J Fluoresc (2015) 25:297–303
DOI 10.1007/s10895-015-1508-2

ORIGINAL PAPER

Fluorescence Spectroscopy as a Tool for Determination of Coumarins by Multivariate Calibration

Roman Poláček · Pavel Májek · Katarína Hroboňová · Jana Sádecká

Received: 21 October 2014 / Accepted: 2 January 2015 / Published online: 18 January 2015
© Springer Science+Business Media New York 2015

Abstract At present, it is necessary to check the quality of many food products in which the content of coumarins is limited. Since a rapid and simple method for the determination of coumarin (COU), 4-hydroxycoumarin (4HC) and dicoumarol (DC) in tea samples was needed, we developed an alternative option to chromatography, i.e., fluorescence spectroscopy with multivariate calibration. The synchronous fluorescence spectra were recorded at constant wavelength differences 70, 80 and 90 nm from 200 to 400 nm. The different experimental parameters affecting the synchronous fluorescence intensities of the analytes were carefully studied and optimized. Partial least squares (PLS) method and multi linear regression (MLR) were compared on determining the concentrations. The best results were obtained by the PLS method on synchronous fluorescence spectra at $\Delta\lambda$=90 nm. The results from the analysis of herbal tea *Melilotus officinalis* by synchronous fluorescence spectroscopy with PLS model are equivalent with the results from HPLC. Fisher *F*- test and Student's *t*- test confirmed this finding.

Keywords Synchronous fluorescence spectrometry · Multivariate calibration · Coumarin · 4-hydroxycoumarin · Dicoumarol

Introduction

COU is a natural substance found in many plants, especially in sweet clover (*Melilotus officinalis* and *Melilotus albus*), in the tonka bean, in woodruff, vanilla grass, cassia cinnamon (*Cinnamomum cassia*) [1]. It was found also in fruits, e.g., in strawberry, cherry and raspberry. COU is used as a fixative in perfumes, additives for paints and spray, and food flavoring [2]. COUs are considered to be hepatotoxic and administered in very high doses over long periods of time they proved to be carcinogenic in animal experiments. However, recent scientific findings of the European Food Safety Authority (EFSA) indicate that they have no genotoxic mechanism of action [3].

Most coumarins have pharmacological properties and are used in various areas of medicine [4]. COU and its derivatives have great applicability in anticoagulant drugs, which alter the kinetics of blood coagulation. The mechanism of action is due to the chemical similarity to vitamin K1, which in the synthesis of factors II, VII, IX, X and protein C and S, causes the appearance of carboxyl forms of these factors followed by their inability to act adequately in kinetics of coagulation [5]. The plants containing COU convert it into 4HC and subsequently condense to DC (3, 3′-methylene-bis-(4′ hydroxycoumarin)). The conversion of COU to DC is a result of the action of several molds and fungi, including *Penicillium nigricans*, *Penicillium jensi*, *Aspergillus fumigatus*, *Fusarium* and *Mucor* [6]. Following ingestion, DC antagonizes vitamin K epoxide reductase, which is responsible for maintaining the stores of active vitamin K1 in the body. In the absence of sufficient active vitamin K1, vitamin K-dependent clotting factors (II, VII, IX, X) come into play, which subsequently results in the occurrence of hemorrhagic diathesis. Clinical signs include spontaneous hematomas, weakness, tachycardia, tachypnea, pale mucous membranes, and prolonged prothrombin times. DC toxicities were reported for the first time in dead cattle [7]. DC has a much higher anticoagulant ability than COU. DC and its derivatives, like Warfarin, are used as anticoagulant drugs. The European Committee established the maximum content of COU in food and cosmetic products and prohibited the presence of DC [8, 9].

R. Poláček (✉) · P. Májek · K. Hroboňová · J. Sádecká
Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, SK-812 37 Bratislava, Slovakia
e-mail: roman.polacek@stuba.sk

The intensive use of COU and its compounds in recent years has required the development of rapid and robust methods of analysis for foods, cosmetics and health care products [10]. Recently, HPLC [11–13] and GC-MS [14] have been used for simultaneous determination of COU and its derivatives, including DC, in cosmetic products, while in plants only HPLC was applied [15]. Only one fluorescence method has been developed for simultaneous determination of 6-methylcoumarin and 7-methoxycoumarin in cosmetics, using decomposition of excitation-emission fluorescence matrix by parallel factor analysis (PARAFAC) [10].

Fluorescence spectroscopy is a widely used method in quantitative analysis due to its sensitivity, selectivity, and relatively low cost. Synchronous fluorescence spectroscopy (SFS) has several advantages over conventional fluorescence spectroscopy, concerning especially simple spectra, which are sharp and narrow. SFS consists essentially of simultaneous scanning of excitation and emission monochromators keeping a constant wavelength difference ($\Delta\lambda$) between them. SFS serves as a very simple, effective method for obtaining data and for quantitative determination in one run [16]. Much information about fluorophores can be derived from SFS using multivariate analysis. PLS methods applied on synchronous fluorescence spectra have already been used for simultaneous determination of the compound in a complex matrix. In [17] the authors described these methods, where phenol, resorcinol and hydroquinone were determined in smoker environment. Determination of products in pharmaceutical preparations and different biological fluids were reported. Thus salicylic acid and diflunisal in human serum [18], furesomide and triamterene in pills, triamterene in urine [19], quantification of the content of kerosene in petrol [20] and in food industry, caffeine and caramel in cola type drink [21] and caffeine, caramel and riboflavin in energy drinks [21] were established. So far references for simultaneous determination of COU, 4HC and DC by fluorescence spectroscopy are not available.

The aim of the present work was to develop a rapid, simple and low-cost method for simultaneous determination of COU, 4HC and DC by synchronous fluorescence spectroscopy and multivariate calibration using PLS and MLR methods.

Materials and Methods

Material

COU, 4HC and DC pure sample were purchased from Sigma-Aldrich (United States). Herbal tea from sweet clover plant (*Melilotus officinalis*) was obtained in a local market with bioproducts and in pharmacies.

Reagents

Acetonitrile and methanol were obtained from MERC (Germany), with HPLC gradient grade. Citric acid and disodium hydrogen phosphate were purchased from Sigma-Aldrich (United States) and their stock solutions (100 mL) were prepared by dissolving 3.562 g of disodium hydrogen phosphate and 2.101 g of citric acid in water. These solutions were used to prepare Meltraine buffer solutions at the range of pH 2.4–8.0.

Apparatus

Fluorescence spectra were recorded by a Lumina Fluorescence Spectrometer (Thermo Scientific), equipped with a 150 Watt Ozone-free Xenon lamp. Excitation and emission splits were both set at 5.0 nm. Scan speed was 200 nm min^{-1}. PMT Voltage was established at 500 Volts. Synchronous fluorescence spectra were collected by simultaneously scanning the excitation and emission monochromators in the excitation wavelength range at 200–400 nm, with constant differences $\Delta\lambda$ (from 10 to 100 nm, with step 10 nm) between them. A 1 cm quartz cell was used. The obtained data were treated by LUMINOUS SOFTWARE. All calculations were done using Microsoft Office Excel 2010, Statistica version 7.0 (StatSoft, USA, 2004), MATLAB version 7.0 (The MathWorks Inc., USA, 2005) and PLS_Toolbox version 6.0 (Eigenvector Research Inc., USA, 2010).

The digital pH meter Model 215 (Denver Instrument, USA) calibrated with standard buffers was used for checking the pH of buffer solutions.

Standard Solutions

Standard solutions of COU, 4HC and DC were prepared by dissolving 1.0 mg in 50 mL methanol in a volumetric flask. The standard solution of DC was stable for 4 days when kept in a refrigerator at 4 °C. The COU and 4HC standard solutions were stable for 15 days when kept in a refrigerator at 4 °C.

HPLC Reference Method

HPLC analysis was implemented on an Agilent Technologies 1200 series consisting of a binary pump, thermostat, autosampler and diode array detector. Chromatographic column was Symmetry C18 (3.9×150 mm, 5 μm). The mobile phase consisted of a mixture of 99.7 % methanol plus 0.3 % acetic acid (A) and 0.3 % acetic acid (B). The gradient method was used according to the following program: isocratically 50 % B in min 0, followed by a linear increment to 90 % in min 17, increased to 100 % in min 20, followed by a linear return to the initial conditions in min 21. The flow rate was 0.5 mL min^{-1}. The column temperature was held constant at

23 °C and the injection volume was 10 μL. Each sample was injected at least three times. The diode array detector was worked at 280 nm.

Recommended Procedures

Calibration Curves for Individual Analytes

The synchronous fluorescence spectra were recorded in the range 200–400 nm at Δλ=90 nm. The synchronous fluorescence intensity at the synchronous maxima λ_{max} was read for each analyte. Independent calibration curve for each component was created from these maxima. Synchronous maxima are shown in Fig. 1. The regression equations for the data were computed and are shown in Table 1. Also in Table 1 are the values of λ_{max} and linearity range for the calibration curve. An acceptable linearity relation (R^2>0.998) was obtained between the relative fluorescence intensity of synchronous spectra and the concentration for each component. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using slope and intercept of calibration curves.

Figure of Merit

Figure of merit was used to characterize the quality of a given multivariate calibration model. This treatment utilizes net analyte signal calculations (NAS) defined as the part of the measured signal that is unique for the given analyte. NAS enable the estimation of the figures of merit in multivariate calibration models, such as sensitivity (SEN), selectivity (SEL), limit of determination (LOD) and limit of quantification (LOQ) [22]. In Table 2 the Figure of merit of COU, 4HC and DC for PLS calibration model constructed from spectral data measured at Δλ=90 nm are summarized. These results concern models with optimal number of components.

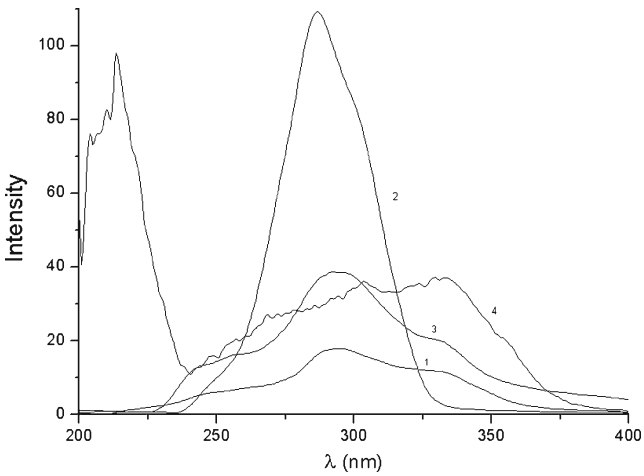

Fig. 1 Synchronous fluorescence spectra for COU (4 mg L^{-1}; 1), 4HC (0.1 mg L^{-1}; 2), DC (0.1 mg L^{-1}; 3) and tea extract (1.5 mL/ 10 mL methanol; 4) measured at Δλ=90 nm

Table 1 Analytical parameters of the SFS method using at Δλ=90 nm

	COU mgL^{-1}	4HC μgL^{-1}	DC μgL^{-1}
λ_{max} (nm)	295	286	300
Concentrate range	2.0–12.0	27.5–275	27.5–275
Coeficient of determination (R^2)	0.9988	0.9985	0.9985
Slope (b)	1.39	0.78	0.20
Intercept (a)	2.14	6.58	6.25
Limit of detection	0.3	6.3	7.3
Limit of quantitation	1.0	20.7	24.2

Procedure for Synthetic Mixtures

The aliquot part of each standard solution and the appropriate part of tea extract were transferred into volumetric flasks diluted to the volume of 10 mL with methanol and well mixed. Measurements of the synthetic sample by procedure of Calibration Curve were then performed. The collected spectra were evaluated by multivariate calibration models.

Procedure for Tea Samples

Herbal tea was mashed to powder and a 0.1 g was transferred into a small conical flask. This sample was extracted with 15 mL of methanol under stirring for 90 min and then the extract was filtered into a 20 mL volumetric flask and filled with the same solvent. Subsequently, 2.5 mL from the sample was transferred into a 10 mL volumetric flask and diluted with methanol to the mark.

Data Analysis

Multivariate calibration is a method of building regression models between independent variables (X, in this case concentration of each analyte, simultaneously or singly), and dependent variables (Y, in this study synchronous fluorescence intensity at Δλ=70, 80 and 90 nm). It usually involves a calibration step and a prediction step. In the calibration step

Table 2 Analytical figures of merit by PLS model onto spectral data measured at Δλ=90 nm

Component	SEN ($L\mu g^{-1}$)	SEL	LOD (μgL^{-1})	LOQ (μgL^{-1})
Coumarin	12.9	0.4	700	2100
4-hydroxycoumarin	7.6	0.8	7.9	23.8
Dicoumarol	3.3	0.7	9.1	27.4

the relationship between spectra and component concentrations is estimated from an unknown sample spectrum. Of the available multivariate calibration procedures, PLS and MLR methods are widely used for multicomponent analysis.

Based on preliminary HPLC results for the concentration of COU, 4HC and DC in tea samples, two different sets (i.e., calibration and prediction sets) of synthetic samples were prepared for multivariate analysis. The experimental design of orthogonal arrays OA9 [23] was used to create calibration and prediction sets, which consist of the three-level, four-factor. Nine calibration solutions were prepared, containing COU (2.0–12.0 mg L^{-1}), 4HC (27.5–275.0 μg L^{-1}), DC (27.5–275.0 μg L^{-1}) and tea extract (0.5–2.0 mL). Prediction sets consisted of nine standard solutions with exact concentration. They contained analytes in the concentration range 41.3–123.8 μg L^{-1} for 4HC and DC, 2.8–6.8 mg L^{-1} for COU, and 0.75–1.75 mL for tea extract. In the same way, three synthetic sample solutions were mixed in methanol. These samples were used for comparison of PLS and MLR methods.

The solutions measured at $\Delta\lambda$=70, 80 and 90 nm from 200 to 400 nm were selected for multivariate analysis, because this spectral interval contains maximum relevant information about the analytes. Each run represents 401 spectral points per spectrum. Autoscale preprocessing was performed for calibration, prediction and sample datasets.

PLS is a method for relating the variations in one or several response variables (concentration data) to the variations of several predictors (spectral data), with explanatory or predictive purposes. MLR models are simpler and easier to interpret than PLS, which uses for regression latent variables without physical meaning. On the other hand, MLR calibration is more dependent on the selection of spectral variables [24]. More information on PLS regression was discussed in [24–26], and on MLR in [24, 27].

Results and Discussion

Emission and Excitation Spectra of Coumarins

The excitation and emission spectra of the coumarins in methanol are shown in Fig. 2. The maximum excitation and emission wavelengths were $\lambda_{ex/em}$=300/372 nm for COU, $\lambda_{ex/em}$= 290/375 nm for 4HC and $\lambda_{ex/em}$=290/365 nm for DC. It is clear that spectra of 4HC and DC are highly overlapped in the excitation mode, while COU is separated. However, the emission spectra of COU and 4HC have very close maxima and the maximum of DC is apart from the other analytes. Therefore simultaneous analysis of COU, 4HC and DC performed only by conventional fluorimetry is not appropriate.

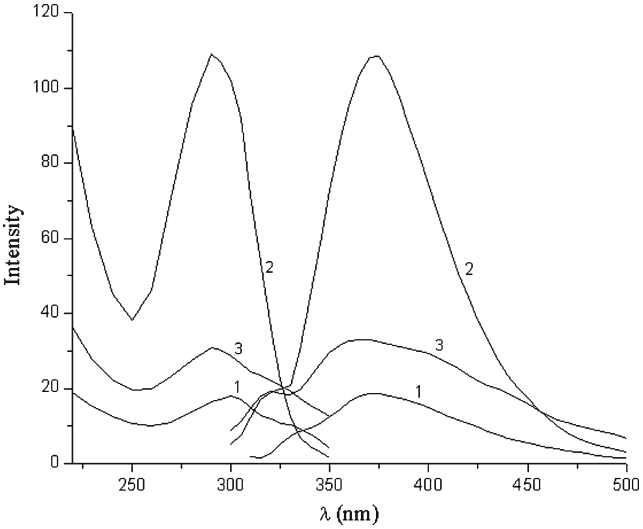

Fig. 2 Excitation and emission spectra of COU (4 mg L^{-1}; 1), 4HC (0.1 mg L^{-1}; 2) and DC (0.1 mg L^{-1}; 3) measured in methanol

Optimization of Experimental Conditions

Since the influence of pH, $\Delta\lambda$ and the type of the diluting solvent play an important role in this experiment, these parameters were carefully studied and optimized. The optimized factor was changed individually, while the others were constant.

Influence of pH

The influence of pH on synchronous fluorescence intensity (spectra) of all three compounds was studied by citrate buffer which covers a wide range of pH (2.4 to 8.0). It was found that pH exerts different influences on analytes. For DC the best fluorescence intensity was a strong acidic environment, but for 4HC the greatest intensity was achieved in the alkaline area of pH. COU has the strongest fluorescence intensity in the weakly acidic region of pH. The fluorescence intensity of the compounds in buffer and in methanol was compared and was found to be lower in buffer solutions. Therefore, for simplicity of the method, no buffer was used during the study.

Effect of Diluting Solvent

Analytes were dissolved in two different solvents, acetonitrile and methanol. Methanol and acetonitrile gave approximately the same synchronous fluorescence intensity at $\Delta\lambda$=90 nm. For this study, methanol was preferably chosen as a solvent, because λ_{max} for each compound was better separated than in acetonitrile.

Table 3 Prediction characteristics of multivariate calibration models for synthetic samples (mean value±standard deviation, n=5)

Δλ (nm)	COU	4HC	DC	COU PLS	4HC	DC	COU MLR	4HC	DC
	Added			Found					
	mgL^{-1}	μgL^{-1}	μgL^{-1}	mgL^{-1}	μgL^{-1}	μgL^{-1}	mgL^{-1}	μgL^{-1}	μgL^{-1}
70				7.5±0.1	105.5±1.5	248.2±3.2	11.6±0.2	57.0±1.4	250.1±2.1
				2.2±0.2	186.8±2.4	55.1±2.3	0.5±0.1	253.3±2.2	−61.0±1.2
				5.4±0.1	183.4±2.8	130.8±2.3	1.0±0.1	306.1±2.0	102.4±1.3
80	**7.5**	**110.0**	**192.5**	8.4±0.1	129.0±1.8	202.6±3.5	10.5±0.1	221.6±2.1	132.5±1.4
	3.0	**192.5**	**220.0**	1.2±0.2	153.8±2.62	95.1±2.2	−0.3±0.1	308.6±2.3	147.1±2.0
	5.5	**220.0**	**110.0**	5.7±0.1	178.4±2.45	141.7±2.6	−2.6±0.1	81.4±1.2	216.3±2.3
90				7.6±0.1	108.4±1.7	232.7±2.5	8.7±0.1	128.0±1.5	199.6±2.4
				3.1±0.1	171.3±1.5	182.8±2.3	2.8±0.1	165.7±1.7	247.5±2.4
				5.4±0.1	200.3±1.8	103.4±1.8	5.6±0.1	188.5±1.6	98.6±1.4
				Recovery (%)					
70				100.3±0.6	95.9±1.5	128.9±1.7	154.0±2.7	51.8±1.3	129.9±1.1
				73.6±5.2	97.0±1.3	25.1±1.0	16.7±1.2	131.6±1.1	−27.7±0.6
				97.3±1.2	83.4±1.3	118.9±2.0	18.4±0.8	139.1±0.9	93.1±1.1
80				112.6±0.2	117.3±1.6	105.3±1.8	139.8±1.5	201.4±1.9	68.8±0.7
				41.6±5.6	79.9±1.4	43.3±1.0	−9.3±0.7	160.3±1.2	66.8±0.9
				103.8±2.4	81.1±1.1	128.8±2.3	−47.7±1.2	37.0±0.6	196.7±2.1
90				101.4±0.6	98.6±1.5	120.9±1.3	116.1±1.3	116.3±1.3	103.7±1.2
				103.2±2.5	89.0±0.8	83.1±1.0	93.9±2.4	86.1±0.9	112.5±1.1
				98.1±1.5	91.0±0.8	93.9±1.6	102.0±1.4	85.7±0.7	89.6±1.3

Selection of Optimum Δλ

The optimum Δλ value is necessary for obtaining a good selectivity and sensitivity by the synchronous fluorescence scanning technique. It has also an effect on the shape of the spectrum, band width and signal value. The value of Δλ is usually selected empirically, the maximum of fluorescence intensity is not always taken into account because a great

Table 4 Results of multivariate methods for synthetic mixtures measured at Δλ=90 nm

	PLS			MLR		
	COU	4HC	DC	COU	4HC	DC
No LVs[a]	3	3	3			
% of variance spectral block	93.47	94.17	93.93			
% of variance concentration block	99.13	99.62	97.68			
RMSEC[b]	0.057	0.014	0.032	2.096E-16	1.206E-17	1.955E-17
RMSECV[c]	0.199	0.118	0.235	0.199	0.118	0.235
SEP[d]	0.189	0.025	0.015	0.465	0.016	0.017
Pred Bias[e]	0.104	−0.004	0.005	−0.205	−0.012	0.011
R^2 Cal[f]	0.991	0.996	0.973	1.000	1.000	1.000
R^2 CV[g]	0.969	0.972	0.964	0.370	0.402	0.843
R^2 Pred[h]	0.957	0.963	0.945	0.335	0.283	0.706

[a] *No LVs* - number of latent variables, [b] *RMSEC* - the root mean squares regression error, [c] *RMSECV* - the root mean squares regression error of cross-validation, [d] *SEP* - standard error of prediction, [e] *Pred Bias* - prediction bias, [f] R^2 *Cal* - coefficient of determination, [g] R^2 *CV* - coefficient of determination of cross-validation, [h] R^2 *Pred* - coefficient of determination of prediction

Table 5 PLS model characteristics for spectral data measured at Δλ= 90 nm

	PLS		
	COU	4HC	DC
No LVs[a]	3	3	3
% of variance spectral block	93.53	93.22	89.28
% of variance concentration block	98.97	96.09	96.79
RMSEC[b]	0.030	0.020	0.011
RMSECV[c]	0.025	0.035	0.017
SEP[d]	0.006	0.028	0.004
Pred Bias[e]	0.003	0.010	0.003
R^2 Cal[f]	0.961	0.990	0.968
R^2 CV[g]	0.974	0.987	0.919
R^2 Pred[h]	0.988	0.988	0.977

[a] *No LVs* - number of latent variables, [b] *RMSEC* - the root mean squares regression error, [c] *RMSECV* - the root mean squares regression error of cross-validation, [d] *SEP* - standard error of prediction, [e] *Pred Bias* - prediction bias, [f] R^2 *Cal* - coefficient of determination, [g] R^2 *CV* - coefficient of determination of cross-validation, [h] R^2 *Pred* - coefficient of determination of prediction

intensity does not mean the best selectivity. In this study Δλ was measured in the range from 10 to 100 nm with 10 nm intervals. The best resolution of λ_{max} of the analytes studied is at Δλ=70, 80 and 90 nm, which were chosen for multivariate calibration. The fluorescence intensity for all compounds in Δλ=70–90 nm was approximately at the maximum. In the range of Δλ=10–70 nm, the intensity increased and at Δλ> 90 nm it decreased. The maximal spectral discrimination of standards and spectra of tea extract at Δλ=90 nm are shown in Fig. 1. The λ_{max} values for 4HC, COU and DC were 286, 295 and 300 nm, respectively.

The large spectral overlapping of COU, 4HC and DC does not allow to use univariate linear calibration, therefore multivariate calibration was performed.

Multivariate Analysis

PLS and MLR regression were chosen since they have different approaches for the creation of the regression model. PLS and MLR methods were compared by results from calibration and prediction set as well as a synthetic mixture. These sets consist of spectral and concentration matrices. Spectral matrices have the dimension 9×401 for calibration and prediction and 3×401 for sample, while concentration matrices of calibration and prediction set have the dimension 9×3 and the sample dimension was 3×3. These matrices were used for building calibration and prediction models. The leave-one-out validation method was used to select the optimal number of latent variables. By this procedure, three latent variables were determined for all models and concentration of each analyte was found from the calibration model including all calibration samples with the optimal number of factors.

The determined concentrations and the recovery from prediction of synthetic samples for definite calibration models and specific Δλ are included in Table 3.

The results for collected synchronous spectra at Δλ= 70 nm showed that the MLR model did not properly predict the concentrations of each component in the synthetic mixtures. The PLS model predicted the concentrations of COU and 4HC well but for DC the values were not satisfactory.

The PLS model built from synchronous spectra at Δλ= 80 nm shows good prediction ability for 4HC. MLR and PLS models are not suitable for determination of the rest of the analytes.

Using both models based on synchronous spectra for Δλ=90, the best compliance for known and predicted values of the concentration of the synthetic samples was obtained. The prediction power of the PLS model is better for COU and 4HC than the MLR model, but for the prediction of DC, MLR is the useful one. Large residuals of predicted values for COU and 4HC from the MLR model support the conclusion that the PLS calibration model is more accurate for determination of all components in the mixture, as shown in Table 4 for characteristics of calibration, cross-validation and prediction models. This claim is evident from comparison of the coefficient of determination of calibration (R^2Cal), coefficient of determination of cross-validation (R^2CV) and the coefficient of determination of prediction (R^2Pred) for both models. Significant differences especially for R^2CV and R^2Pred values pointed to the unsuitability of the MLR calibration model at Δλ= 90 nm for prediction of the COU and 4HC component.

Table 6 Analysis of herbal tea and spiked herbal tea with coumarins (mean value±standard deviation, n=5) measured at Δλ= 90 nm

Sample	COU PLS	4HC	DC	COU HPLC	4HC	DC
	mgL^{-1}	μgL^{-1}	μgL^{-1}	mgL^{-1}	μgL^{-1}	μgL^{-1}
1	3.8±0.1	<LOD	<LOD	3.9±0.1	<LOD	<LOD
2	3.8±0.1	113.4±1.3	80.1±1.4	3.8±0.1	111.1±1.2	80.8±1.7
3	3.7±0.1	174.1±1.6	109.7±1.6	3.7±0.1	173.1±1.4	110.1±1.2
4	3.7±0.1	201.2±1.7	138.4±1.3	3.7±0.1	200.9±1.6	138.8±1.1

Application of the Method to Tea Samples

The proposed method has been applied for the determination of COU, 4HC and DC in herbal tea samples, spiked with these compounds. On the basis of previous results, in this study the synchronous fluorescence spectra measured at $\Delta\lambda$=90 nm were chosen to build a PLS model since they provide the best results. The calibration, cross-validation and prediction characteristics (Table 5) indicate good prediction ability of the PLS method for simultaneous determination of analytes. Table 6 shows the comparison among the unknown and the added concentrations of the compounds studied in herbal tea, and their concentrations predicted by the respective calibration model and HPLC method.

The significance of variance for all results (five runs) obtained by SFS/PLS and HPLC was tested by Fisher *F*- test on 95 % confidence level (p=0.05) and the corresponding Student's two-sided *t*- test assuming equal means was performed. No significant differences between variances in the results from synchronous fluorescence spectroscopy with PLS and conventional HPLC method were found by the Fisher *F*- test (p value was in the interval <0.11; 0.29>). The p-value (df=8, <0.09; 0.92>) from Student's *t*-test indicated no significant differences in determination of coumarins by synchronous fluorescence spectroscopy with multivariate calibration and conventional HPLC method. Based on these results, we assume that the proposed spectrofluorimetric method for simultaneous determination of COU, 4HC and DC in tea is appropriate.

Conclusion

A simple method based on synchronous fluorescence spectroscopy combined with multivariate calibration using the standard addition method was developed for simultaneous determination of COU, 4HC and DC in herbal tea (*Melilotus Officinalis*). We found that synchronous fluorescence measurements at $\Delta\lambda$=90 nm on herbal tea combined with PLS multivariate calibration exhibited excellent statistical characteristics for calibration and prediction. The traditional chromatographic methods for determination of these compounds in herbal tea are relatively expensive and inherently slow. The proposed method, which is rapid and less expensive, can be used as a screening method for COU and the prohibited compounds 4HC and DC in herbal tea before it is applied for chromatography.

Acknowledgments This publication was supported by the Competence Center for SMART Technologies for Electronics and Informatics Systems and Services, ITMS 26240220072, funded by the Research&Development Operational Programme from the ERDF and the Slovak Research and Development Agency under the contract No. APVV-0797-11.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Kim E, Park SB (2010) In: Demchenko AP (ed) Advanced fluorescence reposters in chemistry and biology I: fundamentals and molecular design. Springer, Berlin, pp 149–186
2. Raters M, Matissek R (2008) Eur Food Res Technol 227:637–642
3. EFSA, in Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on request from the commission related to coumarin, adopted on 6 October 2004 EFSA J. 104:1–36
4. Oldenburg J, Rost S, Seidel H, Watzka M, Mueller-Reible CR (2008) Med Gen 20:230–235
5. Hamerschlak N, Rosenfeld LGM (1996) Arq Bras Cardiol 67:209–213
6. Edwards WC, Burrows GE, Tyr RJ (1984) Okla Vet Med Assoc 36: 30–32
7. Campbell HA, Link KP (1941) J Biol Chem 138:21–33
8. Commission Regulation (EU) No 344/2013 of the European Parliament and of the Council of 4 April 2013 on cosmetic products. Official J of the Euro Union L 114:53
9. Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. Official J of the Euro Union L 354/34:15
10. Nie JF, Wu HL, Zhu SH, Han QJ, Fu HY (2008) Talanta 75:1260–1268
11. Xi HW, Ma Q, Wang C, Bai H, Liu X, Wang Y (2010) J Instrum Anal 12:1168–1172
12. Li J, Li N, Wu T, Wang C (2005) Life Sci Instrum 5:34–38
13. Zhao XY, Fu XF, Wang P, Li J, Hu XZ (2011) J Anal Sci 1:49–52
14. Zhao XY, Lin YF, Hu XZ, Wang P, Fu XF, Ji L (2010) Chin J Anal Lab 3:76–79
15. Wu Y, Han X, Zhang J-H, Yan F-L (1998) Grassl China 1:36–38
16. Patra D, Mishra AK (2002) Trends Anal Chem 21:787–798
17. Pistonesi MF, Di Nezio MS, Centurion ME, Palomeque ME, Lista AG, Fernandez BS (2006) Talanta 69:1265–1268
18. de la Peiia M, Moreno MD, Durhn-Me I, Salinas F (2006) Talanta 43: 1349–1356
19. Luis ML, Fraga JMG, Jiménez AI, Jiménez F, Hernández O, Arias JJ (2004) Talanta 62:307–316
20. Divya O, Mishra AK (2007) Talanta 72:43–48
21. Žiak Ľ, Májek P, Hroboňová K, Čacho F, Sádecká J (2014) Food Chem 159:282–286
22. Galera MM, Zamora DP, Vidal JM, Frenich AG, Espinosa-Mansilla A, de la Pena AM, Lopez FS (2003) Talanta 59:1107–1116
23. Tamhane C (2012) In: Balding DJ et al (eds) Statistical analysis of designed experiments: theory and applications. Wiley- Interscience, New Jersey, pp 300–351
24. Brereton RG (2000) Analyst 125:2125–2154
25. Wise BM, Gallagher NB, Bro R, Shaver JM, Windig W, Koch RS (2006) PLS toolboox 4.0 for use with MatlabTM. Eigenvector Research Inc, Wenatchee, pp 137–167
26. Wold S, Sjostrom M, Eriksson L (2008) Chemometr Intell Lab Syst 58:109–130
27. Brown SH (2009) Ala J Math 34:1–3