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Analysis of 20 Free Amino Acids in a Shanghai Drinking Water Treatment Plant Using Solid-Phase Extraction Coupled with Liquid Chromatography-Tandem Mass Spectrometry

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Abstract: Numerous studies have demonstrated that amino acids serve as important precursors of odor-causing disinfection byproducts (O-DBPs), which may lead to taste and odor issues in drinking water after disinfection. Monitoring these O-DBP precursors is critical for safeguarding public health and mitigating odor-related risks. Therefore, this study conducted long-term monitoring of free amino acids (FAAs) levels in a drinking water treatment plant (DWTP) in Shanghai. A highly sensitive analytical method based on solid-phase extraction coupled with liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS) was developed, demonstrating excellent linearity and precision. The method achieved quantification limits (LOQs) ranging from 0.02 to 0.55 nM, enabling the detection of trace-level amino acids. In the raw water, the total concentration of free amino acids ranged from 7 to 70 μg/L, with the highest level observed in March (69.66 μg/L) and the lowest in August (7 μg/L). Arginine (Arg), valine (Val), aspartic acid (Asp), serine (Ser), glutamic acid (Glu), glycine (Gly), and alanine (Ala) were identified as the dominant FAAs in the raw water of the DWTP, with concentrations significantly higher than those of other FAAs.

Keywords: Free amino acids, disinfection byproduct precursor, solid phase extraction, LC-MS/MS.

1. Introduction

Free amino acids (FAAs) are indispensable fundamental molecules in life activities[1]. They serve not only as core building blocks for proteins but also as critical substrates for maintaining organismal growth, metabolic balance, and overall vitality. In natural water bodies, amino acids originate from diverse sources, with the 20 most common FAAs primarily derived from metabolic processes of organisms such as algae and wastewater discharge from human activities. The concentration of FAAs in natural waters is closely linked to algal activity, typically reaching higher levels during periods of algal blooms[2]. FAAs generally exist at concentrations in the μg/L range in natural waters, representing a significant component of dissolved organic nitrogen (DON), accounting for 15%~35% of DON in water sources[3-5]. Previous studies detected FAAs in East Taihu Lake, where concentrations of 20 FAAs ranged from 0 to 9 μg/L. Most FAAs peaked in autumn, with a total FAAs concentration of approximately 30 μg/L, compared to 0 ~3 μg/L in other seasons[6]. Reported data from Paris's three drinking water treatment plants showed FAAs concentrations in raw water ranging from 0 to 19 μg/L[7], while several plants in western France reported levels between 0 and 30 μg/L[8]. Generally, the concentrations of combined amino acids (e.g., proteins and peptides) are nearly tenfold higher than those of FAAs.

FAAs serve as pivotal precursors of odorant disinfection byproducts (O-DBPs)[9, 10]. More than ten FAAs, including phenylalanine (Phe), leucine (Leu), and isoleucine (Ile), are known to generate O-DBPs upon chlorination[6]. Studies indicate that certain FAAs produce volatile aldehydes, nitriles, and chloroaldimines (R-CH=NCl) during chlorination[11, 12]. These compounds are chemically stable and exhibit low odor thresholds (in the μg/L range)[11, 12]. For instance, Brosillon et al.[7] detected 12 aldehyde, nitrile, and chloroaldimine byproducts derived from Phe, Leu, Ile, and valine in the effluent and distribution systems of Paris's three DWTPs, with concentrations ranging from 0 to 38 nM. This observation suggests that FAAs may react with disinfectants to form volatile organic compounds, thereby contributing to persistent odor issues in treated drinking water systems post-disinfection. Cai et al.[13] demonstrated that combined amino acids (e.g., proteins and peptides) exhibit lower odor-forming potential than FAAs during chlorination and chloramination, highlighting the importance of focusing on FAAs in water treatment processes.

However, current research on detection methods for FAAs in natural water bodies remains limited. Due to the small molecular weight and strong polarity of amino acids, traditional analytical methods face significant challenges. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is widely used for the sensitive and selective detection of trace compounds due to its advantages of rapidity, high efficiency, and broad applicability[14]. LC-MS/MS equipped with an electrospray ionization (ESI) source operates at lower ionization temperatures, making it suitable for thermally unstable compounds. However, its ionization efficiency is relatively low, limiting its ability to sensitively detect non-charged compounds[15]. FAAs are protonated and exist in ionic form under acidic conditions, making them compatible with ESI ionization. Klepacki et al.[16] successfully quantified 24 FAAs in human plasma using an LC-ESI-MS/MS method.

The concentrations of FAAs in natural water bodies are typically very low, requiring the integration of preconcentration techniques. Solid-phase extraction (SPE) is a well-known preconcentration method capable of achieving sufficient sample concentration for environmental analyses at the ng/L level[17, 18]. Nevertheless, SPE is costly, time-consuming, and prone to analyte loss during loading or washing steps, resulting in generally low recovery rates[19].

2. Materials and Methods

2.1. Reagents and Instruments

The 20 FAAs standards were all purchased from Sigma-Aldrich (standard 99%, USA).concentrated ammonia solution (analytical grade), Phe-d5 (≥98 atom%), ammonium formate (HPLC grade), formic acid (MS grade), methanol (HPLC grade), and acetonitrile (HPLC grade) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Concentrated hydrochloric acid (analytical grade) was acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ultrapure water (18.2 MΩ·cm resistivity) was produced using a Millipore Milli-Q water purification system (USA). Benzenesulfonic acid-modified polymer cation-exchange SPE cartridges (Chromabond HR-XC, 3 mL/500 mg, Macherey-Nagel GmbH & Co. KG, Germany) was employed for amino acid enrichment. An Agilent ZORBAX Eclipse Plus C18 column (2.1 × 150 mm, 3.5 μm, USA) was selected for chromatographic separation.

Solid phase extraction was performed using a 12-position Visiprep DL manifold (Supelco, USA). Sample extracts were concentrated under nitrogen using an nitrogen evaporator (Yiheng Instrument Co., Ltd, Shanghai, China) maintained at constant temperature (40℃). LC-MS/MS (SCIEX 6500+, SCIEX, USA) was used for separation and quantification of the FAAs.

2.2. Sample Information

This water plant uses the water from Qingcaosha Reservoir as its water source. Qingcaosha Reservoir is located on Qingcaosha, an alluvial sandbank to the northwest of Changxing Island in Shanghai. It covers a total area of 66.26 square kilometers and has an effective storage capacity of 438 million cubic meters. It is one of the main drinking water sources in Shanghai. This study collected samples and conducted analyses on this water plant from December 2023 to November 2024.

2.3. Solid phase extraction Procedure

Filter aqueous samples through 0.45 μm hydrophilic membrane filters and acidify to pH 1.3 using concentrated hydrochloric acid, then spike with 100 nM Phe-d5 as an internal standard for recovery correction. Proceed by conditioning the SPE cartridge with 2.5 mL methanol and pre-equilibrating it with 4.5 mL of 0.1 M hydrochloric acid. Connect the sample reservoir to the SPE cartridge using a large-volume autosampler, then load the sample while maintaining a vacuum-controlled flow rate of 1.5 mL/min (±0.2 mL/min). Wash the cartridge with 2.5 mL of washing solution (4.5% methanol/95.5% 0.1 M HCl, v/v), then elute the target compounds with 5 mL of methanolic ammonia solution (5% ammonia in methanol). Concentrate the eluate to dryness under a gentle nitrogen stream at 40°C, and finally reconstitute the residues in 1 mL of 30% aqueous methanol (v/v).

All samples obtained from solid-phase extraction (SPE) may be stored temporarily at -20°C and should be analyzed by LC-MS/MS within 48 hours.

3. Results and Discussion

3.1. Optimisation of tandem mass spectrometry conditions for additional analytes

The key mass spectrometry parameters affecting detection accuracy comprise two components: ion source parameters and compound-specific mass spectrometry parameters. The optimized ion source parameters were as follows: ion spray voltage 5.0 kV, ion source temperature 550°C, curtain gas 30 psi, nebulizer gas 55 psi, auxiliary heating gas 40 psi, and collision gas 8 psi. The optimized mass spectrometry parameters for the target amino acids are presented in Table 1.

Table 1. Spectrometry Parameters of FAAs

FAAs		Q1 (m/z)	Q3 (m/z)	CE (V)	DP (V)
Ala	QNT	90	44	15	40
	QL	90	45	32	40
Arg	QNT	175	70	35	19
	QL	175	116	20	19
Asn	QNT	133	74	21	40
	QL	133	87	12	40
Asp	QNT	134	74	19	40
	QL	134	88	18	40
Gln & Lys	QNT	147	84	23	40
	QL	147	130	14	40
Glu	QNT	148	84	17	40
	QL	148	56	29	40
Gly	QNT	76	30	19	40
	QL	76	31	32	40
His	QNT	156	110	19	40
	QL	156	83	35	15
Leu & Ile	QNT	132	69	23	30
	QL	133	86	15	30
Met	QNT	150	104	15	40
	QL	150	133	14	13
Orn	QNT	133	70	25	10
	QL	133	116	13	10
Phe	QNT	166	120	19	30
	QL	166	103	38	30
Phe-d5	QNT	171	125	19	30
	QL	171	154	14	15
Pro	QNT	116	70	21	40
	QL	116	43	51	40
Ser	QNT	106	60	15	40
	QL	106	88	13	10
Thr	QNT	120	103	18	40
	QL	120	77	25	40
Trp	QNT	205	188	15	40
	QL	205	146	18	40
Tyr	QNT	188	91	55	14
Val	QNT	118	55	19	13
	QL	118	72	45	45

Note. QNT = quantitative group; QL = qualitative group; CE= Collision energy; DP= Declustering potential; Ala = Alanine; Arg = Arginine; Asn = Asparagine; Asp = Aspartic acid; Gln = Glutamine; Glu = Glutamic acid; Gly = Glycine; His = Histidine; Leu = Leucine; Ile = Isoleucine; Met = Methionine; Orn = ; Phe = Phenylalanine; Pro = Proline; Ser = Serine; Thr = Threonine; Trp = Tryptophan; Tyr = Tyrosine; Val = Valine.

3.2. Chromatographic optimization

The optimization of chromatographic conditions primarily involved the selection of mobile phases, gradient settings, and flow rate. The optimal mass spectrometric response and chromatographic peak shapes were achieved using 1.15% aqueous formic acid as mobile phase A and acetonitrile containing 0.35% formic acid as mobile phase B.

The addition of ammonium formate to the mobile phase enabled the separation of isomeric compounds such as Leu and Ile. However, the concentration of ammonium formate should not exceed 80 mM, as higher concentrations may induce ion suppression and reduce column longevity[20]. In this study, the addition of 20 mM ammonium formate to mobile phase A yielded optimal separation efficiency, successfully resolving Leu and Ile. Nevertheless, Gln and Lys, which share identical molecular weights, exhibit similar physicochemical properties and produce identical fragment ions, could not be effectively separated under the tested conditions.

Column temperature significantly influences separation efficiency. In this experiment, the column oven was maintained at 40°C. An excessive flow rate resulted in excessively narrow peaks, while a low flow rate led to peak broadening. The optimal peak shape was achieved at a flow rate of 0.3 mL/min.

Higher organic solvent proportions were observed to induce strong ion suppression and elevated background noise, likely due to adverse effects on amino acid ionization and separation efficiency. Systematic adjustment of the organic phase ratio revealed that maintaining the organic phase below 20% minimized background noise and stabilized the baseline. Consequently, the organic phase proportion was restricted to less than 20% during analytical runs, though periodic column washing with a high organic phase (>90%) was implemented to remove residual contaminants.

The final optimized gradient elution program (Table 2) achieved complete elution of all 21 amino acids (including Phe-d5) within 9 min. Extracted ion chromatograms (XIC) obtained from the analysis are presented in Figure 1.

Table 2. mobile phase gradient

Time (min)	A (%)	B (%)	Flow rate (mL/min)
0.0	95	5	0.3
2.0	95	5	0.3
7.0	80	20	0.3
7.2	80	20	0.3
7.3	95	5	0.3
9.0	95	5	0.3

3.3. 3.3 Optimisation of the solid-phase extraction procedure

The charge states of amino acids vary significantly with pH, critically influencing their retention on strong cation exchange (SCX) SPE cartridges. Systematic evaluation of pH effects on recovery rates (Table 3) revealed that acidification to pH 1.3 substantially enhanced amino acid recovery. For instance, Leu recovery increased from 45.33% at neutral pH to 126.8% at pH 1.3. This phenomenon is attributed to the protonation behavior of amino acids: all target compounds exhibited net positive charges at pH 1.3, as their isoelectric points (PI) ranged from 2.77 to 6. Under these conditions, protonation of amine groups coupled with suppressed carboxyl group dissociation generated dominant cationic species, thereby strengthening electrostatic interactions with the negatively charged SCX sorbent and improving retention efficiency.

The linearity, limits of detection (LOD), and limits of quantification (LOQ) of the final SPE-LC-MS/MS method are summarized in Table 4. The method demonstrated excellent linearity with LOQs ranging from 0.02 to 0.55 nM, indicating its suitability for trace-level analysis.

Table 3. The effect of pH on the recovery yield of FAAs

FAAs	Recovery (%)		
	pH=1.3	pH=3.0	pH=7.0
Ala	94.53	17.26	3.37
Arg	21.21	2.43	0.60
Asn	118.00	132.50	68.89
Asp	10.04	5.42	0.10
Gln & Lys	25.42	19.91	11.28
Glu	9.85	6.63	3.34
Gly	85.29	21.73	2.97
His	59.02	96.00	68.21
Leu	126.80	132.20	45.33
Ile	130.10	158.40	63.47
Met	68.06	8.44	2.87
Orn	79.60	93.79	42.95
Phe	111.80	110.00	74.86
Phe-d5	101.40	75.75	43.36

Table 4. Linearity, limit of detection (LOD), and limit of quantification (LOQ) of the SPE-LC-MS/MS method

FAAs	R^2	Linear range (nM)	LOQ (nM)	LOD (nM)
Ala	0.9994	0.2~100	0.13	0.04
Arg	0.9954	0.2~100	0.09	0.03
Asn	0.9996	0.2~100	0.13	0.04
Asp	0.9988	0.2~100	0.16	0.05
Gln & Lys	0.9996	0.2~100	0.14	0.04
Glu	0.9995	0.2~100	0.18	0.06
Gly	0.9949	0.2~100	0.79	0.24
His	0.9945	0.2~100	0.11	0.03
Leu	0.9978	1~100	0.15	0.04
Ile	0.9992	0.2~100	0.43	0.13
Met	0.999	0.2~100	0.06	0.02
Orn	0.9943	0.2~100	0.18	0.05
Phe	0.9998	0.2~100	0.08	0.02
Phe-d5	0.9991	0.2~100	0.18	0.05
Pro	0.9886	0.2~100	0.11	0.03
Ser	0.9994	0.2~100	0.25	0.08
Thr	0.9990	0.2~100	0.09	0.03
Trp	0.9994	0.2~100	0.13	0.04
Tyr	0.9979	0.2~100	0.11	0.03
Val	0.9983	1~100	1.84	0.55

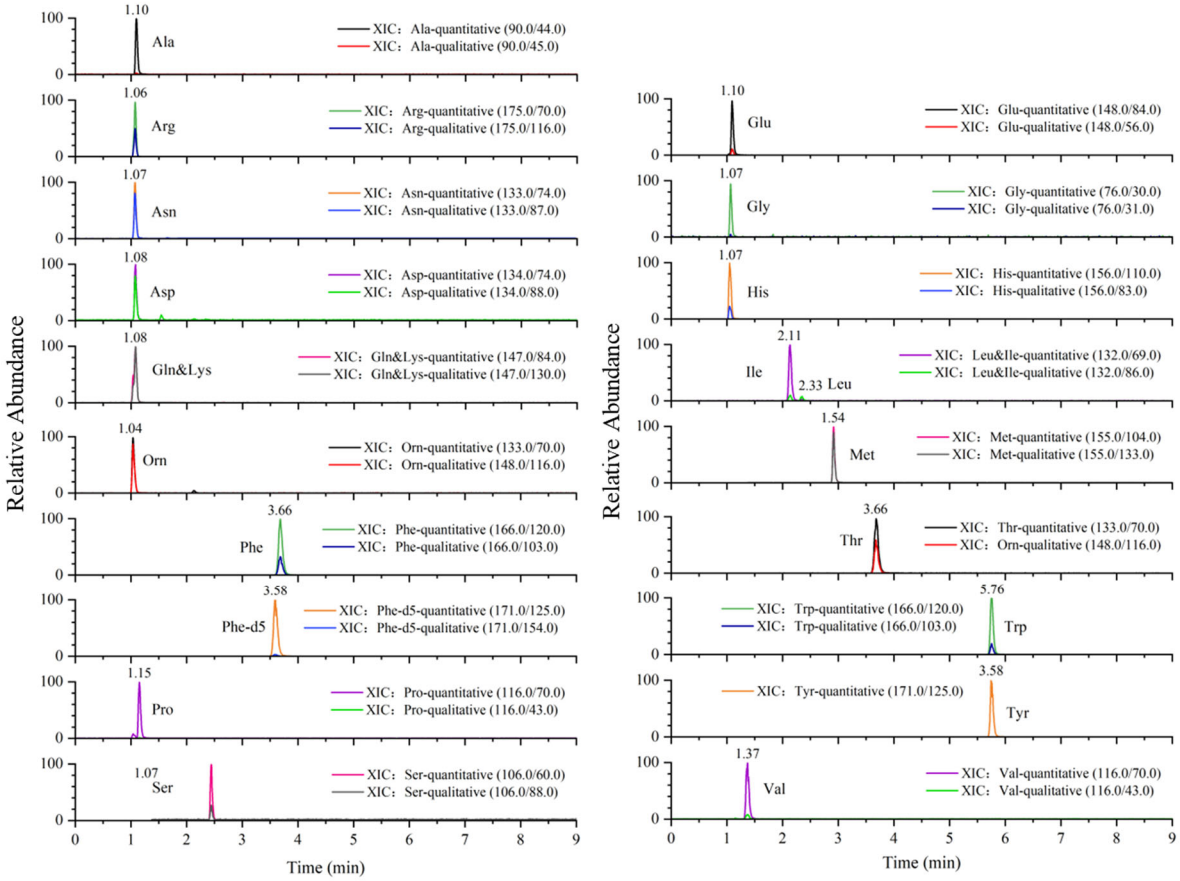

Figure 1. Extracted ion chromatograms (XICs) of 21 FAAs

3.4. Occurrence levels of FAAs in raw water of the DWTP

The figure 2 shows the total concentration of FAAs in the raw water ranged from 7 to 70 μg/L, with a monthly average of 20.05 μg/L. The peak concentration occurred in March (69.66 μg/L), while the lowest was recorded in August (7 μg/L).The primary source of amino acids in drinking water is algal metabolites. In March, the onset of spring and rising temperatures triggered algal blooms, evidenced by visibly high algal concentrations in the raw water, leading to elevated amino acid levels. As the algae entered senescence and reservoir algae control measures were implemented, algal concentrations were effectively maintained at lower levels, resulting in subsequent decreases in FAAs concentrations. During July and August, increased rainfall and the flood season in southern rivers diluted amino acid concentrations, consistent with findings by Chu et al.[21].

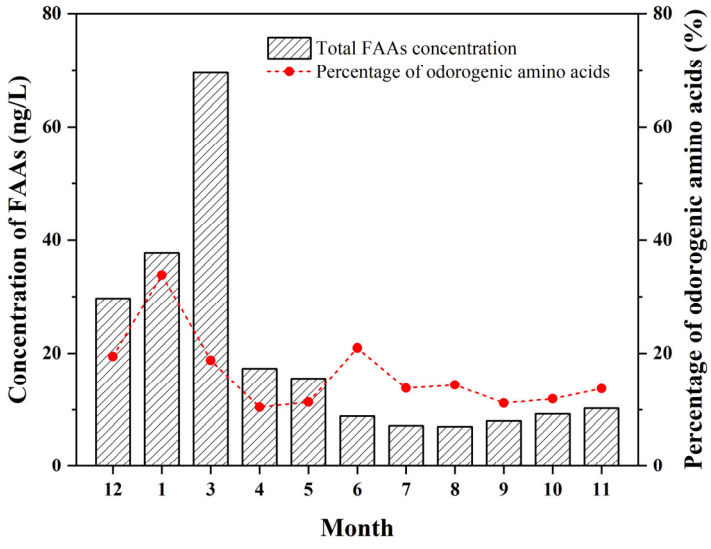

Figure 2. Annual variations in total FAAs concentration and proportion of odorogenic amino acids in raw water at the DWTP

Additionally, as the Qingcaosha Reservoir is situated on an alluvial sandbar at the Yangtze River estuary, it is more susceptible to rainfall effects, which likely contributed to the reduced FAAs concentrations during summer.

Furthermore, Phe, Leu, Ile, and Val were identified as four typical odor-causing amino acids, accounting for 10%~40% (average: 16.35%) of total FAAs. The highest proportion (33.82%) occurred in January, while the lowest (10.44%) was observed in April.

The figure 3 shows the seven most abundant FAAs in DWTP raw water were Arg, Val, Asp, Ser, Glu, Gly, and Ala, with monthly mean concentrations of 4058.19 ng/L, 3534.37 ng/L, 2535.81 ng/L, 2376.97 ng/L, 2060.57 ng/L, 1972.01 ng/L, and 1687.87 ng/L, respectively—significantly higher than other FAAs. In contrast, the three least abundant FAAs were Phe, Asn, and Trp, with mean concentrations of 55.06 ng/L, 71.35 ng/L, and 73.26 ng/L, respectively. Ser reached the peak concentration among all FAAs at 14.44 μg/L during March monitoring. The odor-active compounds Leu and Ile maintained lower but persistent levels, with mean monthly concentrations of 179.35 ng/L and 88.74 ng/L, respectively. Quantitative analysis revealed the following prevalence pattern for odorogenic FAAs: Val> Leu> Ile> Phe, with Val being the only compound consistently detected at μg/L concentrations across all sampling periods.

The composition and abundance of FAAs in natural waters vary seasonally, primarily driven by biological sources (e.g., algae). Differences in regional algal species, microbial communities, and water quality conditions lead to spatial variations in FAAs profiles. The DWTP raw water exhibited rich FAA diversity, with all 20 target FAAs detected monthly. The notable presence of odor-causing amino acids suggests potential risks for O-DBPs formation and associated odor issues.

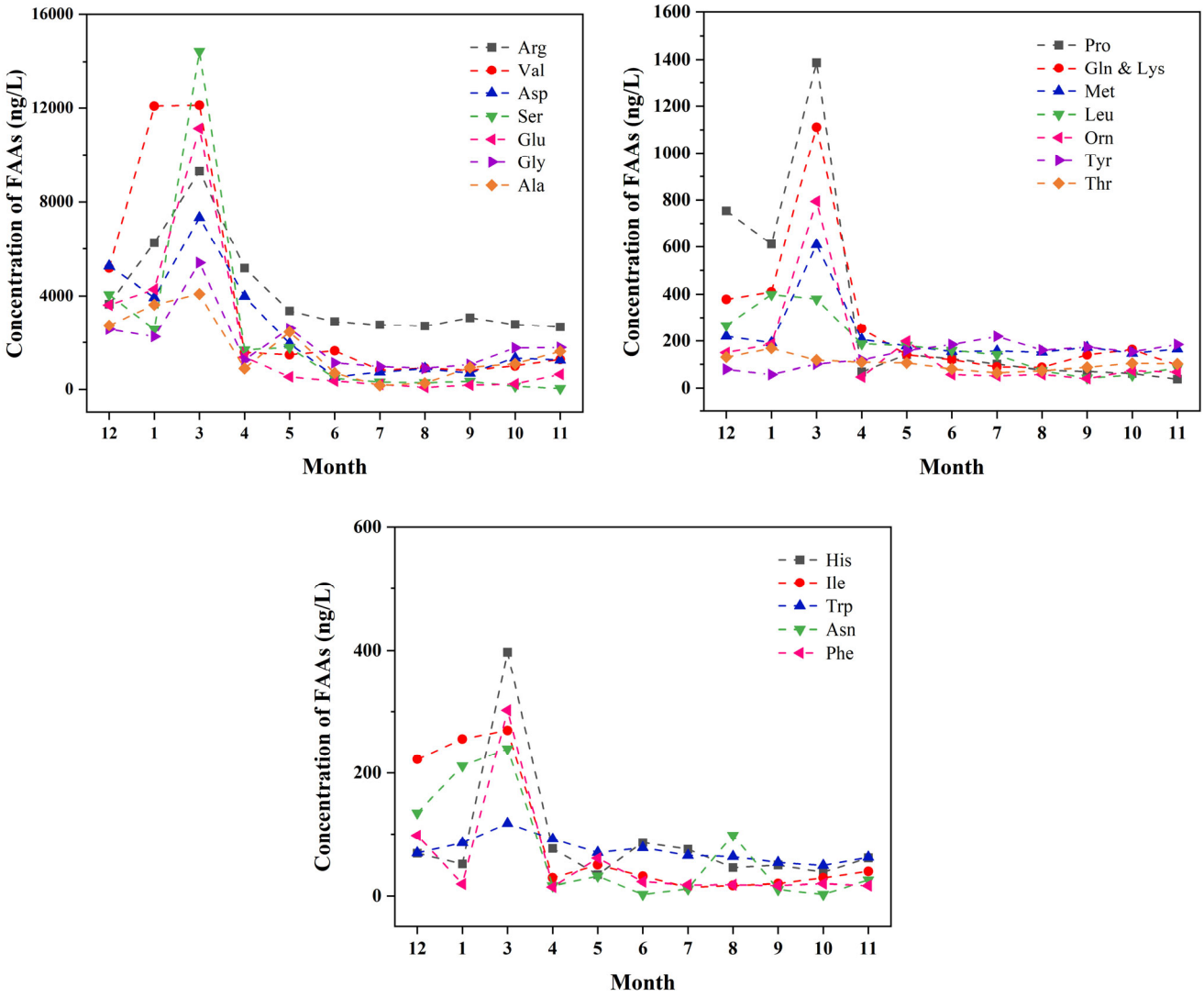

Figure 3. Annual variation profiles of 20 FAAs in raw water at the DWTP

4. Conclusion

This study developed a highly sensitive SPE-LC-MS/MS method for the trace-level detection of FAAs in drinking water, achieving excellent linearity and precision with LOQ as low as 0.02~0.55 nM. Long-term monitoring of a Shanghai DWTP revealed significant seasonal variations in FAAs concentrations (7~70 μg/L), peaking in March due to algal

blooms and declining in August influenced by rainfall dilution. Notably, odor-causing amino acids (Phe, Leu, Ile, Val) constituted 10~40% of total FAAs, highlighting their potential role as precursors for disinfection byproducts (O-DBPs) and associated odor issues. The method's robustness and the observed FAAs dynamics underscore the importance of monitoring these precursors to mitigate public health risks and optimize water treatment strategies. Future research should focus on the direct correlation between FAAs levels and O-DBPs formation to further refine water quality management practices.

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