pg mg^{-1} were clearly detected. However, with the hair matrix together, the peaks of these analytes were masked by the peaks of Compound A and B,

which were five orders of magnitude higher than those of the analytes. This was over the limit of intra-scan dynamic range. Thus a slow mobile phase gradient in LC and multiplexed SIM in MS were used to separate the analytes from the matrix ions. The pH of the mobile phase was changed to elute Compound A, TRI and ETI at different times, as all of these compounds had nominal *m/z* values of 343. Compounds A and B were not identified. They were also found in low concentrations in a blank analysis, and could not be eliminated by washing. Unfortunately, because the SIM condition should be scheduled along with the retention time of the analytes, this meant retrospective analysis of unknown substances could not be performed, which is a major merit of HRAMS.

Quantitative analysis

A typical set of extracted ion chromatograms for hair spiked at 1.0 and 4.0 pg mg^{-1} is shown in Fig. 1. Peaks for ZOP and ALP were not observed at 1 pg mg^{-1}, but all the peaks of the analytes were observed at 4.0 pg mg^{-1}. DIA generated the smallest number of data points per peak owing to the tight peak shape. There were 15-16 data points at 10% of peak height according to the data used for the inter-day quality control at 4 pg mg^{-1} (n = 5). Holčapek et al. recommended that at least 12–15 points per peak should be used for good reproducibility and precision in LC/MS quantification.[16] Accordingly, this acquisition condition was acceptable in terms of the number of data points, although a maximum resolving power of 140,000 was employed.

The equations for the calibration curve and the coefficient of determination are described in Table S1. DMD and DIA could not be calibrated using linear regression because of saturation of the signal intensity at high concentrations. Therefore, quadratic regression was employed for these compounds. Six black hair samples were tested, and

the peaks corresponding to the analytes were not observed (data not shown).

The accuracy and precision were determined at low (1.0 and 4.0 pg mg^{-1}), moderate (100 pg mg^{-1}) and high (2,000 pg mg^{-1}) concentrations (Table 1). According to the criterion for the LLOQ (≤ 20 % accuracy and precision) form the U.S. Food and Drug Administration,[17] the LLOQ of ALP, DMF, ETI and ZOP was 4 pg mg^{-1} and LLOQ of the other analytes was 1 pg mg^{-1}. Comparing the sensitivity on an absolute scale (LLOQ×amount of sample required), the present method is found to be superior to all previous methods (Table 2) despite the shorter extraction time. However, the comparison is not justified as the sample preparation method in this study involves the latest instruments unlike other methods. This is because it has high extraction efficiency, high specificity (from the HRAMS) and high sensitivity (from the quadrupole-Orbitrap mass spectrometer).

Analysis of hair samples from sleep disorder patients

Four hair specimens obtained from patients of with sleep disorders (Donors A–D) were analyzed (Table 3). Daily uses of brotizolam (8.8 pg mg^{-1}), ethyl loflazepate (443 pg mg^{-1} as DMF), FLUN (46.0 pg mg^{-1}) and ZOL (10.2 ng mg^{-1}) were detected for Donor A. Occasional uses of ETI and ZOL (60.0 pg mg^{-1}) were detected for Donor B although the concentration of ETI was below LLOQ. Occasional use of ETI (4.9 pg mg^{-1}) was detected for Donor C, and daily use of ZOP for Donor D. For gray-haired Donor D, the concentration of ZOP was >10 ng mg^{-1} in black hair and 187 pg mg^{-1} in white hair. The concentration of ZOP in the black hair was two orders of magnitude higher than that in white hair, which is consistent with our previous observations for ZOL[14] and confirms the mechanism of incorporation of ZOP involves melanin.

Conclusions

Compared with earlier methods, the present method requires a smaller amount of sample and is complete within a shorter analytical time. This is because of the rapid and effective sample preparation by micropulverized extraction and the sensitive and selective detection with a state-of-the-art Fourier-transform mass spectrometer. These method improvements will be helpful for detecting administration of sedative-hypnotics from hair samples of victims of DFSA.

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Table 1 Accuracy and precision of the spiked samples

Analytes	Accuracy								Precision (relative standard deviation)							
	1 pg mg^{-1}		4 pg mg^{-1}		100 pg mg^{-1}		2000 pg mg^{-1}		1 pg mg^{-1}		4 pg mg^{-1}		100 pg mg^{-1}		2000 pg mg^{-1}	
	Intra-d	Inter-d	Intra-d	Inter-d	Intra-d	Inter-d	Intra-d	Inter-d	Intra-d	Inter-d	Intra-d	Inter-d	Intra-d	Inter-d	Intra-d	Inter-d
alprazolam (ALP)	—	—	−0.37	0.46	7.31	10.63	−4.33	2.70	—	—	14.92	14.02	2.18	4.09	1.28	4.78
brotizolam (BROT)	16.9	20.18	−0.03	7.91	−7.92	5.12	−3.35	6.13	11.58	8.75	5.80	8.71	4.70	3.68	4.20	8.03
N-desmethyldiazepam (DMD)	9.28	7.04	11.04	8.02	−0.06	0.08	−7.14	−7.78	3.17	7.69	4.27	2.41	0.87	2.03	2.34	2.63
N-desmethylfludiazepam (DMF)	28.31	33.04	10.40	10.45	−0.46	−0.39	−9.15	−3.74	4.94	2.03	3.92	2.55	1.37	0.6	1.79	6.10
diazepam (DIA)	−5.22	−7.57	6.35	8.52	10.64	9.73	−10.48	−9.46	9.70	6.49	6.89	5.17	3.00	4.37	3.11	5.98
estazolam (EST)	−0.64	1.91	3.04	3.74	−2.13	−0.85	−4.79	−2.13	7.67	12.43	3.53	1.50	1.12	2.63	2.24	2.14
etizolam (ETI)	33.23	36.26	10.93	15.71	4.16	10.89	1.86	6.66	6.13	1.89	5.88	7.57	4.70	2.45	4.73	2.64
flunitrazepam (FLUN)	−6.80	−5.77	0.55	7.82	2.08	4.75	−0.45	1.61	11.51	8.61	6.16	6.16	2.62	3.22	1.49	3.14
nitrazepam (NIT)	−9.43	−5.00	12.00	14.06	6.48	8.72	−5.29	−0.74	3.18	2.63	2.42	1.84	1.95	1.98	1.80	4.46
ramelteon (RAM)	4.63	9.86	14.52	13.66	12.39	8.54	−1.71	−2.06	4.46	5.04	4.49	3.20	3.30	4.53	2.40	3.18
triazolam (TRI)	0.07	−1.28	10.07	9.36	6.78	6.99	−7.06	−7.22	10.09	4.64	3.42	3.73	1.06	1.66	1.44	1.10
zolpidem (ZOL)	−5.08	−6.69	1.48	4.27	3.36	5.68	−6.56	−4.03	10.53	2.09	5.64	2.63	3.43	2.59	1.83	3.34
zopiclone (ZOP)	—	—	3.37	6.76	−2.30	−0.65	−8.52	−6.11	—	—	2.63	2.67	1.09	1.66	1.07	2.26

n = 5 (intraday), n = 1 each for 5 days (interday). —: Peak not found. Intra-d: intraday. Inter-d: interday.

Table 2 Comparison of LC/MS methods for quantification of sedative-hypnotics in hair

Reference	Extraction time	Mass analyzer*	Model of mass spectrometer	Sample amount (mg)	LLOQ (pg mg^{-1})			LLOQ × sample amount (pg)		
					DIA	FLUN	ZOL	DIA	FLUN	ZOL
Villain et al. (2005) [2]	overnight	Triple Q	Waters Quattro Micro	20	1	–	0.5	20	–	10
Nielsen et al. (2010) [6]	18 h	TOF	Waters LCT Premier XE	10	50	50	50	500	500	500
Vogliardi et al. (2011) [7]	overnight	LIT-Orbitrap	Thermo LTQ Orbitrap	50	5	5	–	250	250	–
Xiang et al. (2011) [3]	1 h	Triple Q	AB Sciex API4000	20	1	1	–	20	20	–
Favretto et al. (2011) [8]	5 min	LIT-Orbitrap	Thermo LTQ Orbitrap	2.5	200	200	100	500	500	250
Kim et al. (2011) [9]	16 h	Triple Q	AB Sciex 3200 Qtrap	10	25	25	25	250	250	250
Rust et al. (2012) [10]	3 h	Triple Q	AB Sciex 5500 Qtrap	30	1.6	10	1.6	48	300	48
Lendoiro et al. (2012) [11]	overnight	Triple Q	Waters Quattro Micro	50	5	5	5	250	250	250
Wietecha-posłuszny et al. (2013) [12]	10 min	TOF	Bruker MicrOTOF-QII	45	45	–	–	2970	–	–
Kronstrand et al. (2013) [13]	overnight	Q-TOF	Agilent 6540	20	50	–	50	1000	–	1000
Miyaguchi (2013) [14]	10 min	Triple Q	Thermo LTQ Orbitrap XL	10	–	–	1	–	–	10
Montesano et al. (2014) [18]	18 h	Triple Q	Waters TQ Detector	10	2	5	2	20	50	20
Present study	10 min	Q-Orbitrap	Thermo Q Exactive	5	1	1	1	5	5	5

* Q, quadrupole; TOF, time-of flight; LIT, linear ion trap.

Table 3 Analysis of hair samples from patients with sleep disorders

Donor	Sex	Age	Color	Exposure	Concentration[a]
A	Male	56	Black	BROT (0.25 mg day^{-1})	8.8 pg mg^{-1}
				Ethyl loflazepate (2 mg day^{-1})	443 pg mg^{-1} as DMF
				FLUN (2 mg day^{-1})	46.0 pg mg^{-1}
				ZOL (10 mg day^{-1} as tartrate)	10.2 ng mg^{-1}
B	Male	40	Black	ETI	<4.0 pg mg^{-1}
				ZOL	60.0 pg mg^{-1}
C	Male	42	Black	ETI	4.9 pg mg^{-1}
D	Female	69	Black[b]	ZOP (3.75-7.5 mg day^{-1})	>10 ng mg^{-1}
			White[b]		187 pg mg^{-1}

[a] $n = 2$. [b] The gray-haired sample was separated to black and white before analysis.

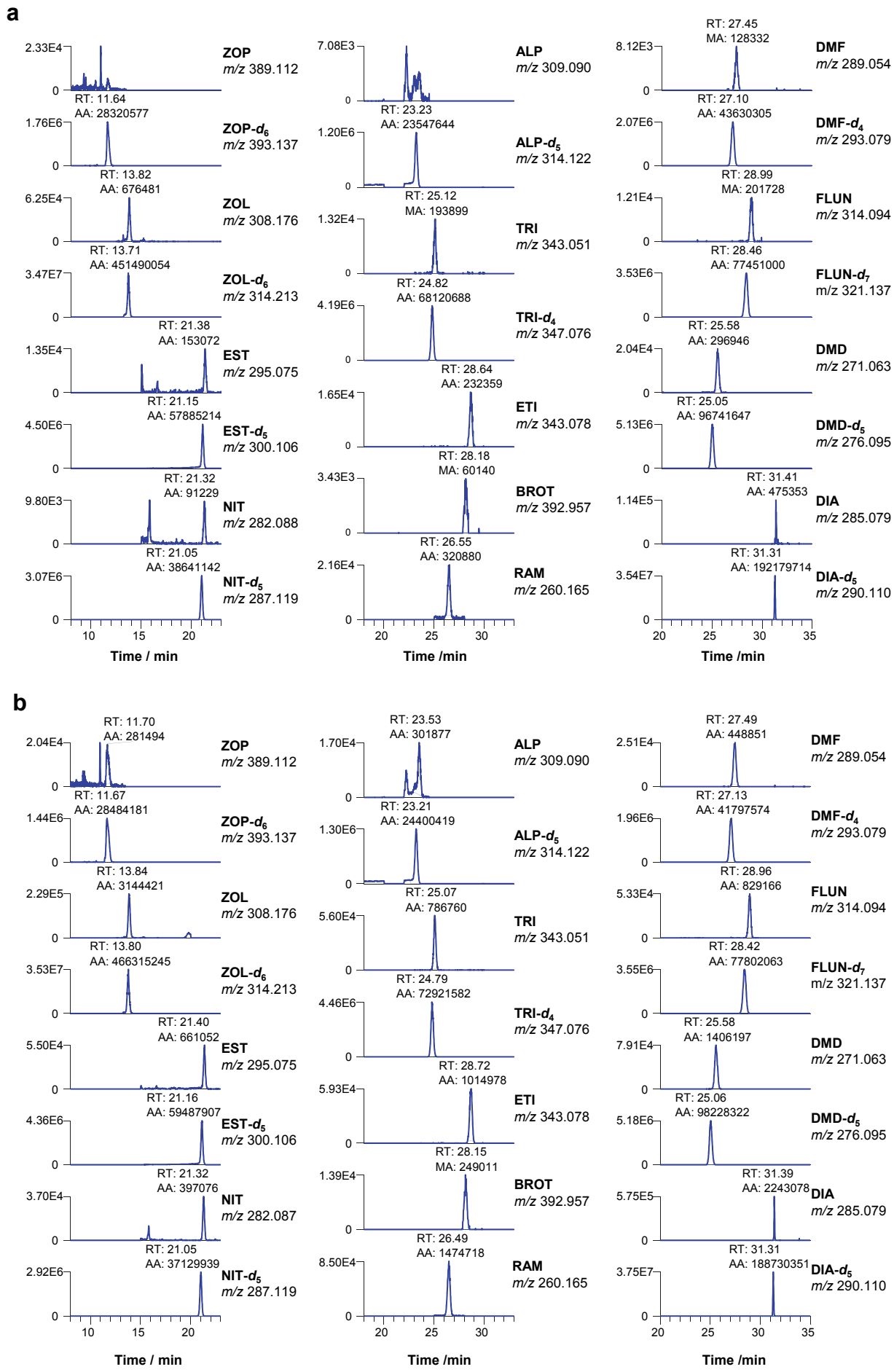

Fig. 1