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A New Concept for Testing Chromatographic Peak Purity: Application to Peak Purity Determination of Main Ingredients in Tea Extracts

MARWA S. MONEEB[1,*], ISMAIL I. HEWALA[1], HATEM A. ELMONGY[2] and ABDEL-AZIZ M. WAHBI[1]

[1]Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt
[2]Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Damanhour, Damanhour, Egypt

*Corresponding author: Fax: +20 3 4873273; Tel: +20 3 4871317; E-mail: marwamoneeb@yahoo.com

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A new concept was applied to test the spectral purity of chromatographic peaks. The proposed concept is based on the application of the orthogonal functions (P1 and P2) to the absorption spectra recorded at five different time intervals throughout the eluted chromatographic peak. Ratios of strong peaks of the p1- and p2- convoluted absorption spectra were computed for the investigated samples and for standard solutions of the corresponding reference materials. Such ratios are considered spectrophotometric characteristics for each compound, independent of concentration. For pure peaks, the calculated orthogonal ratios at different time intervals were similar to those of spectrum apex with RSD values less than 1 %. This new concept was applied to determine the peak purity of the main alkaloids in tea extracts which contain different interfering structurally related substances. The proposed concept could be considered a universal technique for peak purity assessment in any quality control laboratory.

Keywords: Peak purity, HPLC, Tea, Orthogonal functions.

INTRODUCTION

The verification of the chromatographic peak purity is an essential requisite of a separation analysis. That is to ensure that no co-eluting impurity contributes to the peak's response of the separated species. The investigation and confirmation of peak purity should be performed before quantitative information from a chromatographic peak is used for further calculations[1]. Neglecting peak purity confirmation leads to erroneous quantitative results due to hidden impurities under the investigated peak. The presence of such impurities remains undiscovered, so important information might be lost. Validated analytical methods usually include the peak purity check as a major item in the list of their method validation criteria including selectivity (peak purity determination), linearity, limits of detection and quantitation, quality of data (accuracy and precision) and ruggedness[1].

Recently, the rapid-scanning detectors based on the linear photodiode array provide a useful solution for peak purity testing challenges. They have the ability of on-line acquisition of UV-visible spectra during the peak's elution that enables flexible selection of wavelengths for optimum detection sensitivity of each component. These detectors record several signals at different wavelengths in parallel; and extract signals from a 3-D data matrix, containing spectral data and separation signals. Moreover, the spectral data can be manipulated by a variety of digital algorithms to ascertain the purity of an eluting peak. The combination of absorbance spectra (zero, first, second, …order) and retention time data provides a powerful data set, not only for direct solute identification, but also for assessment of chromatographic peak purity. Digital algorithms for manipulating such spectral data include derivative chromatograms in time domain method[2,3], derivative spectra overlay in time domain method[4], derivative spectra in the wavelength domain[5], absorbance ratio method[4,6], spectral overlay in wavelength domain method[4], relative absorption spectra in wavelength domain[5] and plots of log A *versus* the wavelength in wavelength domain[4,7,8].

Glenn's method of orthogonal function[9] has been extensively used to eliminate interference in spectrophotometric analysis. An absorption curve f(λ) can be expanded in terms of orthogonal functions as follows:

$$f(\lambda)= p_0P_0+p_1P_1+p_2P_2+p_3P_3+\cdots+p_nP_n \quad (1)$$

f(λ) denotes the absorption of the sample at (n+1) wavelengths. P_j are the orthogonal polynomials given in standard works on numerical analysis[10] and p_j, are their respective coefficients [where j stands for the order of the polynomial (0, 1, 2, 3, …n)]. Thus p_0P_o is known as the constant component; p_1P_1 is the linear component; p_2P_2 is the quadratic component, *etc*. of

the curve. The coefficients, p_j are linearly related to concentration[9,11]. Thus:

$$p_j = \alpha_j C_a \quad (2)$$

where: α_j is the coefficient of P_j for the A (1 %, 1 cm) of the pure compound a. C_a is the concentration. In the presence of irrelevant absorption, each coefficient is the sum of two terms. Thus,

$$P_j = \alpha_j C_a + p_j (Z) \quad (3)$$

where: Z denotes contribution from irrelevant absorption[11].

The interference can be corrected for by using a suitable polynomial, number of points, wavelength range and intervals. Agwu and Glenn[12] developed the ratio pj/p_0 for testing the purity of several pharmaceutical compounds, where j = 2, 3, 4,….n and p_0 is the coefficient of the constant component. These ratios are independent of concentration and are sensitive to the presence of interferents that contribute to p_0. Orthogonal coefficients ratio has been used to test peak purity in spectrophotometric analysis[13]. It has also been used to test band purity of drugs in the infrared region[14]. Ratios of strong peaks of the p1- and p2- convoluted absorption spectra are considered spectrophotometric characteristics for each compound, independent of concentration[15].

In this study, orthogonal functions (P1 and P2) in time domain and orthogonal function ratios were used for the first time to test the spectral purity of eluted chromatographic peaks. The orthogonal convoluted curves of eluted peak spectra at different time intervals of the peak were generated and overlaid for investigation of peak purity. The orthogonal ratios of strong peaks of the p1- and p2- convoluted absorption spectra were computed for the investigated samples and for standard solutions of the corresponding reference materials. Such ratios are considered spectrophotometric characteristics for each compound, independent of concentration. These ratios can inspect the variations in peak homogeneity and ascertain its purity. The pure peaks share the same spectral features, hence, produce uniform ratios and perfectly superimposed convoluted curves.

The aim of the present work is to introduce a new concept for testing the spectral purity of the chromatographic peaks through the application of the orthogonal functions (P_1 and P_2). The orthogonal functions and the ratios of the convoluted absorption spectra were computed at the peaks optima at different time intervals of the eluted peak to identify the investigated compounds and ensure their purity. The proposed new concept was applied to determine the peak purity of the main ingredients in black, green and white tea extracts. The tea extracts are examples of multicomponent matrices containing different interfering structurally related substances. The control of the peak purity of investigated compounds in such matrices is a crucial requisite prior to quantitation to ensure the absence of interference from other biosynthesized structurally related compounds. Caffeine (CF) and theobromine (TB) as the main methylxanthine alkaloids of tea extracts[16] were separated using HPLC-DAD. The purity of the separated components was tested using the proposed concept and compared to the purity data obtained using the Agilent Chemstation Software.

EXPERIMENTAL

Pharmaceutical grade of caffeine and theobromine were kindly supplied by Amriya for Pharmaceutical Industries, Alexandria, Egypt. Acetonitrile (HPLC grade) was purchased from Merck. Sodium acetate, glacial acetic acid, anhydrous sodium sulphate, lead acetate and sodium carbonate were purchased from Sigma Aldrich, Egypt and were all of analytical grade. Black, green and white tea samples were purchased from a local market.

The HPLC-DAD system (Agilent Technologies, Palo Alto, CA, USA) consisted of quaternary pump G1311A which comprises a solvent cabinet, vacuum degasser G1322A, a four-channel gradient pump and a photodiode array. The chromatographic system is equipped with thermostated column compartment G1316A and manual injector that uses a Rheodyne 7725i 7-port sample injection valve fitted with a 20 µL sample loop. All are Agilent 1200 series.

The chromatographic separations were performed using the chromatographic conditions described in the BP under the monograph of theobromine "test for related substances"[17]. Separation was performed on Zorbax C18 column (250 × 4.6 mm, 5 µm). The column was thermostated at 25 °C during analysis. Agilent chemstation software for LC was used for data acquisition and analysis.

The mobile phase consisted of acetonitrile-acetate buffer (0.16 M) (7:93 v/v) adjusted to pH 4.7 ± 0.1 with glacial acetic acid (system I). The mobile phase was modified to acetonitrile-acetate buffer (pH 4.7; 0.16 M) (12:88 v/v) (system II) for application of the new concept. The separation was carried out at a flow rate of 1 mL min^{-1} and injection volume of 20 µL. The wavelength of detection was set at 272 nm using photodiode array detector. The mobile phases used were degassed and filtered through a 0.45 µm membrane filter (Millipore, Milford, MA, USA) prior to use. The samples solutions were filtered using 0.45 µm disposable filters.

Preparation of the stock and standard solutions: Stock solutions of caffeine and theobromine were prepared by weighing separately 10 mg of each reference standard into a 100 mL volumetric flask. The powder was dissolved in acetate buffer pH 4.7. The dissolution of theobromine necessitates sonication for 10 min at 50 °C. The solutions were diluted to volume with the same solvent. The caffeine working standard solution (50 µg mL^{-1}) was prepared by transferring 50 mL aliquot of the stock solution into a 100 mL volumetric flask and then diluted to volume with acetate buffer. The theobromine working standard solution (20 µg mL^{-1}) was prepared by transferring 20 mL aliquot of the stock solution into a 100 mL volumetric flask and then diluted to volume with acetate buffer.

Preparation of sample solutions: Samples of 1 g of black, green and white tea were separately weighed and boiled for 0.5 h in 100 mL of distilled water. Evaporation was compensated during boiling. The obtained infusions were then filtered and each filtrate was quantitatively transferred into a 100 mL volumetric flask and diluted to volume with water. A 0.5 mL aliquot of lead acetate (10 % w/v) was added to a 25 mL aliquot of each sample to precipitate tannins and the mixture was stirred for 3 min at room temperature. The solutions were filtered and 0.1 mL of sodium carbonate (10 %

w/v) was added to the filtrates to remove excess lead acetate. The mixtures were stirred for 2 min at room temperature then filtered and volumes were adjusted to 100 mL with distilled water.

Software and treatment of data: The linear and quadratic orthogonal functions (P1 and P2) were computed for the absorbance readings extracted from the UV absorption spectra of eluted peaks using a BASIC program[18]. The absorbance readings of caffeine and theobromine were selected in the range of 210-310 nm at 1 nm interval and extracted at five different points throughout the eluted peak. The five points included the peak start, maximum ascending slope, peak maximum, maximum descending slope and peak end (Table-1). P1 and P2 orthogonal functions were computed at 1 nm intervals using 6-points orthogonal polynomials. The convoluted orthogonal absorption curves were generated and they were all overlaid for the same chromatographic peak. Orthogonal function ratios were computed at the peak optima of the p1- and p2- convoluted orthogonal absorption curves.

TABLE-1
FIVE SELECTED POINTS THROUGHOUT THE ELUTED PEAKS OF CAFFEINE AND THEOBROMINE

No.	Selected points	Compound retention time (min)	
		Caffeine	Theobromine
1	Peak start	8.59	3.88
2	Maximum ascending slope	8.66	3.94
3	Peak maximum	8.73	4.11
4	Maximum descending slope	8.81	4.35
5	Peak end	8.88	4.47

RESULTS AND DISCUSSION

The new concept was tested by its application to the assessment of the spectral purity of the peaks obtained from the HPLC of the standard solutions of caffeine, theobromine and their mixture using the BP chromatographic conditions (system I). The chromatograms of caffeine (50 μg mL^{-1}) and theobromine (20 μg mL^{-1}) reference standard solutions were separately recorded (Figs. 1A and 2A). The UV-absorption spectra were extracted at the five selected points across each peak (Figs. 1B and 2B). The p1- and p2- convoluted orthogonal absorption curves of the extracted spectra were computed (Figs. 1C, D and 2C, D). The wavelengths of orthogonal optima were recorded. Caffeine and theobromine being structural isomers, they share the same spectral characteristics[16]. Ratios of strong peaks of the p1- and p2- convoluted absorption spectra were computed (Table-2). The chromatogram of a mixture containing caffeine (50 μg mL^{-1}) and theobromine (20 μg mL^{-1}) is shown in Fig. 3. The spectral purity of the investigated compounds in their mixture was assessed using the new method. Both peaks were proved to be spectrally pure. The UV-absorption spectra extracted at different time intervals throughout each eluted peak are similar to that extracted at the peak apex and similar to those obtained for the peak of the pure standard. The p1- and p2- convoluted orthogonal absorption curves of the spectra obtained at different time intervals of the eluted peak share the same spectral features; intersect at the same wavelengths; and the ratios of the orthogonal (p1 and p2) optima of the UV-absorption spectra extracted at different time intervals of the eluted peaks are similar to those of spectrum apex with RSD % values less than 1 % (Table-3).

The results were in good agreement with those obtained from the application of the Agilent Chemstation software. The Agilent Chemstation software depends on the comparison of spectra at fixed points on the chromatographic peak with spectrum apex or any other reference spectra selected by the user using a similarity factor[1] which is defined as:

$$\text{Similarity} = r \times 1000 \quad (4)$$

$$\text{where, } r = \frac{\sum_{i=1}^{i=n}[(A_i - A_{av})\cdot(B_i - B_{av})]}{\sqrt{\sum_{i=1}^{i=n}(A_i - A_{av})^2 \cdot \sum_{t=1}^{t=n}(B_i - B_{av})^2}} \quad (5)$$

and A_i and B_i are measured absorbances in the first and second spectrum on the chromatogram, respectively at the same wavelength; n is the number of data points and A_{av} and B_{av} are the average absorbances of the first and second spectrum, respectively[1].

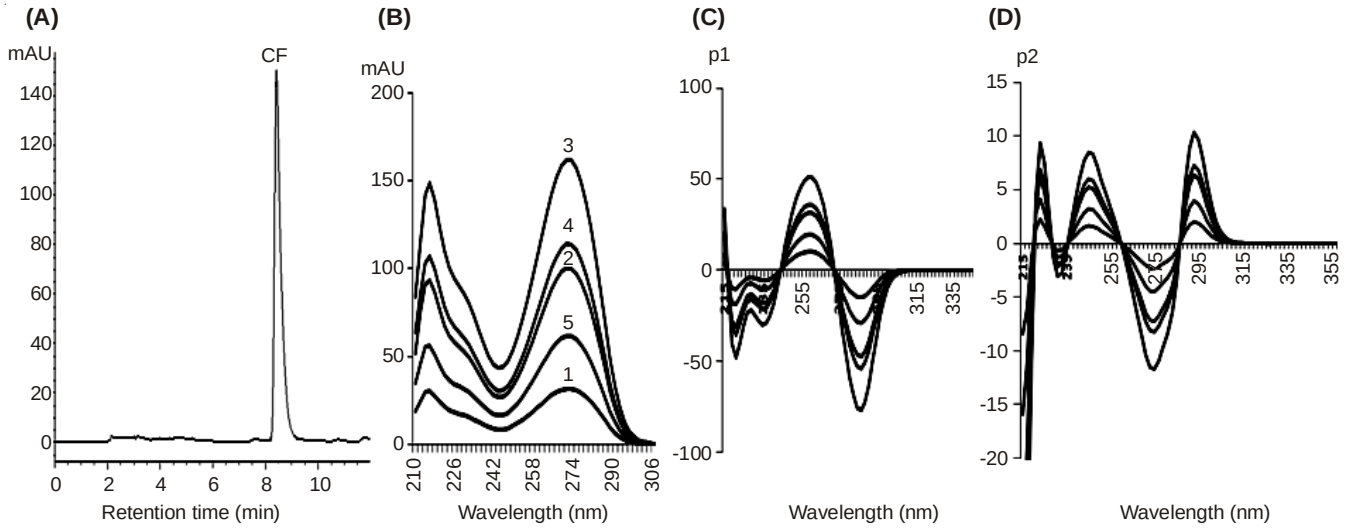

Fig. 1. (A) HPLC chromatogram of (50 μg mL^{-1}) caffeine standard solution using the mobile phase system I; (B) its spectra extracted at the five selected points (indicated in Table-1); (C) the corresponding p1- convoluted orthogonal spectra and (D) the p2- convoluted orthogonal spectra

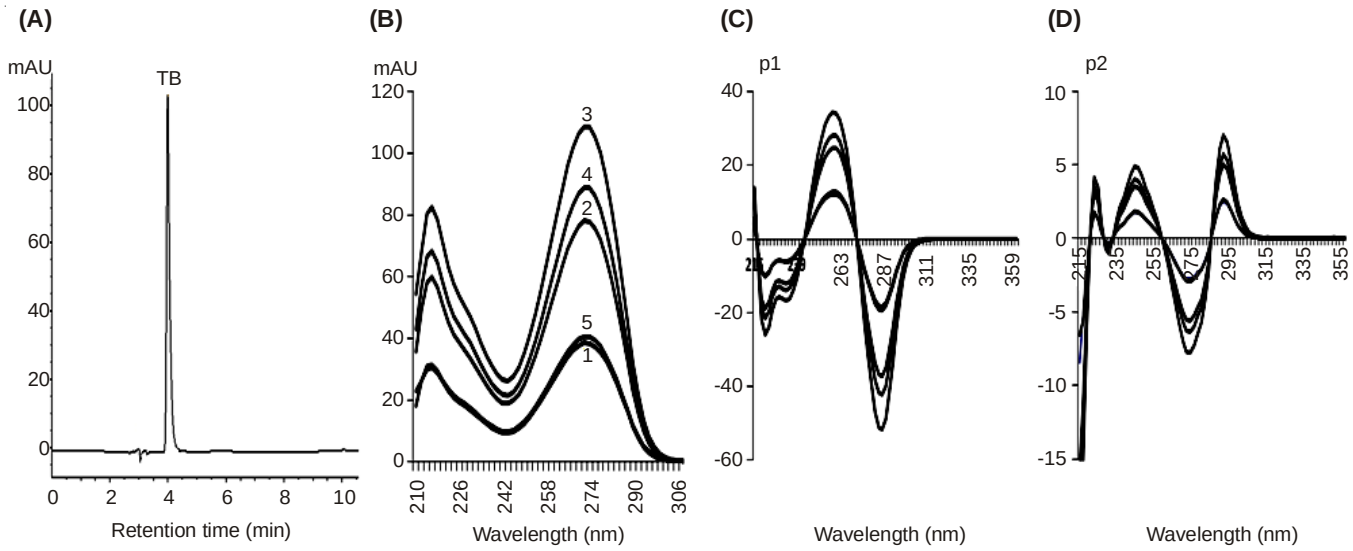

Fig. 2. (A) HPLC chromatogram of (20 μg mL^{-1}) theobromine standard solution using the mobile phase system I; (B) its spectra extracted at the five selected points (indicated in Table-1); (C) the corresponding p1- convoluted orthogonal spectra and (D) the p2- convoluted orthogonal spectra

TABLE-2
RATIOS OF p1- AND p2- ORTHOGONAL OPTIMA OF THE SPECTRA OF CAFFEINE AND THEOBROMINE REFERENCE STANDARDS EXTRACTED AT THE FIVE SELECTED POINTS THROUGHOUT THE ELUTION TIME OF THE HPLC PEAKS

Selected points[a]	Compound			
	Caffeine		Theobromine	
	$p1_{287/259}$[b]	$p2_{275/293}$[b]	$p1_{287/259}$[b]	$p2_{275/293}$[b]
1	1.502±0.023	1.099±0.086	1.499±0.011	1.096±0.066
2	1.504±0.011	1.116±0.101	1.503±0.067	1.099±0.015
3	1.503±0.012	1.121±0.011	1.504±0.081	1.100±0.071
4	1.503±0.033	1.127±0.021	1.503±0.019	1.103±0.075
5	1.498±0.021	1.122±0.019	1.503±0.088	1.109±0.097
Mean	1.502	1.117	1.502	1.101
SD	0.002	0.009	0.0017	0.004
RSD (%)	0.133	0.806	0.116	0.363

[a]Selected points indicated in Table-1; [b]Mean of 3 replicates ± SD.

TABLE-3
RATIOS OF p1- AND p2- ORTHOGONAL OPTIMA OF THE SPECTRA OF A MIXTURE CONTAINING CAFFEINE AND THEOBROMINE REFERENCE STANDARDS EXTRACTED AT THE FIVE SELECTED POINTS THROUGHOUT THE ELUTED PEAK USING SYSTEM I

Selected points[a]	Compound			
	Caffeine		Theobromine	
	$p1_{287/259}$[b]	$p2_{275/293}$[b]	$p1_{287/259}$[b]	$p2_{275/293}$[b]
1	1.502±0.015	1.123±0.046	1.506±0.042	1.086±0.071
2	1.505±0.056	1.130±0.032	1.496±0.077	1.094±0.056
3	1.501±0.035	1.123±0.034	1.491±0.032	1.105±0.072
4	1.505±0.043	1.132±0.076	1.495±0.036	1.107±0.022
5	1.501±0.025	1.124±0.015	1.502±0.047	1.111±0.011
Mean	1.503	1.126	1.498	1.100
SD	0.002	0.004	0.005	0.009
RSD (%)	0.122	0.340	0.333	0.818
% Deviation[c]	0.067	0.806	0.267	0.091

[a]Selected points indicated in Table-1; [b]Mean of 3 replicates ± SD; [c]% Deviation from the pure standard = [(|Mean of the ratio in the mixture–mean of the ratio of the pure standard|) / mean of the ratio of the pure standard] × 100. (Mean of the ratio of the pure standard is indicated in Table-2).

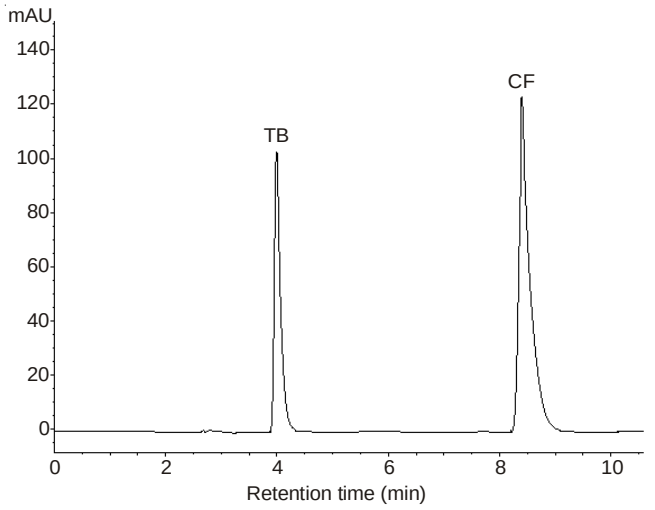

Fig. 3. HPLC chromatogram of a mixture containing (50 μg mL^{-1}) caffeine (CF) and (20 μg mL^{-1}) theobromine (TB) using the mobile phase system I

A similarity factor of 0 indicates no match and that of 1000 indicates identical spectra. Generally, values very close to the ideal similarity factor (greater than 995) indicate that the spectra are very similar, values lower than 990 but higher than 900 indicate some similarity and underlying data should be observed more carefully[1].

A threshold limit shows the effect of noise on a given similarity factor. In essence, a threshold value is a similarity factor with background noise contribution. The determination of noise threshold is performed automatically based on the standard deviation of pure noise spectra at a specified time with a user selectable number of spectra, usually at 0 min with 14 spectra. The threshold limit gives the range for which spectral impurity lies within the noise limit[1].

For the mixture of caffeine and theobromine reference standards, the purity factors for caffeine and theobromine were 999.986 and 999.954, respectively. The automatically computed threshold limits were 999.896 and 999.861,

respectively. Both purity factors lie within the corresponding threshold limits indicating pure peaks.

The use of the HPLC system I in the analysis of black, green and white tea extracts showed two distinctive peaks corresponding to caffeine and theobromine (Fig. 4). However, upon the application of the proposed concept to test the purity of the separated peaks, it was found that the caffeine peak was found to be spectrally pure in all the investigated tea extracts. This is because all terms of pure peak according to the proposed concept were met. The UV absorption spectra extracted at different time intervals throughout the eluted peak are similar to that extracted at the peak apex. The p1- and p2- convoluted orthogonal absorption curves obtained at different time intervals intersect at the same wavelengths. The ratios of the orthogonal p1- and p2- optima of the UV absorption spectra extracted at different time intervals of the eluted peak are similar to those of spectrum apex with RSD % values less than 1 % (Tables 4-6). The theobromine peak was found to be impure in all extracts, as the UV-absorption spectra extracted at different time intervals throughout the eluted peak intersect with that extracted at the peak apex as shown in green tea

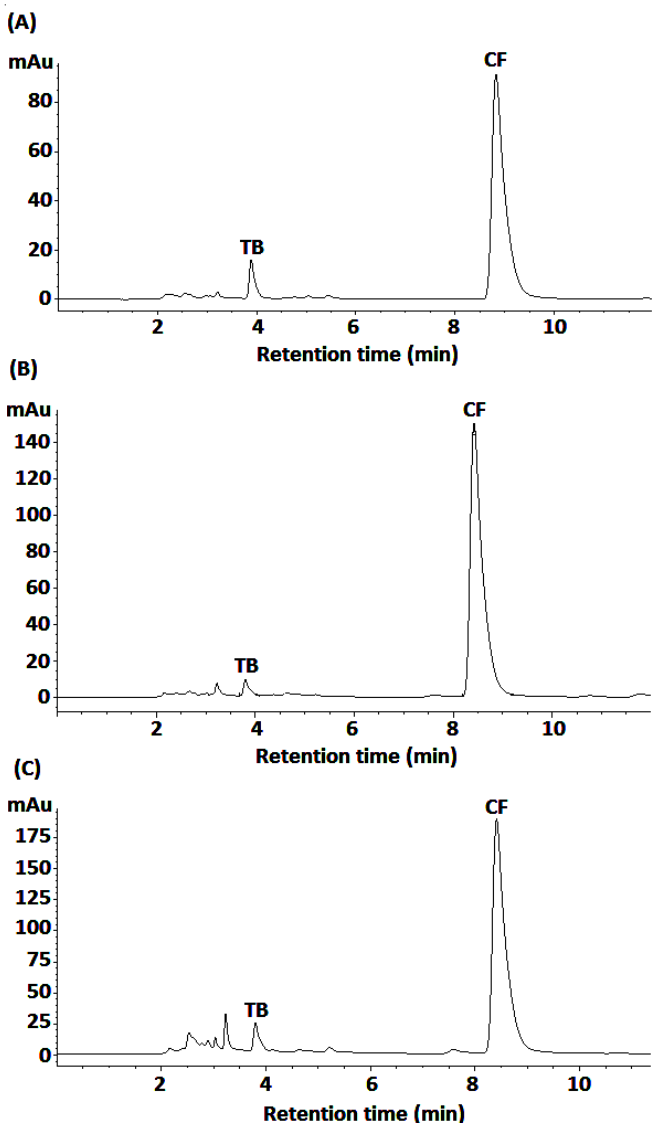

Fig. 4. HPLC chromatograms of (A) black tea extract; (B) green tea extract and (C) white tea extract using mobile phase system I

TABLE-4
RATIOS OF p1- AND p2- ORTHOGONAL OPTIMA OF THE SPECTRA OF BLACK TEA EXTRACT EXTRACTED AT THE FIVE SELECTED POINTS THROUGHOUT THE ELUTED PEAK USING SYSTEM I

Selected points[a]	Compound			
	Caffeine		Theobromine	
	$p1_{287/259}$[b]	$p2_{275/293}$[b]	$p1_{287/259}$[b]	$p2_{275/293}$[b]
1	1.505±0.081	1.122±0.058	1.546±0.055	1.156±0.069
2	1.505±0.014	1.125±0.071	1.426±0.062	1.264±0.051
3	1.504±0.044	1.123±0.046	1.411±0.061	1.135±0.022
4	1.502±0.036	1.122±0.044	1.515±0.022	1.217±0.041
5	1.497±0.015	1.125±0.016	1.388±0.094	1.235±0.015
Mean	1.503	1.123	1.457	1.201
SD	0.003	0.001	0.062	0.048
RSD (%)	0.199	0.089	4.245	4.038
% Deviation[c]	0.067	0.537	3.000	9.083

[a]Selected points indicated in Table-1; [b]Mean of 3 replicates ± SD; [c]% Deviation from the pure standard = [(|Mean of the ratio in the mixture – mean of the ratio of the pure standard|) / mean of the ratio of the pure standard] ×100. (Mean of the ratio of the pure standard is indicated in Table-2).

TABLE-5
RATIOS OF p1- AND p2- ORTHOGONAL OPTIMA OF THE SPECTRA OF GREEN TEA EXTRACT EXTRACTED AT THE FIVE SELECTED POINTS THROUGHOUT THE ELUTED PEAK USING SYSTEM I

Selected points[a]	Compound			
	Caffeine		Theobromine	
	$p1_{287/259}$[b]	$p2_{275/293}$[b]	$p1_{287/259}$[b]	$p2_{275/293}$[b]
1	1.503±0.022	1.122±0.031	1.483±0.049	1.102±0.017
2	1.504±0.015	1.123±0.091	1.461±0.017	1.217±0.088
3	1.503±0.052	1.125±0.072	1.473±0.088	1.202±0.061
4	1.501±0.021	1.129±0.019	1.360±0.089	1.131±0.077
5	1.501±0.017	1.131±0.055	1.409±0.108	1.150±0.058
Mean	1.502	1.126	1.432	1.160
SD	0.001	0.003	0.044	0.043
RSD (%)	0.067	0.266	3.039	3.721
% Deviation[c]	0.000	0.806	4.660	5.359

[a]Selected points indicated in Table-1; [b]Mean of 3 replicates ± SD; [c]% Deviation from the pure standard = [(|Mean of the ratio in the mixture – mean of the ratio of the pure standard|) / mean of the ratio of the pure standard] ×100. (Mean of the ratio of the pure standard is indicated in Table-2).

TABLE-6
RATIOS OF p1- AND p2- ORTHOGONAL OPTIMA OF THE SPECTRA OF WHITE TEA EXTRACT EXTRACTED AT THE FIVE SELECTED POINTS THROUGHOUT THE ELUTED PEAK USING SYSTEM I

Selected points[a]	Compound			
	Caffeine		Theobromine	
	$p1_{287/259}$[b]	$p2_{275/293}$[b]	$p1_{287/259}$[b]	$p2_{275/293}$[b]
1	1.504±0.067	1.123±0.077	1.497±0.061	1.117±0.081
2	1.504±0.071	1.122±0.072	1.500±0.077	1.119±0.066
3	1.502±0.096	1.122±0.058	1.701±0.084	1.153±0.071
4	1.500±0.073	1.118±0.052	1.966±0.099	1.263±0.011
5	1.491±0.083	1.136±0.077	1.497±0.102	1.100±0.021
Mean	1.500	1.124	1.632	1.150
SD	0.005	0.006	0.184	0.059
RSD (%)	0.333	0.534	11.275	5.130
% Deviation[c]	0.133	0.627	8.655	4.450

[a]Selected points indicated in Table-1; [b]Mean of 3 replicates ± SD; [c]% Deviation from the pure standard = [(|Mean of the ratio in the mixture – mean of the ratio of the pure standard|) / mean of the ratio of the pure standard] ×100. (Mean of the ratio of the pure standard is indicated in Table-2).

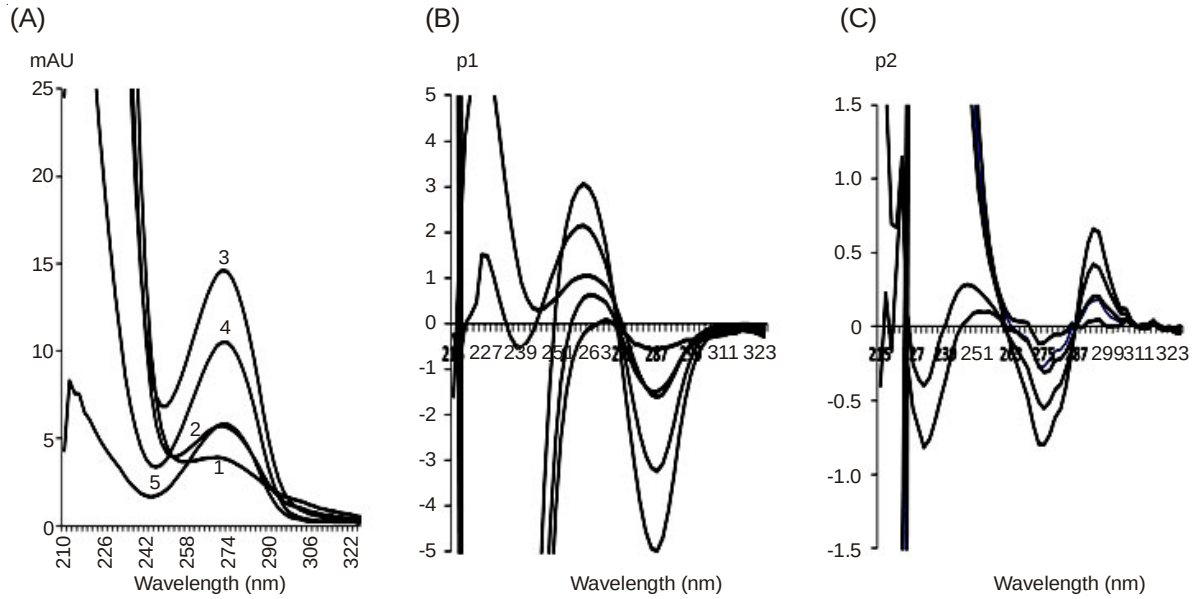

Fig. 5. (A) Spectra of theobromine at the five points (indicated in Table-1) extracted from HPLC of green tea extract, (B) their p1- convoluted orthogonal spectra and (C) their p2- convoluted orthogonal spectra

extract as a representative example in Fig. 5. The p1- and p2-convoluted orthogonal absorption curves of the spectra obtained at different time intervals of the eluted peak intersect at different wavelengths (Fig. 5). Furthermore, the ratios of the orthogonal (p1 and p2) optima of the UV-absorption spectra extracted at different time intervals of the eluted peaks differ from those of spectrum apex with RSD % values higher than 3 % (Tables 4-6). The Agilent Chemstation software method has been applied. It showed that the caffeine peaks were pure as the purity factors were 999.998, 999.987 and 999.989 that lie within the automatically computed threshold limits 999.994, 999.983 and 999.987 for black, green and white tea extracts, respectively. While the theobromine peaks were impure as the purity factors were found to be 999.769, 999.886 and 999.864 exceeding the automatically computed threshold noise limits 999.989, 999.987 and 999.977 for black, green and white tea extracts respectively. This indicates impurity.

The mobile phase was modified in order to separate any interference with the theobromine peak. When the modified HPLC system II was applied to the analysis of the investigated tea extracts, a new small peak appeared next to the theobromine peak (Fig. 6). This was suggested to be a co-eluting component with theobromine when system I was used. The purity of theobromine peak was checked again using the proposed concept and it was found to be pure.

The orthogonal ratios could be also used to confirm the identity of chromatographic peaks by comparing the mean orthogonal ratios of the investigated compounds to that of the corresponding pure reference materials. In all the studied cases the percentage deviation did not exceed 1 % for the pure caffeine peak; and was found to be ≥ 3 % for the impure theobromine peak (Tables 4-6). This confirms the validity of these ratios in the identity verification of compounds.

Conclusion

The proposed concept could be successfully applied to the spectral purity assessment of HPLC peaks. Unlike Agilent

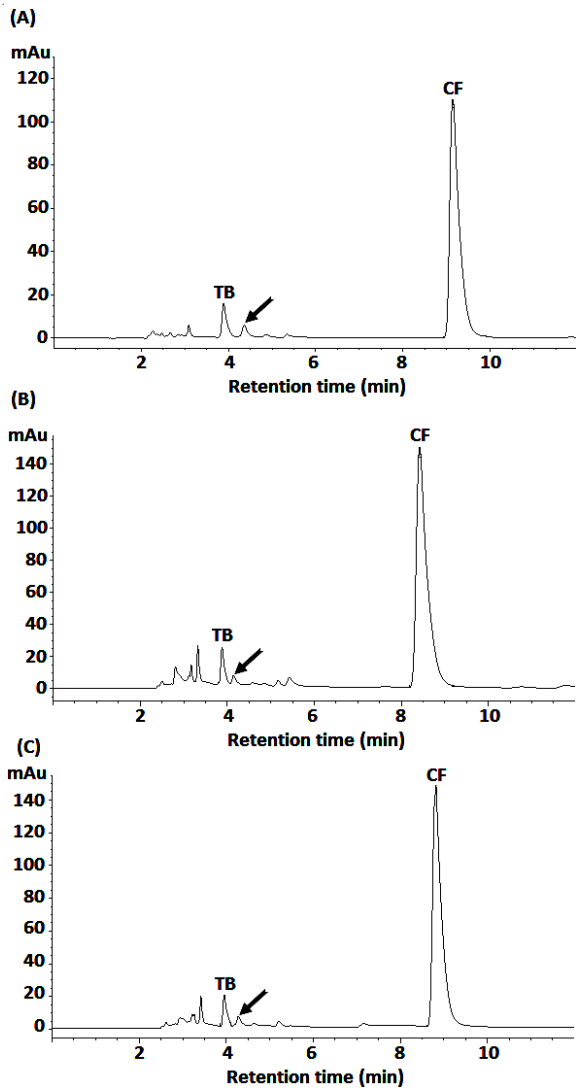

Fig. 6. HPLC chromatogram of (A) black tea extract; (B) green tea extract and (C) white tea extract using mobile phase system II showing a resolved peak near theobromine peak

Chemstation software method, the proposed concept is non-confusing and its results are easily and rigorously interpreted. Even in situations where the purity results are free from sources of confusion, the proposed method remains superior to the Chemstation method as it does not only detect spectral interference and confirm the purity of the resolved peaks, but also constitutes a useful tool for the identification of the resolved components. The proposed concept could be considered a universal technique for peak purity assessment in any quality control laboratory.

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