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Chemometric resolution of coeluting peaks of eleven antihypertensives from multiple classes in high performance liquid chromatography: A comprehensive research in human serum, health product and Chinese patent medicine samples

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ABSTRACT

A novel chemometric-assisted high performance liquid chromatography method coupled with diode array detector (HPLC-DAD) was presented for the simultaneous determination of eleven antihypertensives from multiple classes in most concerned matrix systems. With the aid of second-order calibration which enables specific information of analytes to be well extracted, the heavily overlapping profiles between analytes and the coeluting interferences can be successfully separated and thus accurately quantified. A great advantage of the novel strategy lies in the fact that the analysis could be carried out with the same isocratic mobile phase (methanol/KH_2PO_4: 58:42, v/v, pH 2.60) in a short time regardless of the changes of matrices, such as human serum, health product and Chinese patent medicine. Both qualitative and quantitative results indicate that the hybrid strategy that using HPLC-DAD coupled with second-order chemometric method would be a high performance approach for the purpose of simultaneously quantifying multiple classes of antihypertensives in complex systems. Additionally, the analytical strategy can potentially benefit drug monitoring in both therapeutic research and pharmaceutical quality control. Moreover, the accuracy and reliability of the proposed methodology has been evaluated using several statistical parameters such as root mean squared error of prediction (RMSEP), figures of merit (FOM) and reproducibility of inter-day analysis.

1. Introduction

High blood pressure, widely called hypertension, is a cardiac chronic disease with a symptom of sustaining rise in systemic arterial blood pressure. It is evident that the elevated blood pressure plays a central role in the pathogenesis of both coronary heart disease and stroke which are the main contributors to the global burden of disease [1–9]. Therefore, the adequate control of hypertension is one of the biggest challenges facing the public health worldwide. The pharmacological treatment options for hypertension can be mainly divided into two categories depending on the severity of the symptoms: monotherapy and combination therapy [6]. In principle, the joint of complementary medicines can not only help to meet the target blood pressure, but also decrease the morbidity and mortality of complications. In practice, many doctors as well as the World Health Organization have been beginning to appreciate the positive outcome and feedback from combining the available antihypertensives [2–7].

Generally, the commonly prescribed western antihypertensive drugs involve diuretics, angiotensin-converting enzyme inhibitors, beta blockers, angiotensin II antagonists, calcium antagonists, alpha blockers, etc. [2,6]. Western medicine treatment is widely adopted due to its rapid onset and high efficiency. Along with the benefits of medication, several risky side effects, such as abuse, unforeseen long term complications, and interaction mechanisms of combining taken drugs, cannot be ignored. More recently, Chinese herbal medicines made from natural herbs have seen an increasing usage as complementary or alternative therapies due to their little side effects. The popularity usages of Chinese herbal medicines demand an urgent necessity for their safety assessment, especially when adulterations in Chinese herbal medicines have been repeatedly reported in various occasions. Therefore, a comprehensive research of co-administrated antihypertensives, in particular, quantification of these compounds in both biological matrix and pharmaceutical preparations is a work of great significance.

Up to now, routine analyses of antihypertensive have been frequently performed by high performance liquid chromatography (HPLC) coupled with different detectors, for instance, ultraviolet

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detector (UV) [10–13], fluorescence detector (FD) [14,15], amperometric detector (AD) [16], diode array detector (DAD) [17–19] and mass spectrometry (MS) [20–22]. However, most works have been focused on quantifying a small amount or one kind of antihypertensives in biological matrix or Chinese herbal matrix. To the best of our knowledge, little work has been published for the analysis of multiple antihypertensives from various classes in different concerned analytical systems involving biological matrix and pharmaceutical preparations. Besides, most of the developed methods were built upon complicated chromatographic conditions. Usually, internal standard, gradient elution and/or long time chromatographic runs were required.

Although tedious pretreatments have been performed before the HPLC analysis, chromatographic separation with acceptable resolutions could not always be achieved owing to either structural similarity among the analytes and coeluting interferences from matrices or the limited time range of per chromatographic run. Since it is essential for the analysis of multiple compounds in complex systems with proper resolution, many efforts have been devoted to enhance the separation capability of chromatographic-based techniques, which is, of course, a troublesome task owning to the lack of generality. Fortunately, with the aid of second-order calibration which makes full use of the information collected in multi-way data array, the separation ability of routine chromatographic-based techniques can be further enhanced by employing 'mathematical separation' to partially substitute the 'physical or chemical separation'. Recently, the strategy that employs hyphenated instruments coupled with second-order calibration algorithms has been successfully applied in a wide range of fields [23–31].

In the present work, eleven antihypertensives were initially assayed using HPLC-DAD coupled with second-order chemometric method based on alternating trilinear decomposition (ATLD) algorithm. The investigated antihypertensives were Triamterene, Indapamide, Propranolol, Furosemide, Carvedilol, Bisoprolol, Doxazosin, Reserpine, Amlodipine, Captopril and Losartan. Compared with other published methods, this proposed strategy manifests several merits: first of all, the combination of chemometric method with HPLC-DAD strategy is originally applied to comprehensively quantify multiple classes of antihypertensives in most concerned biological and Chinese herbs matrix systems (i.e. human serum, health product and Chinese patent medicine samples). Secondly, the introduction of chemometric method, e.g. second-order calibration, enables the separation of analytes in different matrices to be carried out with the same simple isocratic mode and the analysis procedure to be significantly improved. Thirdly, the widely used multiple antihypertensives originating from different complicated analytical systems can be simultaneously analyzed using the same isocratic chromatographic condition, which would be convenient for the purpose of clinical and toxicological monitoring as well as routine pharmaceutical quality control. In addition, the work shown in this paper can be expected as a valuable example of the rapid analysis of multiple antihypertensives in different matrices. The proposed method was evaluated in terms of root mean squared error of prediction (RMSEP), figures of merit (FOM) and reproducibility of inter-day analysis.

2. Experiment

2.1. Chemicals and solutions

Triamterene (TRI, 99%), Indapamide (IND, 99%), Propranolol (PRO, 99%), Carvedilol (CAR, 99%), Bisoprolol (BIS, 99%), Doxazosin (DOX, 99%), Reserpine (RES, 99%), Amlodipine (AML, 99%), Captopril (CAP, 99%), Losartan (LOS, 99%), were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Changsha, China); Furosemide (FUR, 98%) was provided by Adamas Reagent Co., Ltd. (Beijing, China) and all chemicals were used as received. The structure formulas of studied eleven antihypertensives were shown in Fig. 1. Human serum was obtained from Yuanhengjinma Bio-technology Development Co., Ltd. (Beijing, China) and stored at −20 °C in the refrigerator. Luobuma (*Apocynum venetum* leaf, AV) tea and Du-zhong (*Eucommia ulmoides* Oliv., EU) Pingya tablet were purchased from a local pharmacy and stored at room temperature. Methyl alcohol (TEDIA Company, USA) was HPLC-grade, potassium dihydrogen phosphate and concentrated hydrochloric acid were analytical-grade.

All stock solutions (around 1 mg mL^{-1}) were prepared by dissolving the corresponding standards in methanol, stored in brown volumetric flask at −20 °C and were stable at least 3 months (except RES which was freshly prepared).

2.2. Apparatus and chromatographic condition

A 3K30 ultracentrifuge with cooling system (Sigma, USA), a Milli-Q water purification system (Millipore, USA) and a grinder (Zhejiang Yiligongmao, China) were used.

The separation was performed on a LC-20AT liquid chromatographic system (Shimadzu, Japan) coupled with a WondaSil C18 column (5 μm, 150 mm × 4.6 mm, GL Sciences Inc., Japan), a manual injector with a 10 μL loop and a diode array detector. Spectra data were recorded at the range of 190–450 nm. The mobile phase was isocratic and consisted of methanol and 10 mM KH_2PO_4 (pH 2.60) at the ratio of 58:42 (v/v). The flow rate was maintained at 1.0 mL min^{-1} and the column temperature was set to be 30 °C. In all cases, recorded datasets were analyzed in Matlab version 6.5 (Mathworks, Inc.) environment and the programs were developed in the laboratory on a Dell computer (Intel(R) Core2, Dell China) using windows XP (Microsoft) software.

2.3. Sample pretreatment

2.3.1. Human serum samples

Before usage, the drug free human serum was thawed at 36 °C in water bath. The treatment of serum can be summarized as follows: firstly, 2 mL human serum was transferred into a 10 mL centrifugal tube, and then 6 mL of methanol was added and the mixture was sonicated for 30 min [14]. Secondly, the tube was centrifuged for 15 min at 6000 rpm, the supernatant was transferred into another tube. The residue was added another 6 mL methanol, mixed, sonicated, centrifuged and separated in the same way. Thirdly, the extracts were mixture and then evaporated to dryness under nitrogen at 40 °C in water bath. Finally, the residue was reconstituted, adjusted pH to 2.60 (using 2 M HCl) and diluted to 2 mL with mobile phase. An aliquot of 10 μL resulting solution was injected into the HPLC system.

2.3.2. AV samples

AV tea was first smashed, an amount of 5.00 g sample was transferred into a 50 mL centrifuge, 30 mL of methanol was added and then the mixture was treated in the same way as human serum samples described above. At last, the residue was redissolved with 5 mL mobile phase.

2.3.3. EU samples

The treatment of EU was the same as AV samples described above.

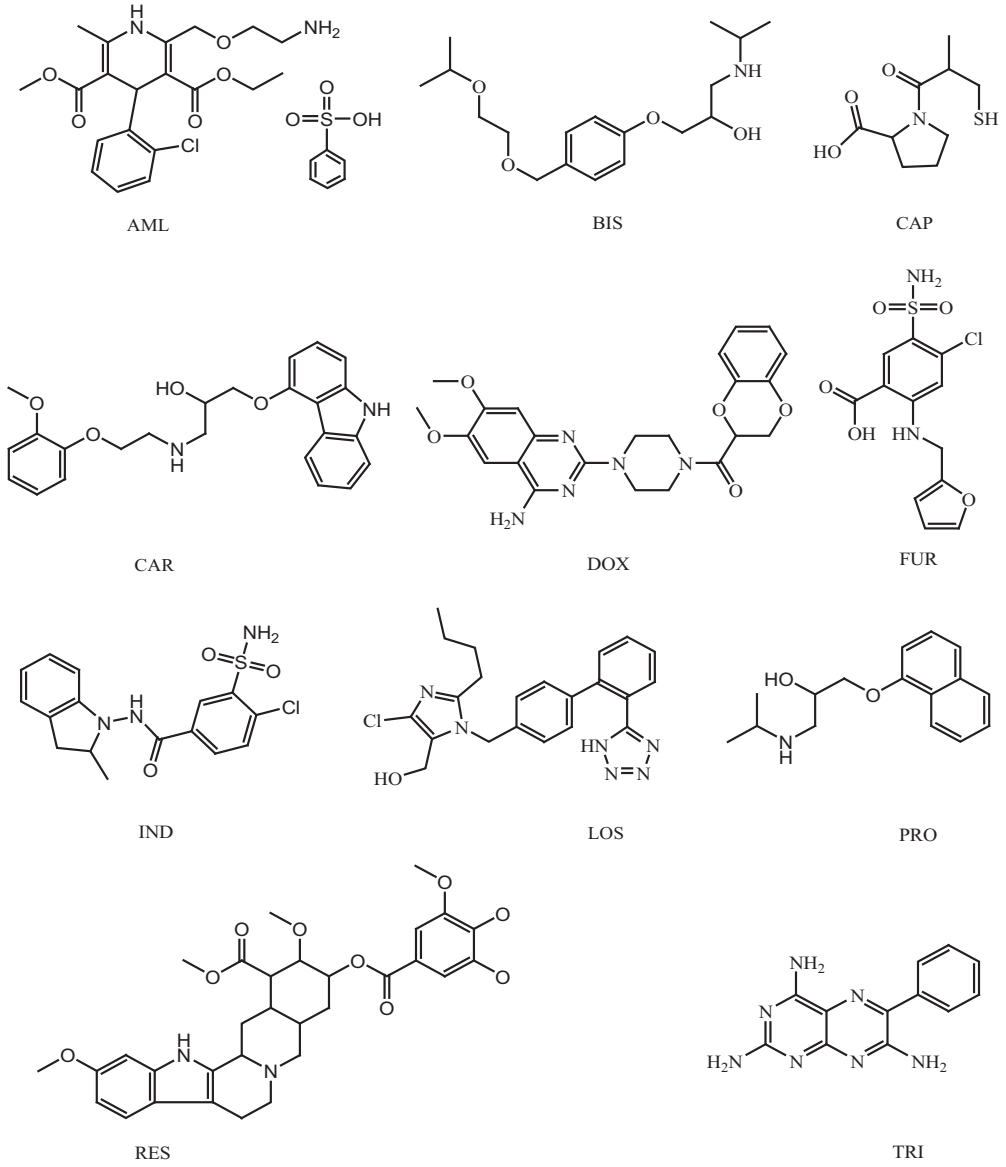

Fig. 1. Structure formulas of the studied eleven antihypertensives.

Table 1
Retention time (t_R), linear range and corresponding determination coefficient (R^2) for the studied eleven antihypertensives in calibration samples.

Samples	TRI	CAP	BIS	PRO	FUR	IND	DOX	CAR	RES	AML	LOS
t_R (min)	2.36	2.85	3.13	3.72	3.94	4.34	4.51	4.65	6.50	7.51	8.94
Linear range ($\mu g\,mL^{-1}$)	0.19–1.89	0.90–9.00	0.36–3.60	0.41–2.85	0.38–3.42	0.36–3.24	0.42–3.78	0.48–3.02	0.80–8.00	1.51–19.58	0.88–8.80
R^2	0.9977	0.9991	0.9959	0.9997	0.9998	0.9987	0.9999	0.9996	1.0000	0.9996	0.9992

2.4. Sample preparation

2.4.1. Calibration samples and validation samples

All working solutions were freshly prepared by diluting stock solutions with the mobile phase. The calibration samples were prepared by firstly mixing different amount of working solutions, and then diluting to 10 mL with the mobile phase. The corresponding concentrations of studied eleven antihypertensives in the calibration samples were summarized in Table 1. Five validation samples without interference were also designed in order to check the accuracy of the proposed strategy, and their concentrations were identical with the following prediction samples.

2.4.2. Prediction samples

Three groups of prediction samples (human serum, AV and EU samples) were designed and the concentrations of analytes were within the linear ranges of calibration samples. For each group, 5 samples were prepared by spiking suitable volume of working solutions to the aforementioned exacts, and the concentrations of prediction samples for each of the three groups were shown in Tables 2, 3 and 4, respectively.

All solutions were filtered through 0.45 μm cellulose membrane filter (Shanghai Jicheng Co., Ltd., China) before being injected into the HPLC system.

Table 2
Quantitative results of the studied eleven antihypertensives in human serum samples.

Samples	TRI ($\mu g\,mL^{-1}$)		CAP ($\mu g\,mL^{-1}$)		BIS ($\mu g\,mL^{-1}$)		PRO ($\mu g\,mL^{-1}$)		FUR ($\mu g\,mL^{-1}$)		IND ($\mu g\,mL^{-1}$)	
	Add	Found	Add	Found	Add	Found	Add	Found	Add	Found	Add	Found
t01	0.28	0.28	9.00	9.59	3.60	3.06	1.43	1.36	0.76	0.72	0.72	0.76
t02	0.66	0.63	7.20	7.66	3.06	3.29	2.24	2.22	1.52	1.44	1.44	1.35
t03	1.04	1.04	5.40	5.27	2.52	2.74	2.65	2.62	1.90	1.94	1.80	1.66
t04	1.42	1.40	4.50	4.41	1.80	1.65	0.81	0.75	2.66	2.63	2.52	2.29
t05	1.79	1.90	2.70	2.47	1.26	1.16	1.83	1.85	3.42	3.55	3.24	2.97
Average recovery (%)	99.8 ± 3.9		100.0 ± 6.4		96.9 ± 10.5		97.4 ± 3.4		98.9 ± 4.0		94.9 ± 6.1	

Samples	DOX ($\mu g\,mL^{-1}$)		CAR ($\mu g\,mL^{-1}$)		RES ($\mu g\,mL^{-1}$)		AML ($\mu g\,mL^{-1}$)		LOS ($\mu g\,mL^{-1}$)	
	Add	Found	Add	Found	Add	Found	Add	Found	Add	Found
t01	3.78	3.80	1.91	2.03	7.60	7.39	2.01	1.94	1.32	1.38
t02	2.94	2.99	0.95	0.91	6.00	5.96	6.02	5.98	3.08	3.14
t03	2.10	2.26	2.86	2.98	4.40	4.35	10.04	10.03	4.84	4.72
t04	1.26	1.22	2.39	2.37	2.80	2.63	14.06	14.08	6.60	6.27
t05	0.42	0.42	1.43	1.48	1.20	1.13	18.07	18.05	8.36	8.75
Average recovery (%)	101.3 ± 4.1		101.9 ± 4.2		96.7 ± 2.6		99.2 ± 1.4		100.7 ± 4.3	

Table 3
Quantitative results of the studied eleven antihypertensives in AV samples.

Samples	TRI ($\mu g\,mL^{-1}$)		CAP ($\mu g\,mL^{-1}$)		BIS ($\mu g\,mL^{-1}$)		PRO ($\mu g\,mL^{-1}$)		FUR ($\mu g\,mL^{-1}$)		IND ($\mu g\,mL^{-1}$)	
	Add	Found	Add	Found	Add	Found	Add	Found	Add	Found	Add	Found
t01	0.28	0.29	9.00	8.63	3.60	3.49	1.43	1.44	0.76	0.76	0.72	0.73
t02	0.66	0.66	7.20	7.27	3.06	2.87	2.24	2.32	1.52	1.45	1.44	1.42
t03	1.04	1.13	5.40	5.09	2.52	2.33	2.65	2.80	1.90	2.01	1.80	1.85
t04	1.42	1.41	3.60	3.94	1.80	1.77	0.81	0.81	2.66	2.77	2.52	2.56
t05	1.79	1.91	1.80	1.70	1.26	1.17	1.83	1.90	3.42	3.48	3.24	3.48
Average recovery (%)	103.3 ± 4.2		99.0 ± 6.4		94.8 ± 2.8		102.6 ± 2.5		101.5 ± 3.9		102.3 ± 3.3	

Samples	DOX ($\mu g\,mL^{-1}$)		CAR ($\mu g\,mL^{-1}$)		RES($\mu g\,mL^{-1}$)		AML ($\mu g\,mL^{-1}$)		LOS ($\mu g\,mL^{-1}$)	
	Add	Found	Add	Found	Add	Found	Add	Found	Add	Found
t01	3.78	3.71	1.91	1.78	7.60	6.89	2.01	2.10	1.32	1.40
t02	2.94	2.98	0.95	0.99	6.00	5.49	6.02	6.17	3.08	3.05
t03	2.10	2.17	2.86	2.91	4.40	4.04	10.04	10.90	4.84	5.10
t04	1.26	1.27	2.39	2.51	2.80	2.93	14.06	14.57	6.60	7.06
t05	0.84	0.82	1.43	1.46	1.20	1.20	18.07	17.74	8.36	8.56
Average recovery (%)	100.2 ± 2.4		101.4 ± 4.8		95.7 ± 6.3		103.5 ± 3.8		104.0 ± 3.3	

Table 4
Quantitative results of the studied eleven antihypertensives in EU samples.

Samples	TRI ($\mu g\,mL^{-1}$)		CAP ($\mu g\,mL^{-1}$)		BIS ($\mu g\,mL^{-1}$)		PRO ($\mu g\,mL^{-1}$)		FUR ($\mu g\,mL^{-1}$)		IND($\mu g\,mL^{-1}$)	
	Add	Found	Add	Found	Add	Found	Add	Found	Add	Found	Add	Found
t01	0.28	0.29	9.00	8.50	3.60	3.90	1.43	1.42	0.76	0.76	0.72	0.72
t02	0.66	0.63	7.20	6.89	3.06	3.30	2.24	2.21	1.52	1.46	1.44	1.42
t03	1.04	0.91	5.40	5.69	2.52	2.38	2.65	2.60	1.90	1.95	1.80	1.73
t04	1.42	1.40	3.60	4.44	1.80	1.92	0.81	0.76	2.66	2.66	2.52	2.38
t05	1.79	1.80	1.80	2.79	1.26	1.12	1.83	1.77	3.42	3.53	3.24	3.09
Average recovery (%)	96.9 ± 6.2		99.5 ± 4.7		101.2 ± 9.0		97.3 ± 2.1		100.3 ± 2.8		97.1 ± 2.5	

Samples	DOX ($\mu g\,mL^{-1}$)		CAR ($\mu g\,mL^{-1}$)		RES($\mu g\,mL^{-1}$)		AML ($\mu g\,mL^{-1}$)		LOS ($\mu g\,mL^{-1}$)	
	Add	Found	Add	Found	Add	Found	Add	Found	Add	Found
t01	3.78	3.72	1.91	1.92	7.60	7.70	2.01	2.08	1.32	1.23
t02	2.94	2.96	0.95	1.01	6.00	6.08	6.02	6.33	3.08	3.03
t03	2.10	2.01	2.86	2.99	4.40	4.42	10.04	10.09	4.84	4.40
t04	1.26	1.24	2.39	2.46	2.80	2.89	14.06	13.47	6.60	6.41
t05	0.84	0.83	1.43	1.42	1.20	1.19	18.07	18.03	8.36	8.15
Average recovery (%)	98.4 ± 1.8		102.8 ± 2.7		101.1 ± 1.6		100.9 ± 3.6		95.4 ± 3.2	

2.5. Theory

In HPLC-DAD measurements, two-dimensional data array can be obtained for each sample in a single run and thus three-way data array can be obtained by arraying different groups of samples (e.g. calibration and validation samples, calibration and prediction samples) along the third direction. Generally, each individual element in the three-way data array is the response of compounds involved in the matrix, can be mathematically expressed as:

$$x_{ijk} = \sum_{n=1}^{N} a_{in} b_{jn} c_{kn} + e_{ijk}, \quad (i = 1, 2, \ldots, I; \quad j = 1, 2, \ldots, J; \quad k = 1, 2, \ldots, K) \tag{1}$$

where x_{ijk} is the recorded data of the kth sample, at the ith number of elution time data points and jth number of wavelengths points, a_{in}, b_{jn} and c_{kn} represent the resolved profiles of the nth component at ith number of elution time data points, jth number of wavelengths points and kth sample, respectively, and e_{ijk}, is the residual not accounted for by the model.

2.5.1. Alternating trilinear decomposition (ATLD) algorithm

ATLD algorithm developed by Wu et al., is one of the most commonly used second-order algorithms, and is particularly suitable for handling with chromatographic-based datasets. ATLD algorithm alternately minimizes the following three objective functions to update the qualitative profiles (**A** and **B**) and the relative concentrations (**C**) of individual components:

$$\sigma_1 = \sum_{k=1}^{K} ||\mathbf{X}_{..k} - \mathbf{A}\,\mathrm{diag}(\mathbf{c}_{(k)})\mathbf{B}^T||_F^2 \tag{2}$$

$$\sigma_2 = \sum_{i=1}^{I} ||\mathbf{X}_{i..} - \mathbf{B}\,\mathrm{diag}(\mathbf{a}_{(i)})\mathbf{C}^T||_F^2 \tag{3}$$

$$\sigma_3 = \sum_{j=1}^{J} ||\mathbf{X}_{.j.} - \mathbf{C}\,\mathrm{diag}(\mathbf{b}_{(j)})\mathbf{A}^T||_F^2 \tag{4}$$

ATLD uses the Moore–Penrose generalized inverses strategy which bases on singular value decomposition, and thus has the advantages of being insensitive to number of component, fast convergence and fully exploiting the second-order advantage. More detailed information for ATLD can be found in Refs. [30,32].

3. Result and discussion

3.1. Optimization of the chromatographic condition

Optimization of separated operating conditions of high performance liquid chromatography will do great benefit to the analysis procedure. It is well known that the retention behavior of an analyte depends on its physical/chemical property as well as several characteristics of the employed chromatographic systems, such as the composition of chromatographic column, mobile phase, and the pH value of mobile phase. Methanol–water mobile phase is one of the most commonly used mobile phases for reversed-phase high performance liquid chromatography. Several methanol-based mobile phases were tested, involving methanol–water, methanol–formic acid aqueous, and methanol–phosphate buffer. Finally, according to the short separation time and peak symmetry, methanol–KH_2PO_4 (10 mM) at the ratio of 58:42 (v/v) was selected. On the other hand, the pH value of the mobile phase is another key factor in the separation procedure of ionizable compounds [33–36]. Thus, a low-pH mobile phase (pH 2.60) was preferred due to the amphoteric properties of the studied eleven antihypertensive drugs.

3.2. Quantification of eleven antihypertensives in concerned matrix systems

To provide a general observation, the typical chromatographic plot for calibration sample c08 which contains the eleven antihypertensives was shown in the first row of Fig. 2. Apparently, all of the studied eleven antihypertensives could be eluted completely within 10 min. In addition, the entire chromatographic profiles of eleven drugs can be roughly divided into five groups according to their elution ranges: (I) a single analyte, TRI, eluted in the region 2.20–2.50 min; (II) two analytes, CAP and BIS, coeluted in the region 2.60–3.40 min; (III) the third group, PRO and FUR eluted in the region 3.50–4.20 min with acceptable resolution; (IV) three analytes, IND, DOX and CAR eluted together within 1 min (4.18–4.95 min), whose chromatographic profiles were seriously overlapped and only two elution peaks could be observed in this region; (V) the last three analytes, RES, AML and LOS eluted, which achieved successful separation. Meanwhile, some qualitative and quantitative parameters such as retention time, linear range and corresponding coefficient for each of the analytes obtained from the calibration samples were summarized in Table 1. Determination coefficients ($R^2 > 0.99$) confirmed that reasonable calibration curves were set up over the used concentration. All results indicate clearly that the aforementioned type of analytical system poses great challenge to chromatographers. Apart from the overlapping peaks among the analytes, it is worth noting that unexpected interferences which originate from background matrix may occur in the real samples and overlap any of the peaks shown in Fig. 2. If there are coeluting interferences existing, the specific information space of analyte might be misdetermined by classical univariate method. Fortunately, with the aid of second-order calibration methods such as ATLD algorithm, these problems can be mostly if not completely overcome. The underlying philosophy of this advantage lies in the fact that the decomposition of trilinear method is always unique, which allows relative chromatographic, spectral and concentration profiles of analytes to be extracted apart from those of uncalibrated interferences.

In the field of second-order calibration coupled with chromatography applications, one should be aware of the presence of time shifts, which are caused by the very nature of chromatographic techniques, such as the changes of temperature, sampler, and composition of mobile phase. One consequence of this is that these time shifts disturb the trilinear structure of the data array and thus may result in invalid results for analytes. Therefore, time shifts should be checked and corrected before trilinear methods being applied. Up to now, several methods have been reported for the purpose of aligning time shifts [37–39]. In this study, the chromatographic profiles (recorded under 220 nm) from five different type data sets were displayed in Fig. 3 (first row). It can be seen clearly from the first row of Fig. 3 that time shifts among calibration sample (c08), validation sample (t05), spiked human serum sample (s05), spiked AV sample (a05) and spiked EU sample (e05) can hardly be observed, which indicate that no significant time shifts occur in the present chromatographic during the analysis procedure.

In addition to time shifts, background drifts may bring trivial treatments for traditional univariate methods. Compared with traditional strategies, second-order chemometric methods have a significant advantage in handling with the baseline drift problems. In principle, the baseline drift can be easily modeled by including extra number of components. For the analytes in group V (RES, AML, LOS) which reached baseline separation in calibration samples, it is advocated to quantify them by univariate calibration methods, for instance, chromatographic peak area vs. concentration. However,

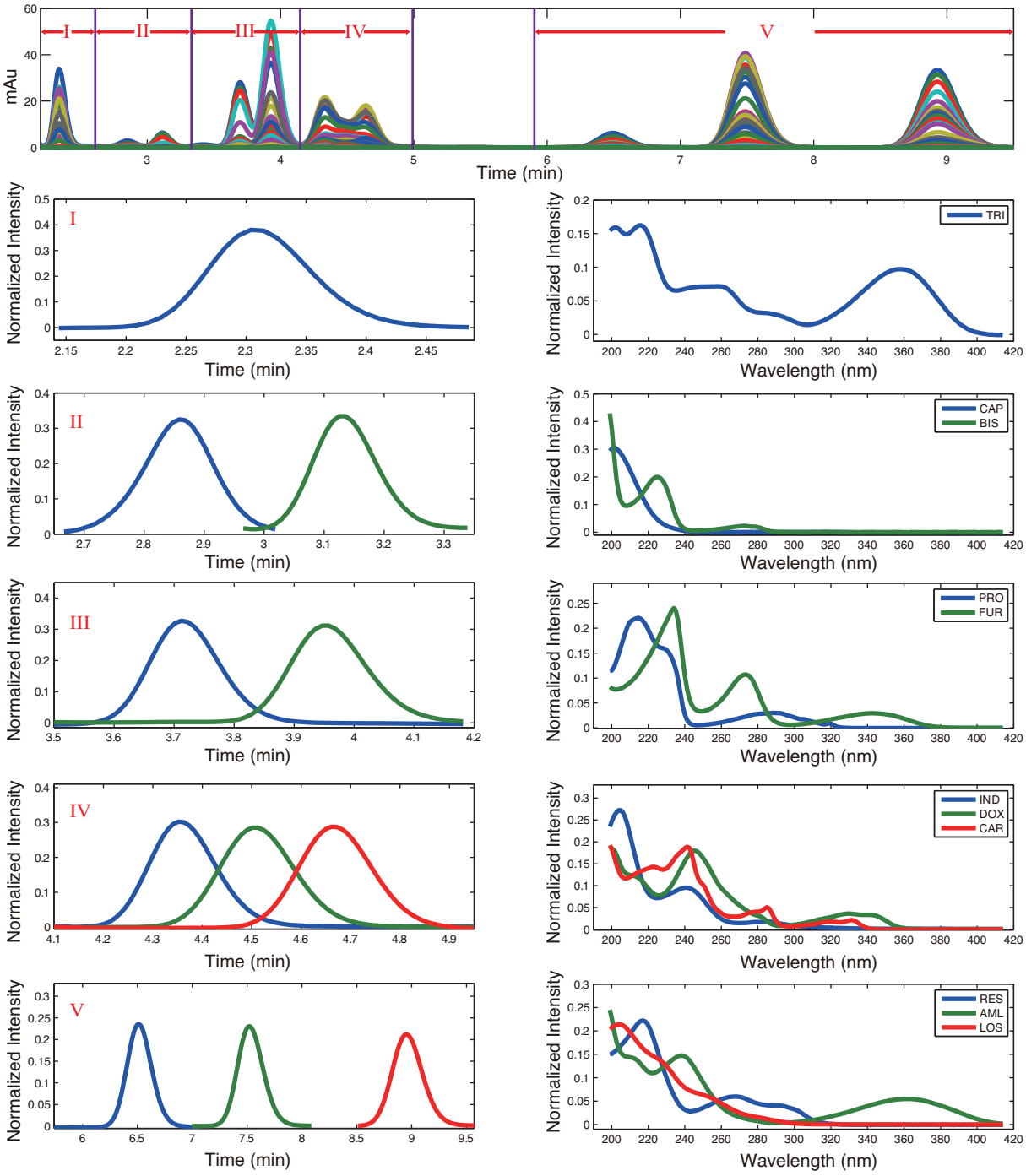

Fig. 2. First row: the recorded chromatographic profiles of calibration sample c08 under multi-wavelength channels. The resolved normalized chromatographic profiles for each of the studied eleven antihypertensives (left column) and the corresponding normalized spectra (right column).

there were baseline drift problem and/or coeluting interferences in the real samples, second-order method would be a more advisable choice. In this work, the sub-dataset corresponding to LOS (the last eluted analyte) was employed as an example to illustrate the benefit of introducing second-order calibration method for solving baseline drift problem (Fig. 3a). The baseline drift effect can be successfully eliminated using excessive factors (see the green line shown in Fig. 3b). (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

Furthermore, selecting appropriate number of components would further favor the performance of the employed algorithm. However, correct number of component could not always be provided by current rank estimation methods and thus algorithms which are insensitive to the number of components are preferable. In this work, Core consistency diagnostic (CORCONDIA) test proposed by Bro [40] and ADD-ONE-UP test developed by Chen et al. [41] were employed to estimate the proper number of component. Principally, almost identical result would be provided by ATLD algorithm as long as the selected number is no less than the real one.

The entire data processing procedure adopted in this work can be summarized as four sequence steps: first, characteristic

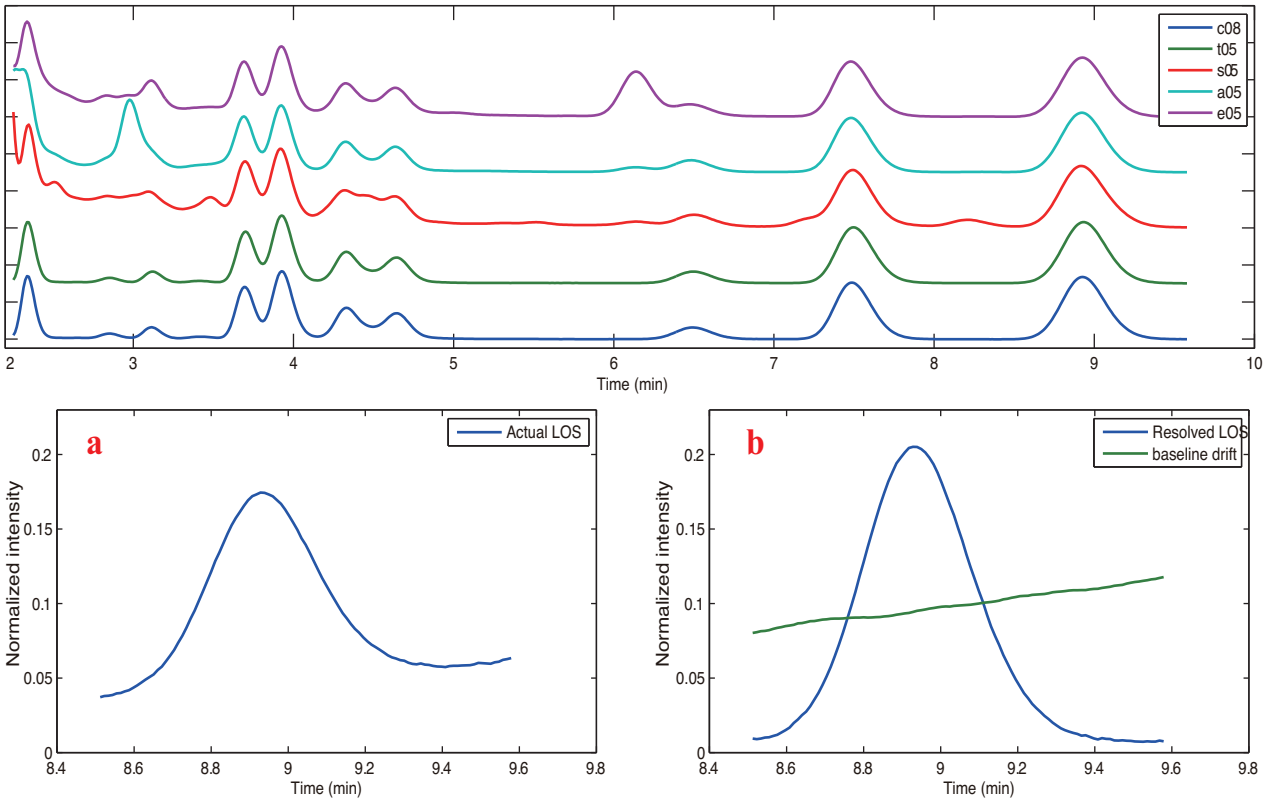

Fig. 3. First row: stacked chromatographic profiles from five different type data sets: calibration sample (c08), validation sample (t05), spiked human serum sample (s05), spiked AV sample (a05), spiked EU sample (e05). The profiles were recorded under 220 nm. Second row: (a) the original chromatographic profile of LOS (recorded under 220 nm) and (b) the corresponding normalized profile after background being corrected.

information for each of the above-mentioned five ranges of retention was carefully selected according to the features of both chromatographic and spectral profiles; second, the proper numbers of components for ATLD algorithm were estimated for each elution range; third, ATLD algorithm was employed to decompose the specific elution ranges with the aforementioned number of components. At last, resolved relative concentrations contributions for each analyte of interest were independently regressed against the corresponding standard concentrations and thus qualitative and quantitative information was received [32]. Specific details were exemplified below.

3.2.1. Validation samples

Prior to analysis, the interference free validation samples were designed to check the accuracy of the trilinear model. Along with the aforementioned data processing technique, satisfactory results were obtained. The average recovery of TRI was 103.6 ± 7.0%, CAP: 97.3 ± 4.2%, BIS: 102.0 ± 5.5%, PRO: 103.0 ± 2.4%, FUR: 99.9 ± 4.3%, IND: 104.8 ± 7.1%, DOX: 99.8 ± 7.4%, CAR: 97.9 ± 7.3%, RES: 98.8 ± 6.0%, AML: 101.1 ± 4.0%, LOS: 99.6 ± 2.1%, respectively. These feasible results indicated trilinear model can be potentially used for three-way data analysis. In the following part, the performance of ATLD on three complicated concerned matrix systems, i.e. human serum samples, health product samples and Chinese patent medicine samples were further investigated.

3.2.2. Human serum samples

Since combination therapy that joins several classes of drugs has been proved to be more favorable than monotherapy [6], monitoring drug concentrations and drug–drug interactions in human serum are important for sensitive patients and pharmacology researches. Therefore, eleven of the widely prescribed antihypertensives were selected and investigated. Fig. 4 (first row) demonstrated the chromatographic profiles of spiked human serum sample s05 under multi-wavelength channels. A visual comparison of Fig. 2 (first row) and Fig. 4 (first row) allows one to assess the high complexity of the Human serum matrix. Obviously, a large number of unexpected interferences coeluted with the analytes all along the elution region, especially for those of TRI, CAP, and BIS which were completely covered by coeluting compounds. Additionally, there was a visible increment in the peak intensities. A possible explanation is that the elution regions of studied eleven antihypertensives were contaminated by co-administrated matrix and the measured signal intensities were the sum responses of analytes and coeluting compounds. All these facts make big challenges for the success implementation of univariate methods. In such cases, second-order methods which do not required complete separation of analytes could be employed.

After decomposing the recorded HPLC-DAD data array (calibration samples plus human serum samples) using ATLD algorithm, qualitative and quantitative results were collected in Fig. 4 and Table 2, respectively. For the convenience of presentation, only the resolved chromatographic and spectral profiles of studied eleven antihypertensives and those of principal interferences were shown, while the profiles corresponding to baseline drift were omitted. A comparison of the retrieved profiles (Fig. 4) with those shown in Fig. 2 indicates that the qualitative information has been successfully extracted even in the presence of serious coexisting interferences in human serum matrix. Furthermore, the average recoveries of eleven analytes (between 94.9 ± 6.1% and 101.9 ± 4.2%) demonstrated that satisfactory results were obtained and the analytical strategy that combing ATLD algorithm with three-way chromatography data can be successfully applied to quantify multiple antihypertensives in human serum samples.

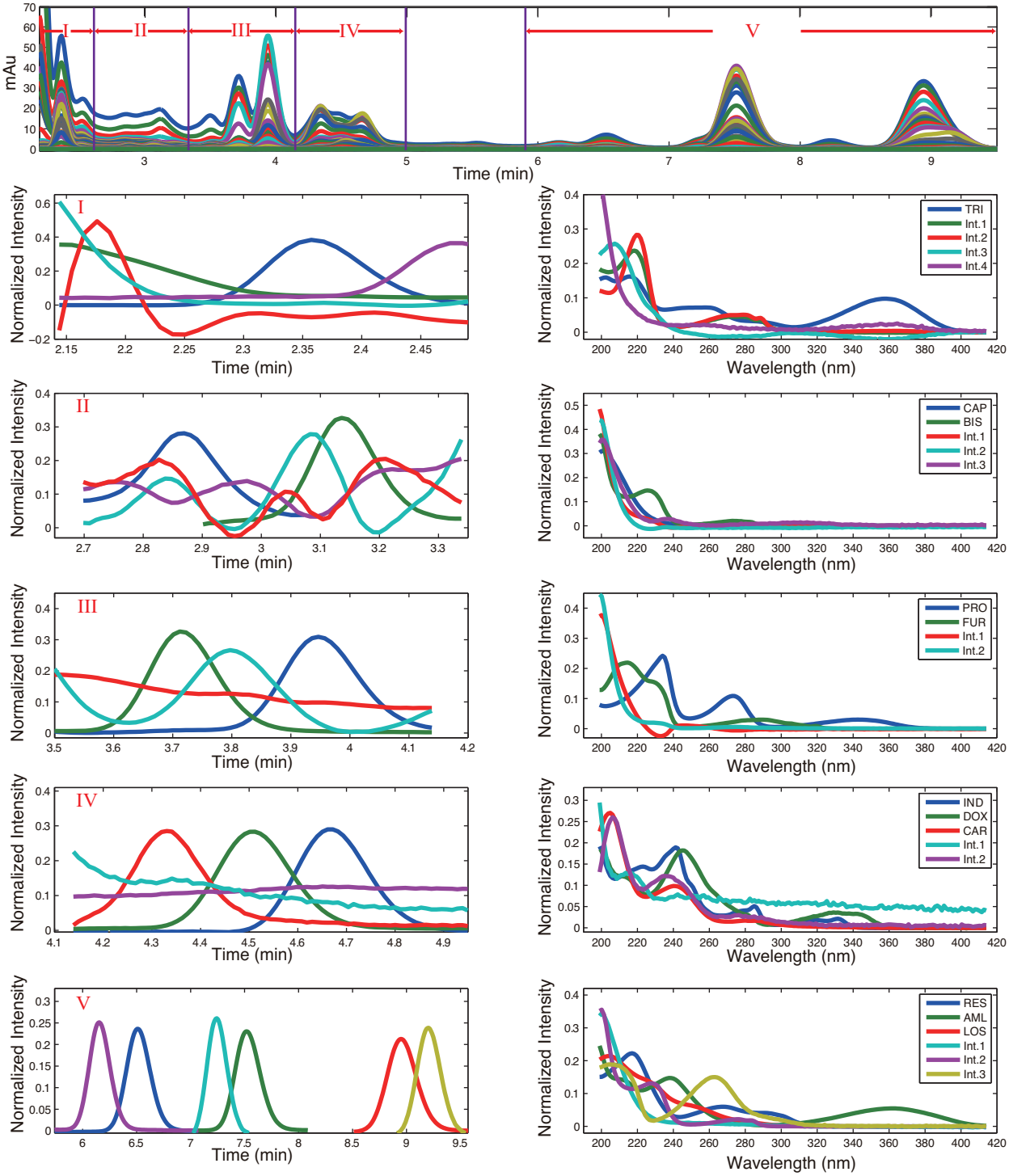

Fig. 4. First row: the recorded chromatographic profiles of human serum sample s05 under multi-wavelength channels. The resolved normalized chromatographic profiles for each of the studied eleven antihypertensives (left column) and the corresponding normalized spectra (right column). Int. represents interference resolved from the matrix.

3.2.3. *A. venetum L. (AV) tea samples*

A. venetum L. (AV), which is known as Luobuma in China, consisted of many bioactive constituents and has been receiving widespread applications as healthy beverage in the treatment of hypertension due to its efficient biological effects [42–44]. Nowadays, health products are widely adopted as supplementary antihypertensive strategy, while the adulteration product has been an emerging issue of concern to the public health. To a patient undergoing therapeutic treatment, misuse of adulterated health product may result in unexpected changes in the blood pressure and might cause harmful effects to patients [2,11]. Consequently, monitoring the qualities of health product in the market is a work of great importance. In this part, the aforementioned eleven antihypertensives which would be illegally added in AV tea were simultaneously analyzed.

The first row of Fig. 5 showed the total chromatographic profiles of spiked AV sample a05 under multi-wavelength channels. Compared with the above two subplots (the first rows of Figs. 2 and 4),

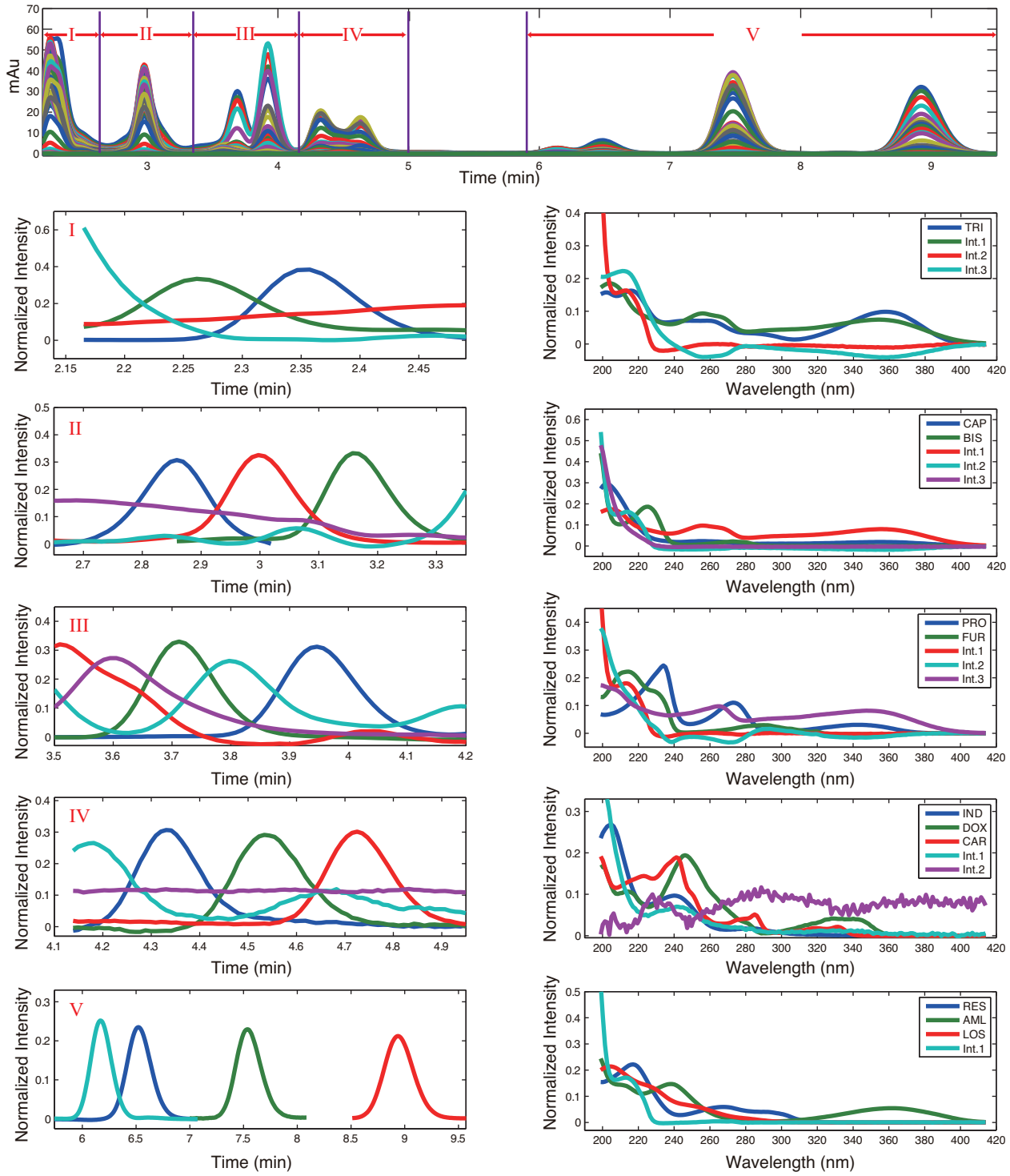

Fig. 5. First row: the recorded chromatographic profiles of AV sample a05 under multi-wavelength channels. The resolved normalized chromatographic profiles for each of the studied eleven antihypertensives (left column) and the corresponding normalized spectra (right column). Int. represents interference resolved from the matrix.

AV matrix has its own characteristics: overlapping peaks accumulated mainly within the first 3.50 min, in other words, the peaks of TRI, CAP and BIS were severely masked by unexpected interferences. With a view to circumvent such dilemma, the data processing procedure was employed to interpret the recorded dataset. Luckily, with the assist of second-order calibration method, both qualitative and quantitative results were retrieved successfully. Resolved profiles and quantitative results were displayed in Fig. 5 and Table 3, respectively.

3.2.4. *E. ulmoides* Oliv. (EU) samples

E. ulmoides Oliv. (EU), widely called Du-zhong in China, is one of the commonly used herbs in Chinese patent medicine. Previous researchers have proved that *E. ulmoides* has many interesting features, such as antihypertensive and anti-oxidative, due to its bioactive compositions [45–48]. Du-zhong Pingya tablet is made from *E. ulmoides* leaves and often used as substitute of antihypertensive medicine. Adulteration of Chinese patent medicine is also a troubling problem and monitoring its quality is a work of

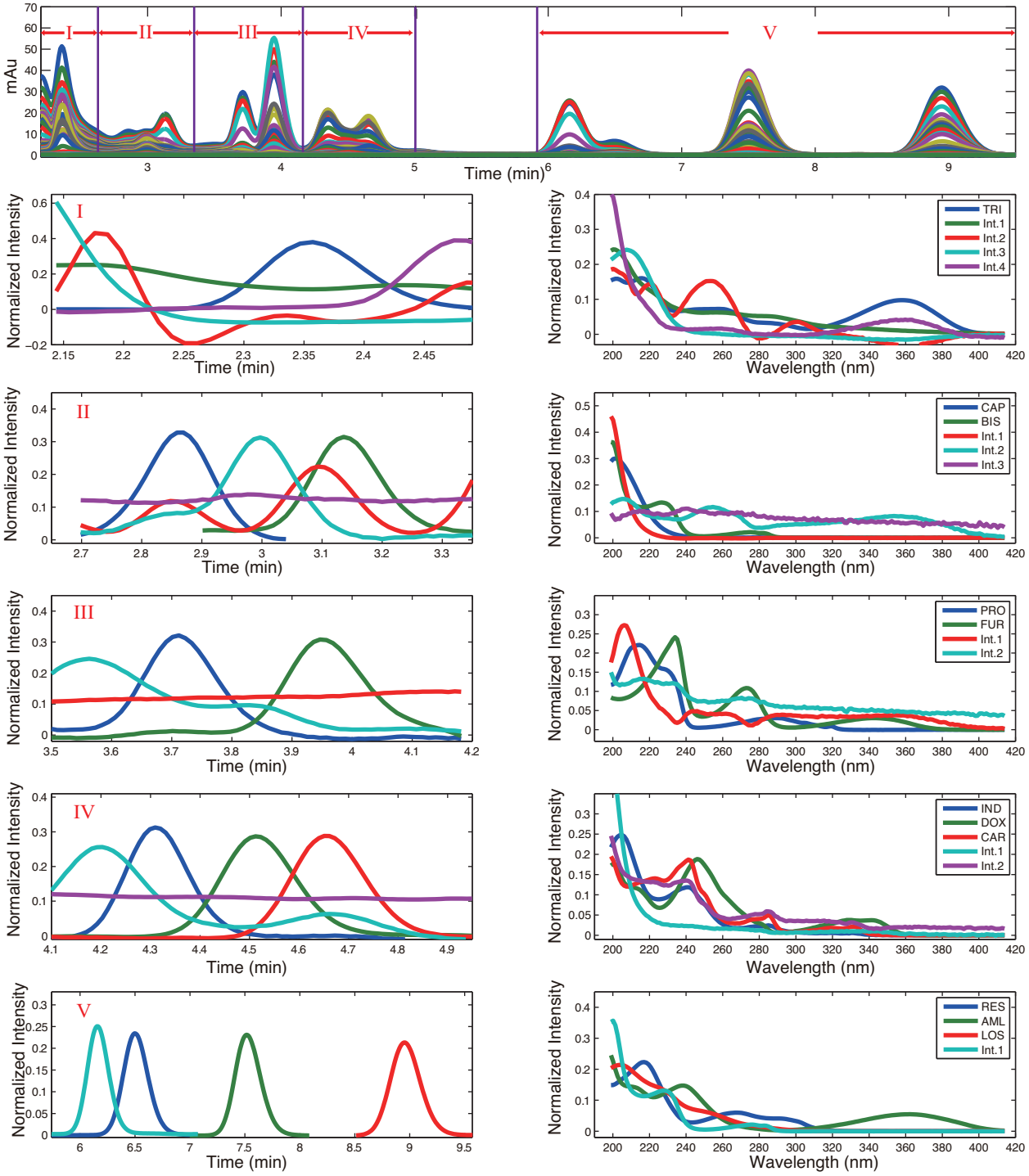

Fig. 6. First row: the recorded chromatographic profiles of EU sample e05 under multi-wavelength channels. The resolved normalized chromatographic profiles for each of the studied eleven antihypertensives (left column) and the corresponding normalized spectra (right column). Int. represents interference resolved from the matrix.

significance as well. Therefore, eleven antihypertensives which would potentially be adulterated in EU samples were simultaneously analyzed. Fig. 6 (first row) presented the chromatographic landscape of spiked EU sample e05 under multi-wavelength channels, and from which we could see the coeluting interferences of EU matrix were mainly occurred in the region 2.00–3.60 min and 5.90–6.40 min. After adopting the aforementioned data processing procedure, corresponding resolved profiles and quantitative results were shown in Fig. 6 and Table 4, which indicated that acceptable results were received.

Though the coeluting interferences originated from human serum, AV and EU matrices are different; a careful overview of the figures (Figs. 4–6) could find that the resolved chromatographic and spectral profiles of the studied eleven antihypertensives are in good agreement, which fully demonstrates the merit of second-order advantage. In addition, the proposed strategy releases the

Table 5
Statistical parameters of the ATLD algorithm in human serum, AV and EU samples ($N = 3$).

Samples	TRI			CAP			BIS			PRO		
	Serum[a]	AV[b]	EU[c]	Serum[a]	AV[b]	EU[c]	Serum[a]	AV[b]	EU[c]	Serum[a]	AV[b]	EU[c]
RMSEP	0.058	9.38	6.90	0.40	37.50	33.00	0.33	20.00	23.00	0.048	11.38	5.00
LOD	0.022	10.00	7.60	3.42	33.75	43.00	0.056	10.00	5.80	0.052	2.25	3.80
LOQ	0.068	30.00	23.00	10.36	102.50	130.00	0.17	30.00	18.00	0.16	7.00	12.00

Samples	FUR			IND			DOX			CAR		
	Serum[a]	AV[b]	EU[c]	Serum[a]	AV[b]	EU[c]	Serum[a]	AV[b]	EU[c]	Serum[a]	AV[b]	EU[c]
RMSEP	0.082	11.25	6.60	0.20	15.00	11.00	0.088	6.75	5.70	0.092	12.13	8.00
LOD	0.17	2.75	4.50	0.12	1.75	8.90	0.12	8.25	0.58	0.066	2.88	0.56
LOQ	0.50	8.38	13.00	0.38	5.25	27.00	0.36	25.00	1.80	0.20	8.63	1.69

Samples	RES			AML			LOS		
	Serum[a]	AV[b]	EU[c]	Serum[a]	AV[b]	EU[c]	Serum[a]	AV[b]	EU[c]
RMSEP	0.14	60.00	8.20	0.042	66.25	34.00	0.26	36.25	27.00
LOD	0.076	21.25	1.02	10.06	6.75	0.66	0.058	2.25	1.08
LOQ	0.22	66.25	3.10	3.20	20.00	2.01	0.18	6.75	3.26

[a] Represents concentrations expressed in $\mu g\,mL^{-1}$ in human serum.
[b] $\mu g\,g^{-1}$ in AV tea.
[c] $\mu g\,g^{-1}$ in EU.

traditional analytical strategy that explores specific separation condition for each system, suggesting that more labor force and toxic organic solvent would be saved. That means the introduction of chemometric method to HPLC makes the analysis of multiple analytes in various/complex systems by a general separation condition possible.

3.3. Method validation

3.3.1. Statistical parameters

Root mean squared error of prediction (RMSEP) [49] and figures of merit (FOM) [50] such as limit of detection (LOD) and limit of quantitation (LOQ) [51] are important parameters which could be used as criterions when comparing or assessing the reliability of analytical results and newly developed analytical strategies. In this work, these parameters were calculated by the following equations:

$$\mathrm{RMSEP} = \sqrt{\sum_{n=1}^{N} \frac{(x - x_{fit})^2}{N - 1}} \quad (5)$$

$$\mathrm{LOD} = 3.3\, s(0) \quad (6)$$

$$\mathrm{LOQ} = 10\, s(0) \quad (7)$$

Here, x and x_{fit} are the actual and predicted concentrations of the analytes, respectively; N is the number of prediction samples; $s(0)$ is the standard deviation of analyte predicted concentration in three different blank samples.

The LODs and LOQs of the studied eleven antihypertensives in three types of matrices, i.e. human serum samples, AV samples and

Table 6
Inter-day precision of the studied eleven antihypertensives in human serum, AV and EU samples ($N = 3$).

Samples	TRI ($\mu g\,mL^{-1}$)			CAP ($\mu g\,mL^{-1}$)			BIS ($\mu g\,mL^{-1}$)			PRO ($\mu g\,mL^{-1}$)			FUR ($\mu g\,mL^{-1}$)			IND ($\mu g\,mL^{-1}$)		
	Add	Mean	RSD%	Add	Mean	RSD%	Add	Mean	RSD%	Add	Mean	RSD%	Add	Mean	RSD%	Add	Mean	RSD%
Serum	0.28	0.28	3.8	2.70	2.58	5.1	1.80	1.81	7.9	0.81	0.76	5.1	0.76	0.72	4.7	0.72	0.72	4.3
	1.04	1.04	3.9	5.40	5.61	8.4	3.06	2.99	7.4	1.83	1.80	3.8	1.90	1.82	4.5	1.80	1.76	8.5
	1.79	1.60	2.3	9.00	8.86	2.0	3.60	3.34	2.9	2.65	2.81	2.9	3.42	3.38	7.8	3.24	3.21	4.0
AV	0.28	0.28	5.5	2.70	2.82	2.7	1.80	1.85	3.5	0.81	0.75	6.4	0.76	0.73	2.9	0.72	0.71	6.8
	1.04	1.05	4.0	5.40	5.44	6.7	3.06	2.76	4.1	1.83	1.87	3.0	1.90	1.85	8.0	1.80	1.80	8.4
	1.79	1.85	3.9	9.00	8.83	7.2	3.60	3.32	4.6	2.65	2.82	1.5	3.42	3.33	6.6	3.24	3.21	3.0
EU	0.66	0.64	3.1	4.50	4.40	1.2	1.26	1.27	9.4	0.81	0.77	3.0	0.76	0.76	5.5	0.72	0.74	2.0
	1.04	1.04	9.4	7.20	6.91	3.6	1.80	1.77	8.4	1.83	1.82	3.7	1.90	1.92	5.1	1.80	1.75	4.7
	1.79	1.70	8.4	9.00	8.65	6.9	3.60	3.73	7.2	2.65	2.68	3.6	3.42	3.37	6.6	3.24	3.10	1.0

Samples	DOX ($\mu g\,mL^{-1}$)			CAR ($\mu g\,mL^{-1}$)			RES ($\mu g\,mL^{-1}$)			AML ($\mu g\,mL^{-1}$)			LOS ($\mu g\,mL^{-1}$)		
	Add	Mean	RSD%	Add	Mean	RSD%	Add	Mean	RSD%	Add	Mean	RSD%	Add	Mean	RSD%
Serum	1.26	1.22	4.9	0.95	0.96	2.9	1.20	1.22	5.8	2.01	1.88	9.3	1.32	1.31	4.8
	2.94	2.90	3.0	1.91	1.80	5.2	4.40	4.35	2.6	10.04	10.17	6.2	4.84	4.90	3.7
	3.78	3.55	5.7	2.86	2.89	4.5	7.60	7.13	6.1	18.07	18.18	7.3	8.36	8.25	2.0
AV	1.26	1.34	4.3	0.95	0.98	2.3	1.20	1.18	6.1	2.01	2.01	2.9	1.32	1.38	2.1
	2.94	2.99	5.6	1.91	1.85	3.2	4.40	4.59	2.9	10.04	10.55	1.8	4.84	4.84	4.2
	3.78	3.72	1.1	2.86	2.91	0.4	7.60	7.46	2.4	18.07	17.43	7.6	8.36	7.84	3.0
EU	0.84	0.79	4.4	0.95	0.96	6.2	1.20	1.17	4.3	2.01	2.00	3.5	1.32	1.36	8.5
	2.10	2.00	2.4	1.91	1.87	4.4	4.40	4.57	3.1	10.04	10.06	0.8	4.84	4.62	4.7
	3.78	3.66	3.3	2.86	2.89	4.0	7.60	7.64	2.3	18.07	17.79	1.7	8.36	7.81	3.7

EU samples, were collected in Table 5. Bearing in mind that the method used a DAD detector and the complexity of the matrices, the LODs of analytes in three systems were acceptable. The statistical parameters indicated that the values were reasonable and the proposed analytical strategy can be used as an alternative method for simultaneously analyzing multiple classes of antihypertensives in various matrices.

3.3.2. Inter-day analysis

In order to test the reproducibility of the proposed method, three different concentration levels, corresponding to low, middle and high levels were prepared. The inter-day precision of the method was performed by replicate injections of prediction solutions over a period of 3 different days in a month.

The reproducibility of inter-day analysis were summarized in Table 6, from which one can find that, average recoveries of studied eleven antihypertensives were close to 100% and the RSDs were found to be no more than 10%. These values indicate clearly the feasibility and reliability of the proposed method.

4. Conclusions

With a propose to develop a general and reliable method for the comprehensive analysis of multiple classes of antihypertensives in most concerned matrix systems, i.e. human serum, health product and Chinese patent medicine samples, the authors proposed a novel analytical strategy that combines three-way chromatographic techniques, HPLC-DAD, with second-order calibration methodology. All of the qualitative and quantitative results, as well as validating statistical parameters demonstrate that the proposed method is capable to quantify analytes of interest in different complicated matrix systems with the same isocratic chromatographic condition. It is apparently that three-way HPLC techniques, such as HPLC-DAD, coupled with chemometric method can provide a more general chromatographic condition and such strategy could be a reliable and efficient platform for the simultaneous analysis of multiple analytes in various/complex matrices.

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