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Cubic smoothing splines background correction in on-line liquid chromatography–Fourier transform infrared spectrometry

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ABSTRACT

A background correction method for the on-line coupling of gradient liquid chromatography and Fourier transform infrared spectrometry (LC–FTIR) is proposed. The developed approach applies univariate background correction to each variable (i.e. each wave number) individually. Spectra measured in the region before and after each peak cluster are used as knots to model the variation of the eluent absorption intensity with time using cubic smoothing splines (CSS) functions. The new approach has been successfully tested on simulated as well as on real data sets obtained from injections of standard mixtures of polyethylene glycols with four different molecular weights in methanol:water, 2-propanol:water and ethanol:water gradients ranging from 30 to 90, 10 to 25 and from 10 to 40% (v/v) of organic modifier, respectively. Calibration lines showed high linearity with coefficients of determination higher than 0.98 and limits of detection between 0.4 and 1.4, 0.9 and 1.8, and 1.1 and 2.7 mg mL^{-1} in methanol:water, 2-propanol:water and ethanol:water, respectively. Furthermore the method performance has been compared with a univariate background correction approach based on the use of a reference spectra matrix (UBC-RSM) to discuss the potential as well as pitfalls and drawbacks of the proposed approach. This method works without previous variable selection and provides minimal user-interaction, thus increasing drastically the feasibility of on-line coupling of gradient LC–FTIR.

1. Introduction

Polyethylene glycols (PEGs) are widely employed as additives in cosmetic products [1] and pharmaceutical formulations [2] taking advantage of their low toxicity, their solubility and viscosity properties and the fact that they cause little or no ocular and dermal irritation [1]. In personal care products including creams, makeup, bath and hair care products, PEGs are often used at high percentage concentrations as solvents, humectants, binders, emulsion stabilizers and viscosity-increasing agents. In pharmaceutical formulations, PEGs have been reported to affect oral absorption as well as renal elimination of different active drug components [2].

Emerging from the omnipresence of PEGs, adequate tools for qualification and quantification become necessary. Liquid chromatography (LC), size-exclusion chromatography (SEC), thin-layer chromatography (TLC), supercritical fluid chromatography (SFC) and capillary zone electrophoresis (CZE) in combination with different detectors are commonly used for polymer analysis and characterization to obtain information about chemical properties as the average molecular mass and the composition of the polymer [3,4]. Since PEGs lack of chromophors, detection can only be carried out in the low UV or using refractive index (RI) detectors [5], entailing a rather unspecific detection or employing derivatization methods [4] increasing the time of analysis.

Using evaporative light scattering detection (ELSD) there is no need for derivatization [6]. Furthermore, this detector is compatible with gradient elution and provides a significant increase in sensitivity as compared with RI detection, but it is also a low-selective detector and shows a non-linear response. Mass spectrometry (MS) detection is highly sensitive and also shows a very high specificity but its field of application focuses on trace level analysis. Besides, MS detectors are still expensive. In contrast, the on-line hyphenation with an infrared (IR) detector can be a useful alternative to standard detectors providing additional information on the analytes and avoiding time consuming sample preparation steps.

Although infrared spectroscopy can be considered as a well-established technique in the field of polymer analysis, only a reduced number of publications on isocratic LC–FTIR can be found in the literature. Most of them apply solvent elimination prior to detection [7–10] and only few applications use the on-line LC–FTIR approach [11–15]. However, on-line detection presents a series of advantages over the off-line approach: (i) on-line coupling can be easily carried out by the use of a standard flow-cell, (ii) non-volatile buffer systems can be employed, (iii) a further hyphenation with

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additional detectors is possible and (iv) IR spectra are obtained without any orientation, crystallization or degradation occurring during the solvent elimination in the off-line measurement [16]. On the other hand, on-line LC–FTIR presents two main drawbacks: (i) the sensitivity of IR detection is limited and (ii) difficulties in on-line measurements arising from the pre-dominating absorption of most of the commonly used mobile phase components [17]. Recent technical advances in instrumentation could help to overcome problems concerning the sensitivity of IR detection [18]. Concerning the second drawback, under isocratic conditions the spectral contribution of the mobile phase components can be compensated by subtracting a reference spectrum measured either at the beginning of the run, or directly before the elution of the analyte of interest. However, due to strong changes in shape and intensity of the absorption bands of the mobile phase components occurring during gradient separations, this strategy is not applicable in gradient LC–FTIR. Furthermore, background compensation has to be very accurate, because these changes might be up to several orders of magnitude higher than those due to the elution of the analytes of interest. Recently a straightforward strategy to perform chemometric background elimination in on-line LC–FTIR systems, called univariate background correction based on the use of a reference spectra matrix (UBC-RSM) was developed [19]. This approach is based on the use of the spectral information of a set of spectra of different eluent compositions named 'reference spectra matrix' (RSM). Several applications of this method have been developed [18–23]. Basically, three constraints have to be fulfilled to apply UBC-RSM: (i) the mobile phase spectra must have a spectral region characteristic of its composition and free from interferences from other eluting compounds, (ii) an appropriate RSM has to be recorded and (iii) high instrument stability is essential.

In the present study, a newly developed background correction method based on the use of cubic smoothing splines (CSS) was tested on on-line gradient LC–FTIR. The new method was compared to results obtained by the UBC-RSM approach. The usefulness of the proposed method was critically assessed using simulated and real reversed phase gradient LC–FTIR data using methanol, 2-propanol and ethanol as organic phase modifiers. The application of these alcohols as mobile phase components is challenging, because they show intense absorption bands in the mid IR region which complicates background compensation. Additionally, the use of 2-propanol and ethanol as organic modifiers in liquid chromatography is of interest, because both alcohols are considered to have a low toxicity and therefore, when substituting acetonitrile or methanol as organic phase modifier, the method can be considered environmentally-friendly.

1.1. Background correction using cubic smoothing splines

The proposed approach applies univariate background correction to each variable (i.e. each wave number) individually. Spectra measured in the region before and after each peak cluster are used as knots to model the variation of the eluent absorption intensity with time using cubic smoothing splines functions, assuming that FTIR detection follows a linear, additive Lambert–Beer behaviour.

The proposed approach can be described in two steps: (i) peak detection and (ii) calculation of the background signal. The first step involves peak detection and the selection of a series of data points where no peaks are present to be subsequently used as knots for the calculation of the splines fit. In the literature as well as in the commercial chromatographic software packages different approaches aiming at peak detection can be found [24,25]. At least theoretically, many of these algorithms could be applied to identify analyte peaks in uncorrected LC–FTIR chromatograms. In spite of that, the strong change in the slope of the chromatograms difficults peak detection

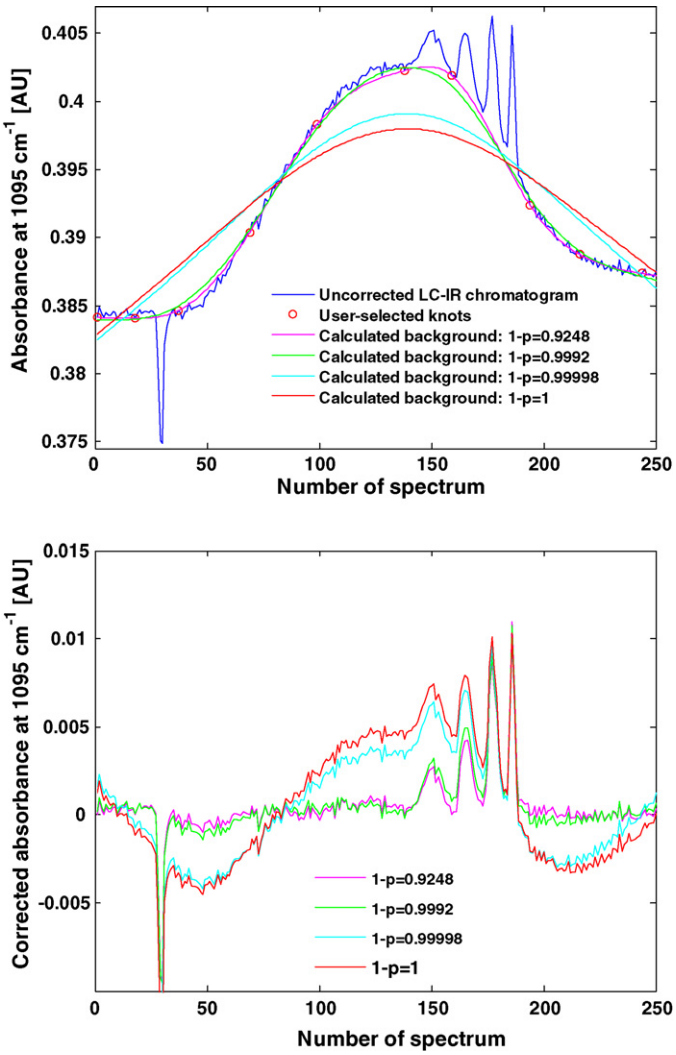

Fig. 1. LC–FTIR chromatograms of a mixture of PEG 2000, PEG 4000, PEG 8000 and PEG 40000 obtained (a) before and (b) after background correction using different smoothing parameters (p) for the calculation of the background signal.

when the mobile phase composition changes rapidly, especially in spectral regions where the eluent absorbs strongly, or when the signal intensity due to the analyte absorption is small. The second step of the approach involves the background correction itself: firstly, using the selected knots a cubic smoothing spline is fitted to model eluent absorption. Then, background correction is accomplished by subtracting the interpolated eluent absorption to the raw LC–FTIR data.

The calculation of the cubic smoothing spline is carried out using the Matlab csaps function [26]. This function uses a smoothing parameter (p) which determines how closely the spline follows the given data (i.e. each considered chromatogram). The csap function is very sensitive to the choice of p, which consequently influences the accuracy of the background correction. Low values of p will lead to splines far away from the underlying trend (see Fig. 1). As described in the Ref. [26], the sensitive range for p is around $1/(1 + \text{epsilon} * k)$, where epsilon is $h3/16$, and h is the average difference between neighbouring sites. A close following of the data when $k = 0.01$ and some satisfactory smoothing when $k = 100$ is expected. This additional 'smoothing factor' k used for the calculation of p has to be selected very carefully. Fig. 1 shows the effect of selecting different values of k between 0.01 and 100, keeping a constant value of epsilon = 1230, on the background correction of

a LC–FTIR injection of a PEG standard solution. A constant value of 0.01 was used throughout this work. Knot selection was performed manually. As a general criterion, every 15th point was selected as knot in baseline regions where no peaks were present. Additionally, a single data point between analyte peaks was also included as knot if peaks were completely resolved.

1.2. Univariate background correction based on the use of a reference spectra matrix (UBC-RSM)

The univariate background correction based on the use of a reference spectra matrix (UBC-RSM) process can be divided into five steps [19]. First, the sample matrix SM (z,c) and the reference sample matrix RSM (k,c), where z and k are the number of spectra included in the SM and RSM, respectively, and c is the number of variables in each spectrum, have to be obtained. This can be carried out experimentally acquiring spectra during the injection of a sample to obtain the SM and measuring spectral changes during the re-equilibration of the chromatographic system after a gradient or during a blank-injection, to obtain the RSM. Secondly, for all obtained spectra an identification parameter (IP), which is characteristic for the mobile phase composition, has to be calculated. In this work, the absorbance ratio (AR) of the absorbance values at two previously selected wave numbers was used as IP (see Eq. (1)):

$$\mathrm{AR}_S = \frac{y^S_{r1}}{y^S_{r2}} \quad (1)$$

where y^S_{r1} and y^S_{r2} are the absorbance values at the wave numbers $r1$ and $r2$ (cm^{-1}) measured in the spectra S ($S = (1, \ldots, z)$) for spectra included in the SM and $S = (1, \ldots, k)$ for those of the RSM. In the third step, for every spectrum of the SM, the most adequate background spectrum included in the RSM, which is the one with the closest AR, is identified and selected for the subsequent calculation of the background corrected spectrum. In step 4 for each spectrum contained in the SM, a correction factor (KF) is calculated. This step is optional and aims at the correction of minor changes in the spectral absorbance intensity during the chromatographic run. KF_S is defined as the ratio of absorbance at a defined wave number (φ) of the sample spectrum S and its corresponding reference spectrum γ selected in step 3, as indicated in Eq. (2):

$$\mathrm{KF}_S = \frac{y^S_\varphi}{y^Y_\varphi} \quad (2)$$

The last step consists of a simple subtraction of the selected RSM spectrum from the corresponding SM spectrum as described in Eq. (3):

$$\mathrm{Corrected}_S = S - \mathrm{KF}_S * S_{\mathrm{RSM}} \quad (3)$$

where $\mathrm{Corrected}_S$ is the background corrected sample spectrum, S is the original sample spectrum, S_{RSM} is its corresponding background spectrum which was selected in step 3 and KF_S is the calculated correction factor for the sample spectrum S. If no KF is applied, KF_S is set to 1.

2. Experimental

2.1. Simulated data

Four chromatographic data sets were simulated using a reference spectra set (S_{pegs}) of PEG 2000, PEG 4000, PEG 8000 and PEG 40000 polyethylene glycol standards. PEG concentration profiles (C_{pegs}) were generated using the gspeak function of the Peak Generation Matlab toolbox [27]. Then, the simulated chromatograms (D_{lc}) were calculated according to:

$$D_{\mathrm{lc}} = C_{\mathrm{pegs}} S^T_{\mathrm{pegs}}$$

The peak heights of the PEG 2000, PEG 4000, PEG 8000 and PEG 40000 simulated peaks were 0.0015, 0.004, 0.0035 and 0.005 with peak-sigma values of 1, 3, 1.5 and 1.5, respectively.

Four different situations were simulated: (i) all four peaks are baseline resolved choosing the peak position for the peak maximum at simulated retention times of 5.8, 6.8, 8.2 and 9.6 min for the four peaks, respectively; (ii) the first and the second peak are overlapping changing the retention time of the second peak to 6.5 min; (iii) the second and the third peak are overlapping changing the retention time of the second peak to 7.7 min and (iv) all four peaks are overlapping choosing retention times of 5.8, 6.5, 7.2 and 7.5 min, respectively. In addition, real experimental noise and changing background conditions were considered by adding uncorrected FTIR spectra acquired during a blank gradient LC run of methanol:H_2O with methanol concentrations ranging from 30 to 90% (v/v) to the four simulated matrices.

2.2. Apparatus and reagents

2.2.1. LC

A Dionex P680 high performance liquid chromatograph (Sunnyvale, CA, USA), equipped with a GENESIS C_4 column (150 × 2.1 mm, 4 μm, 300 Å) from Grace Davison Discovery (Deerfield, IL, USA) and a sample injection loop of 20 μL, was employed for chromatographic separations. Linear gradients were run with methanol:water, 2-propanol:water and ethanol:water mobile phases from 30 to 90, 10 to 25 and 10 to 40% (v/v) of organic solvent in 10, 15 and 10 min, respectively. Water was purified with a Milli-Q system from Millipore (Billerica, MA, USA). All solvents were multisolvent grade and purchased from Scharlau Chemie S.A. (Barcelona, Spain).

2.2.2. FTIR

A Bruker IFS 66/v FTIR spectrometer (Bremen, Germany) equipped with a liquid nitrogen refrigerated mercury–cadmium–telluride detector and a vacuum system was employed for spectra acquisition. The scanner of the interferometer was operated at a HeNe laser modulation frequency of 100 kHz. Spectra were recorded in the range between 4000 and 950 cm^{-1}, with a spectral resolution of 8 cm^{-1} and a zerofilling value of 2. Co-adding 25 scans/spectrum, a spectra acquisition frequency of 15 spectra min^{-1} was provided. On-line hyphenation to the LC system was carried out using a flow-cell with CaF_2 and ZnSe windows and a pathlength of 10 μm placed in the dry-air purged sample compartment of the spectrometer. For instrumental and measurement control as well as for data acquisition, the OPUS software (version 6.5) from Bruker was employed. Background correction of data was run under Matlab 7.7.0 (R2008b) from MathWorks (Natick, Massachusetts, USA) using the csaps Matlab function, in-house written functions and a Matlab GUI file. In-house written Matlab functions and GUI files used in this work are available on-line [28].

2.2.3. Standards

Standard mixtures of polyethylene glycol (PEG) were prepared by mixing pure standards of different molecular weights (2000, 4000, 8000 and 40,000 g/mol) obtained from Fluka (Buchs, Switzerland) and dissolving them in different mobile phases. All solutions were filtered with a 0.22 μm nylon syringe filter prior to their injection into the chromatographic system. These standards were used to test and assess the chromatographic procedures and the background correction process.

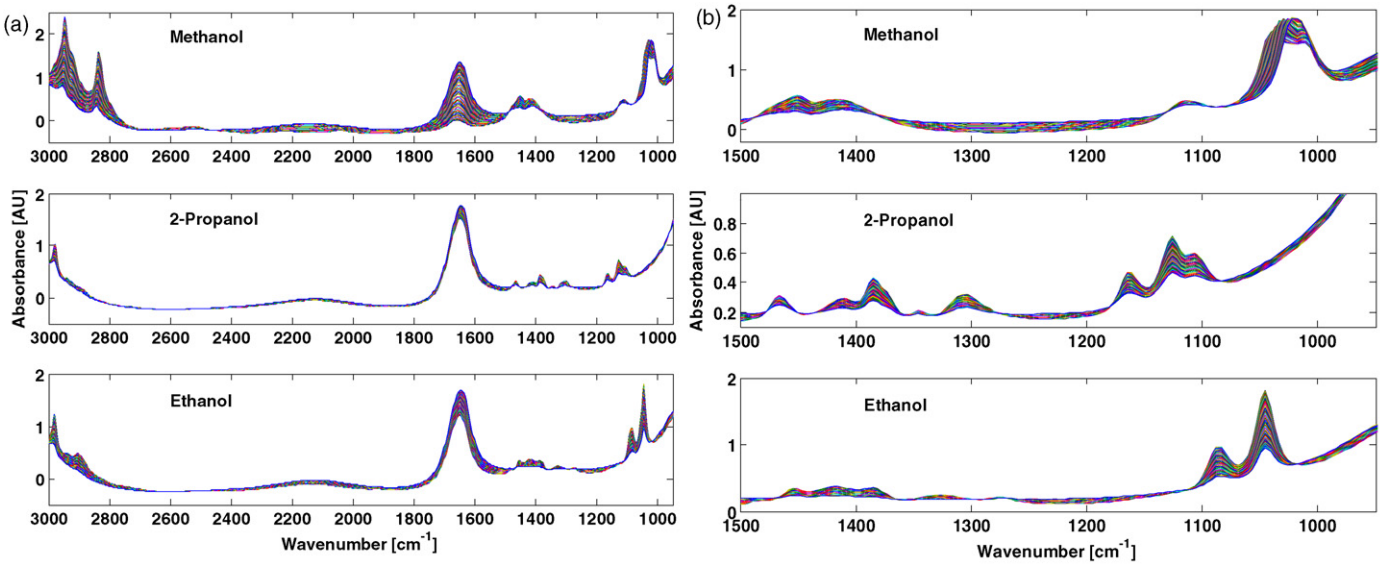

Fig. 2. On-line FTIR spectra measured during LC gradient blank runs of methanol:water, 2-propanol:water and ethanol:water mixtures ranging from 30 to 90, 10 to 25 and 10 to 40% (v/v) organic solvent, respectively, (a) between 3000 and 950 cm^{-1} and (b) between 1500 and 950 cm^{-1}.

3. Results and discussion

3.1. Infrared spectra of mobile phase and PEG

Fig. 2 shows infrared spectra of the three different mobile phase systems used in this work between 3000 and 950 cm^{-1} (a) and 1500 and 950 cm^{-1} (b). In the spectral interval between 2990 and 2835 cm^{-1} vibrational bands are caused by antisymmeric and symmetric CH_3 and CH_2 vibrations [29]. The band around 2100 cm^{-1} emerges due to overtones and combination bands of intermolecular vibrations and OH stretches [30]. In the region between 1470 and 1385 cm^{-1} several overlapping CH_3 and CH_2 deformation bands can be observed [29]. At ~1643 cm^{-1} the strong absorption band due to the HOH bending mode of water molecules is located. Because of saturation effects, the OH stretching absorption of alcohol and water molecules do not show a linear behavior with changing concentrations [30,31]. Additionally, the position of the HOH bending and HOH bending/hydrogen bond libration combination modes shift to higher frequencies with increasing alcohol concentrations. It is clear that due to the changes in shape and intensity of the mobile phase absorption bands during the gradient (see Fig. 2), a constant background cannot be used to correct the spectra of the eluting analytes.

Fig. 3 shows spectra of PEG 4000 solutions in the 3000–950 cm^{-1} range. PEG 4000 was dissolved in mixtures of the three alcohols with water in the same concentrations as at the beginning of the applied gradients, respectively. A set of characteristic analyte bands arises in this interval, the most intense bands being located in the 1100 cm^{-1} region. The ν(C–O) stretching vibration, the in-plane banding vibration of the C–O–H groups and the ν(C–C) stretching as well as the deformational $\delta(-CH_2-)$ vibration contribute to the shape of this band, among others. The shape of this band changes with the molecular weight of the polymer as well as with the water content of solutions [32]. Due to structural similarities between the mobile phase components and the analyte, this band overlaps strongly with mobile phase absorption bands. Additionally, bands due to (C–O–H) in-plane deformations can be observed in the spectral range from 1300 to 1400 cm^{-1}. These bands are almost identical for PEGs of different molecular weights. In the spectral interval between 2924 and 2881 cm^{-1} vibrational bands due to antisymmeric and symmetric CH_3 and CH_2 vibrations can be observed [29]. Furthermore it can be seen that band shapes are affected by the solvent, being slightly different for PEGs dissolved in methanol, 2-propanol and ethanol aqueous solutions.

3.2. Simulated on-line LC–FTIR data

To evaluate the proposed background correction method, it was initially tested on simulated data calculated as described in Section 2. Uncorrected chromatograms of the four simulated situations are depicted in Fig. 4a. Whereas the elution of the analytes can be appreciated as a change of the slope in the chromatogram, the detection of small peaks might become difficult without appropriate background correction.

Peak detection and knot selection were performed manually. The selection of the knots is important as it influences the resulting chromatograms and consequently precision and accuracy and

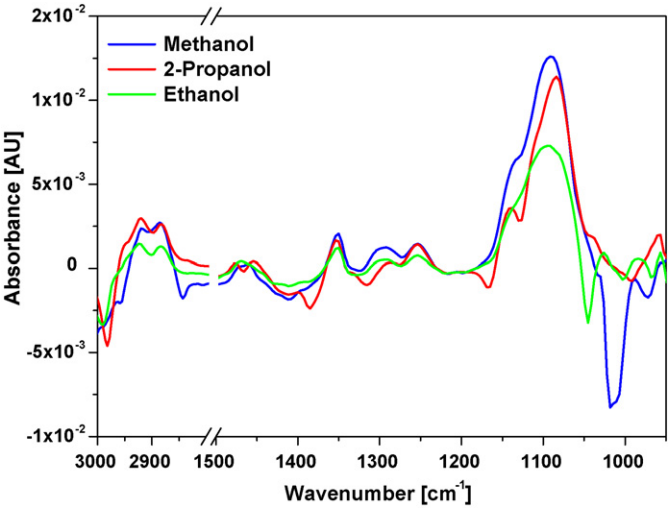

Fig. 3. Spectra of PEG 4000 at 5 mg mL^{-1} in methanol:water, 2-propanol:water and ethanol:water at 30, 10 and 10% (v/v) of organic solvent, respectively, acquired in a continuous flow experiment.

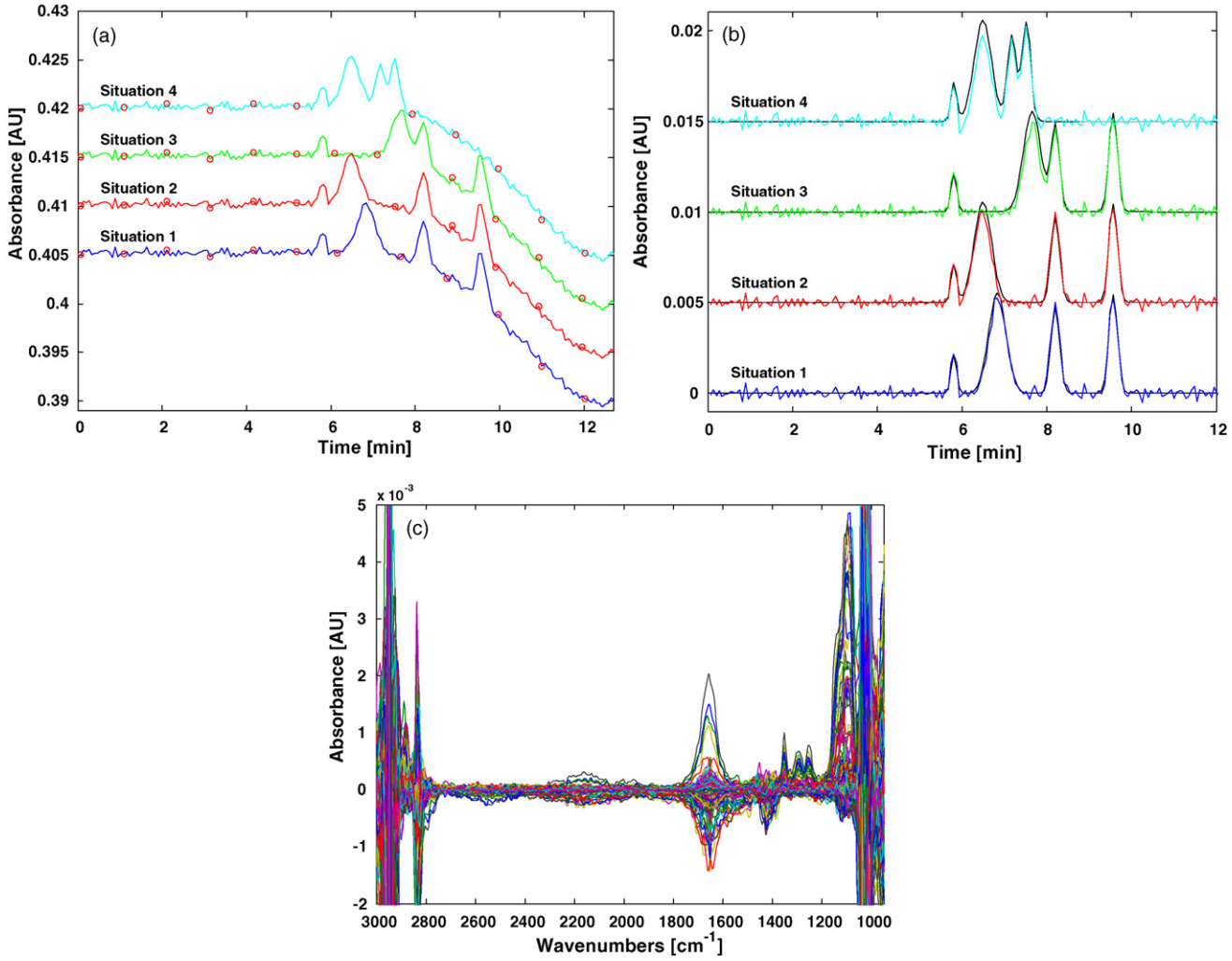

Fig. 4. Simulated chromatographic separations of PEGs. Chromatograms extracted at 1095 cm^{-1} (a) without background correction, (b) after applying the CSS correction method, and (c) background corrected spectra of the simulated data set corresponding to situation 2. *Note*: Red circles in (a) indicate selected knots, black lines in (b) show corresponding simulated chromatograms before adding noise to simplify interpretation. For details about the simulated data sets see text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the quality of the recovered spectra required for qualitative and quantitative analysis. However, an additional advantage of the new background correction based on cubic smoothing splines over other methods is its potential for a high grade of automatization during the whole process leading to a low user-interaction. The use of an appropriate algorithm which reliably identifies the peaks and their beginning and ending will therefore directly reduce the time required for background correction of real LC–FTIR data and thus, it would facilitate the application of on-line LC–FTIR measurements in routine analysis.

As already stated before, the selection of the knots is a critical step of this approach. Fig. 5 shows the influence of the selection of different knots on the recovered chromatograms. The worst results were obtained using every fifth spectrum for correction. The baseline shows a valley during analyte elution, being spectral shapes and chromatographic peak heights strongly modified because some of the data points (knots) used for the background calculation contain significant amounts of signal from the eluting analytes. As it can be seen in Fig. 5, using every fifth spectrum in the interval from 0 to 5.7 min and from 7.9 to 12 min, the results can be improved. Selecting additional spectra at 5.9, 6.9 and 7.3 min different results were found. When analyte peaks overlap strongly, the correction gets worse including the spectrum of the valley between the two peaks in the knots vector. The selection process is also influenced by the chromatographic resolution of the peaks, as peak overlapping complicates the selection process of the knots.

Fig. 4b shows background corrected chromatograms of all simulated situations and the corresponding simulated chromatograms without adding noise. In general it can be observed that even small peaks are now easy to identify in all chromatograms, the baseline shows a random distribution of the noise and a lack of slope. From situation 1 it can be seen that the background correction method works well for baseline resolved peaks. All four peaks were recovered almost without affecting peak shape, height and area (data not shown). Situations 2–4 show that the background correction also works well in case of overlapping peaks if an appropriate knot selection is feasible. On the other hand, Fig. 4c shows the high quality of the background corrected spectra obtained from the simulated data set corresponding to situation 2. As it can be seen, spectra show a lack of sloping baseline drift. Apart from the saturated spectral regions above 2800 cm^{-1} and below 1060 cm^{-1}, due to intense absorption of the mobile phase components, the spectral noise is reduced to an adequate level being 3.4 mAU at 1643 cm^{-1} and 1.0 mAU in the region between 1470 and 1385 cm^{-1} were a strong water absorption band and intense absorption bands of the alcoholic mobile phase component interfere. In other spectral regions the peak-to-peak noise is even lower, like for example from 1360 to 1200 cm^{-1} being 0.3 mAU.

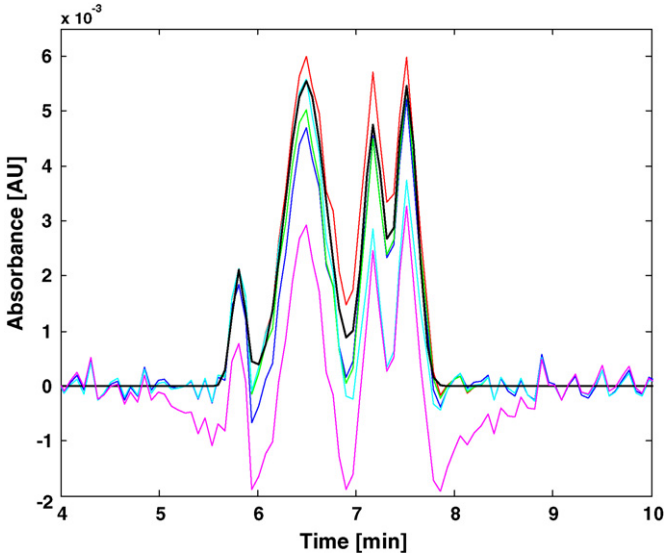

Fig. 5. Chromatograms extracted at 1095 cm^{-1} using a single point baseline correction at 1400 cm^{-1} from situation 4 after applying the CSS method using different knots, pink line: using every fifth point as a knot (see correction step 1), dark blue line: using every fifth spectrum in the interval from 0 to 5.7 min and from 7.9 to 12 min, red line: same knots as blue line adding one spectrum at 5.9 min, green line: same knots as blue line adding two spectra at 5.9 and 6.9 min, light blue line: same knots as blue line adding three spectra at 5.9, 6.9 and 7.3 min, black line shows corresponding simulated chromatograms before noise addition to simplify interpretation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Real on-line LC–FTIR data

Fig. 6 shows spectra acquired during the injection of a standard mixture of four PEGs in a linear methanol:water gradient from 30 to 90% (v/v) methanol before (Fig. 6a) and after (Fig. 6b) background correction. The identification of the analytes is not possible without background correction due to the predominant absorbance of the mobile phase components and the strong changes in both, intensity and shape of the mobile phase bands. It is remarkable that after background correction the identification as well as the quantification of all four analytes is feasible.

To show the potential of on-line LC–FTIR applying background correction for quantitative applications, calibration lines have been established for all three mobile phase systems injecting eight standard mixtures using methanol:water and 2-propanol:water and 10 for the calibration line of the ethanol:water mobile phase system. The analytical figures of merit of the three determinations are summarized in Table 1. Analyte specific chromatograms were extracted measuring the absorbance at 1095, 1088 and 1103 cm^{-1} in methanol:water, 2-propanol:water and ethanol:water, respectively, applying a single point baseline correction at 1400 cm^{-1}. All calibration lines showed high linearity with coefficients of determination higher than 0.98. Limits of detection and limits of quantification were established for the four analytes in the three mobile phase systems and varied respectively between 0.13 and 0.4 and between 0.4 and 1.4 in methanol:water, between 0.3 and 0.5 and between 0.9 and 1.8 in 2-propanol:water and between 0.3 and 0.8 and between 1.1 and 2.7 in ethanol:water. The repeatability was measured as the relative standard deviation (RSD [%]) and ranged between 1.4 and 6.6, 3.5 and 5.3 and 3.3 and 8.7% for methanol:water, 2-propanol:water and ethanol:water, respectively. Best results were obtained using the methanol:water mobile phase system. Results obtained for 2-propanol:water were similar to those of methanol:water. Noise was somewhat higher using ethanol:water because of the higher background absorption of this mobile phase in the spectral range of the analytes signal. However, 2-propanol and ethanol are recommended due to their low toxicity, thus being more environmentally-friendly solvents.

Fig. 7a shows chromatograms extracted after background correction of data obtained during the injection of a mixture of the four PEG standards at a concentration of 5 mg mL^{-1} in the three mobile phase systems, evidencing the adequate chromatographic resolution of the four peaks, the lack of a sloping baseline as well as the random distribution of the noise around the baseline. In accordance with the results obtained from the calibration data, it can be observed that the noise was slightly higher using ethanol:water than for the other mobile phase systems. Using methanol as organic modifier, better peak shapes (higher and thinner) were obtained than with 2-propanol and ethanol. Fig. 7b shows the corrected spectra obtained during the whole chromatographic run. Due to the different chemical structure of the three alcoholic mobile phase components, spectral saturation and noise were affected. Using methanol:water at a maximum methanol concentration spectra get completely obscured around 1030 cm^{-1}, using ethanol:water the strongest absorption is observed around 1045 cm^{-1}. Using 2-propanol:water, spectra get saturated below 950 cm^{-1}. Again, no sloping can be seen in any of the depicted spectra.

To assess the ability to identify PEG spectra in the corrected data sets, the correlation coefficients between spectra extracted from

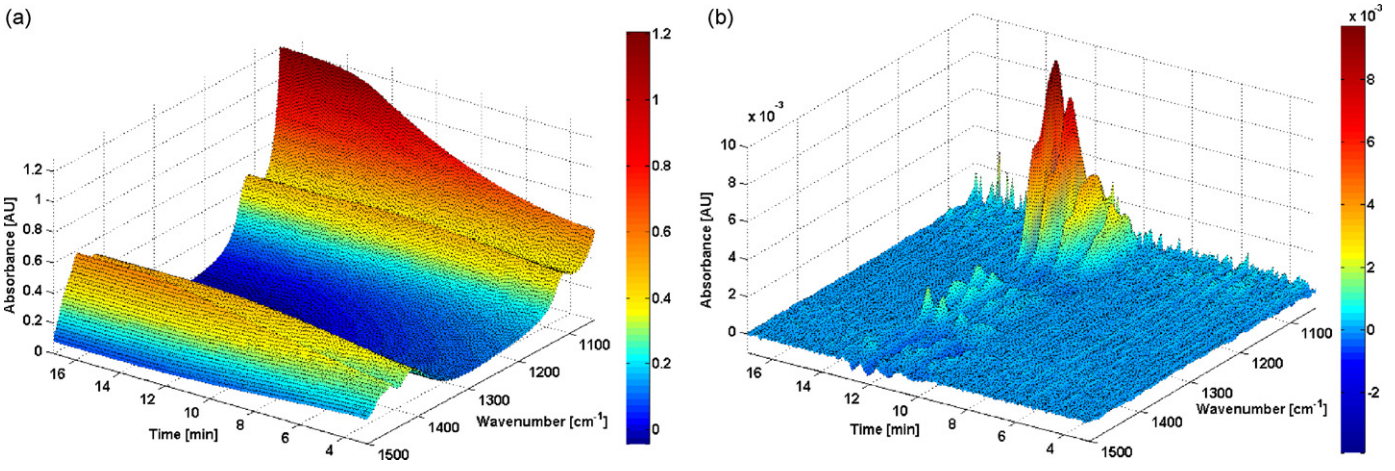

Fig. 6. 3D plots of (a) uncorrected spectra and (b) spectra obtained by applying the CSS method. *Note*: Spectra were acquired during injection of a standard mixture containing 5.1 mg mL^{-1} of PEG 2000, 5.3 mg mL^{-1} of PEG 4000, 5.6 mg mL^{-1} of PEG 8000 and 5.1 mg mL^{-1} of PEG 40000 between 1500 and 950 cm^{-1} and 3 and 17 min.

Table 1
Figures of merit of the on-line LC–FTIR determination of PEGs using cubic smoothing splines background correction.

Mobile phase system	Analyte	Calibration curve[a] $y=(a\pm s_a)*x+(b\pm s_b)$		R^2	Noise (AU)[b]	LOD (mg mL^{-1})[c]	LOQ (mg mL^{-1})[d]	Repeatability (%)[e]
		$a\pm s_a$	$b\pm s_b$					
Methanol:H_2O[f]	PEG 2000	0.00038 ± 0.00002	0.00 ± 0.08	0.98	0.04	0.3	1.1	3.4
	PEG 4000	0.00039 ± 0.00001	0.00 ± 0.06	0.99	0.05	0.4	1.3	4.8
	PEG 8000	0.00040 ± 0.00002	0.01 ± 0.08	0.98	0.06	0.4	1.4	6.6
	PEG 40000	0.00039 ± 0.00002	0.03 ± 0.07	0.98	0.02	0.13	0.4	1.4
2-Propanol:H_2O[g]	PEG 2000	0.00030 ± 0.00001	0.00 ± 0.04	0.989	0.05	0.5	1.8	5.3
	PEG 4000	0.00040 ± 0.00002	−0.09 ± 0.08	0.98	0.06	0.5	1.5	4.3
	PEG 8000	0.00027 ± 0.00001	−0.06 ± 006	0.986	0.02	0.3	0.9	3.7
	PEG 40000	0.000242 ± 0.000005	0.00 ± 0.02	0.998	0.03	0.3	1.1	3.5
Ethanol:H_2O[h]	PEG 2000	0.00031 ± 0.00002	0.05 ± 0.08	0.96	0.08	0.8	2.6	5.9
	PEG 4000	0.00036 ± 0.00002	0.01 ± 0.08	0.98	0.10	0.8	2.7	5.1
	PEG 8000	0.00033 ± 0.00001	−0.02 ± 0.05	0.991	0.08	0.7	2.4	8.7
	PEG 40000	0.000288 ± 0.000007	−0.01 ± 0.03	0.994	0.03	0.3	1.1	3.3

[a] Calibration curve obtained from standard mixtures, being a and b the slope and the intercept of the calibration lines.
[b] Noise measured as the standard deviation of the peak areas of five independent injections of a standard mixture.
[c] Limit of detection established as the concentration at which the signal to noise ratio is higher than 3.
[d] Limit of quantification established as the concentration at which the signal to noise ratio is higher than 10.
[e] Relative standard deviation for five independent measurements.
[f] Analyte specific chromatograms were extracted measuring the absorbance at 1095 cm^{-1} and applying a single point baseline correction at 1400 cm^{-1}.
[g] Analyte specific chromatograms were extracted measuring the absorbance at 1088 cm^{-1} and applying a single point baseline correction at 1400 cm^{-1}.
[h] Analyte specific chromatograms were extracted measuring the absorbance at 1103 cm^{-1} and applying a single point baseline correction at 1400 cm^{-1}.

background corrected chromatograms at the peak apex of each analyte, and reference spectra were calculated. The spectral intervals used for comparison varied for the three considered mobile phases being: 1366–1070 cm^{-1} for methanol:water and 1366–1072 cm^{-1} for 2-propanol:water and ethanol:water. Results found are summarized in Table 2 and as can be seen, recovered PEG spectra allowed the identification of PEGs in all mobile phase systems showing correlation coefficients higher than 92%. Furthermore it can be observed that high spectral similarity avoids the differentiation of the four considered PEGs.

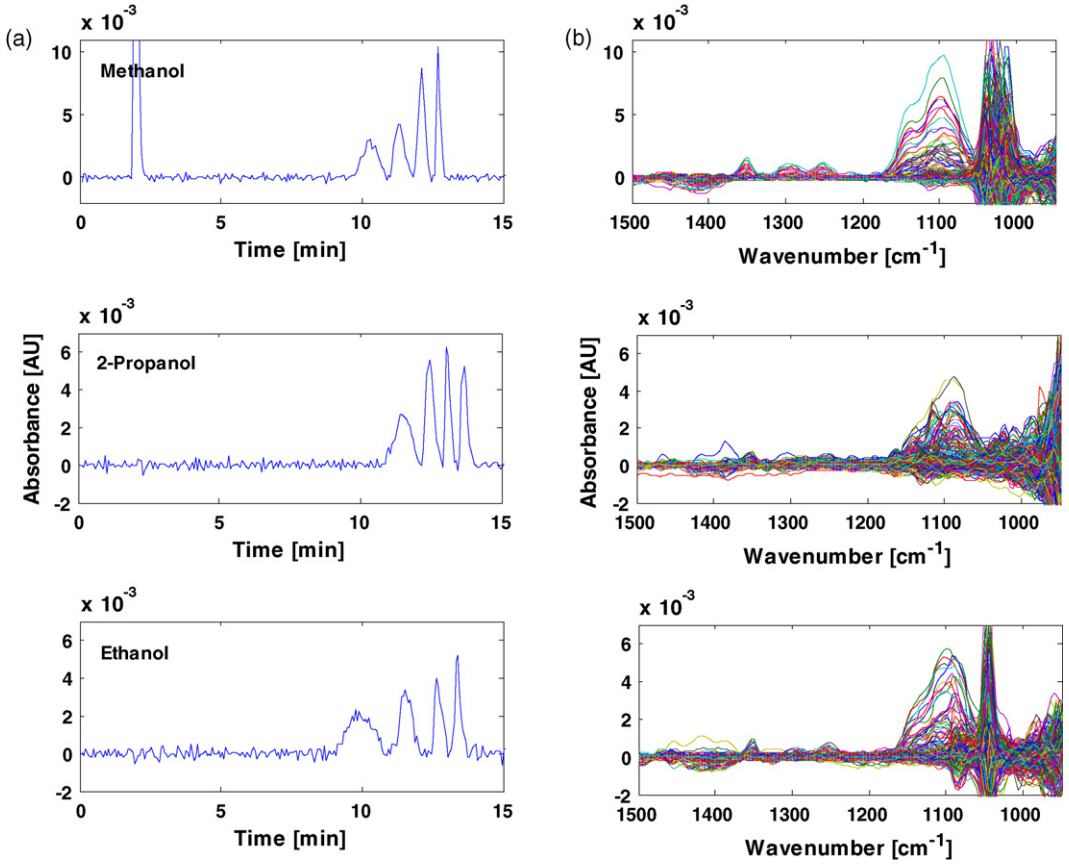

Fig. 7. Chromatographic separations of PEGs in different mobile phase systems. (a) Background corrected chromatograms extracted from data acquired during the injection of a standard mixture containing 5 mg mL^{-1} of each of the four PEG standards in methanol:water, 2-propanol:water and ethanol:water gradients ranging from 30 to 90, 10 to 25 and 10 to 40% (v/v) of organic solvent, respectively and (b) background corrected spectra acquired during the whole chromatographic run. *Note*: Chromatograms were extracted as described in Table 1.

Table 2
Correlation between spectra extracted from background corrected chromatograms and reference FTIR spectra of PEGs.

Mobile phase system	Analyte[a]	Correlation coefficient (%)[b]			
		PEG 2000 Ref[c]	PEG 4000 Ref[c]	PEG 8000 Ref[c]	PEG 40000 Ref[c]
Methanol: H_2O[d]	PEG 2000	96.7	97.1	97.0	98.7
	PEG 4000	99.1	99.1	99.1	98.4
	PEG 8000	97.6	98.0	97.9	99.7
	PEG 40000	97.4	97.7	97.6	99.9
2-Propanol: H_2O[d]	PEG 2000	92.6	89.0	89.6	90.7
	PEG 4000	97.7	96.2	96.4	97.2
	PEG 8000	98.1	98.9	99.0	98.7
	PEG 40000	99.2	97.4	97.6	98.4
Ethanol:H_2O[d]	PEG 2000	98.6	98.5	98.0	98.2
	PEG 4000	99.6	99.6	99.3	99.4
	PEG 8000	99.4	99.6	99.4	99.6
	PEG 40000	97.9	97.9	97.3	97.6

[a] Analyte spectra were extracted during analyte elution from background corrected chromatograms obtained during the injection of a standard mixture containing 5 mg mL^{-1} of each analyte.
[b] Correlation coefficient between two spectra $y1$ and $y2$ is defined as the ratio from the covariance ($Cov(y_1, y_2)$) and the product of the two standard deviations sy_1 and sy_2. According to this definition, a value of percentage of correlation coefficient of 100 indicates identical spectra.
[c] Reference spectra were obtained during FIA-injections of standard solutions of each analyte at a concentration of 5 mg mL^{-1} and using the same mobile phase composition as at the beginning of the gradient in the corresponding mobile phase system.
[d] Spectra were compared in the spectral interval from 1366 to 1070 cm^{-1} for the methanol:H_2O mobile phase system, and from 1366 to 1072 cm^{-1} for the 2-propanol:H_2O and the ethanol:H_2O mobile phase systems.

3.4. Comparison of the proposed method with the UBC-RSM method

The new background correction approach was compared to the UBC-RSM approach, which was successfully applied to methanol:water mobile phase systems [20] using an AR as IP. Fig. 8a shows three chromatograms extracted from the same data set obtained during the injection of a mixture of four PEGs at a concentration of 5 mg mL^{-1}. All chromatograms were extracted at 1095 cm^{-1} applying a single point baseline correction at 1400 cm^{-1}. The first one was extracted before applying any background correction. The second chromatogram was extracted after UBC-RSM background correction, demonstrating the easy identification of all peaks as compared to the uncorrected chromatogram.

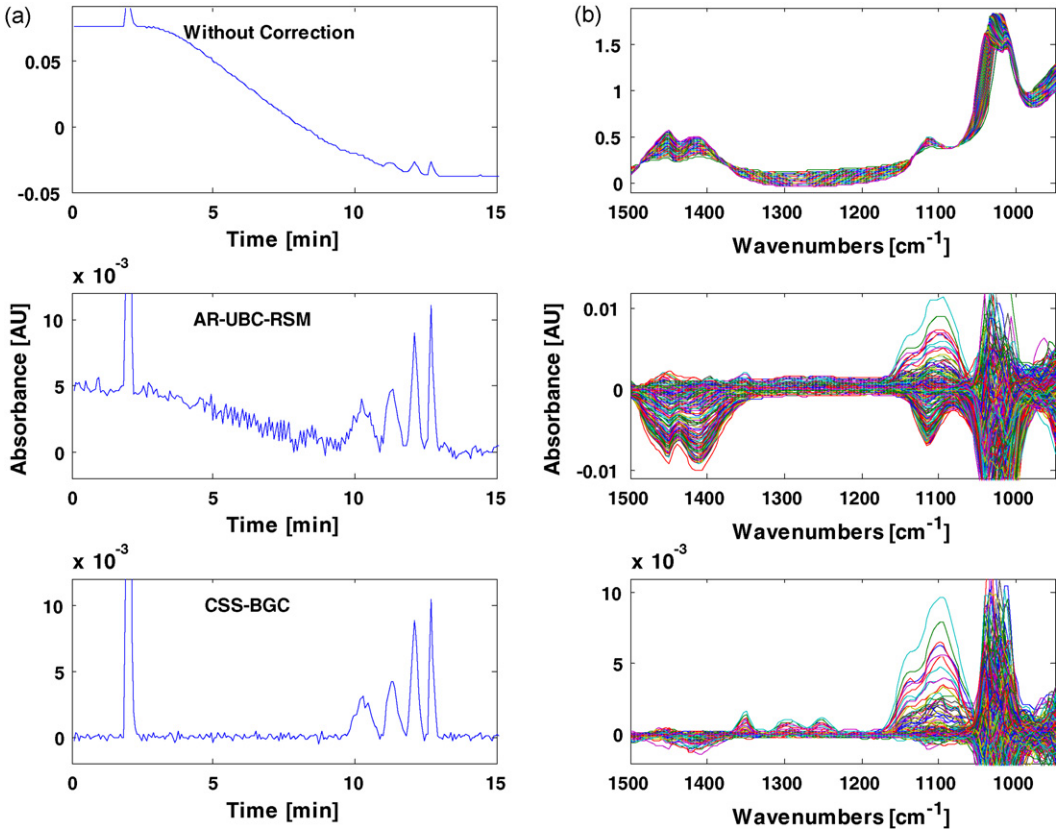

Fig. 8. Chromatographic separations of PEGs using a methanol:water gradient and different background correction methods. (a) Chromatograms extracted from data acquired during the injection of a standard mixture containing 5 mg mL^{-1} of each of the four PEG standards in a methanol:water gradient ranging from 30 to 90% (v/v) methanol without background correction, applying UBC-RSM and CSS correction, respectively and (b) spectra corresponding to the chromatograms shown in (a) acquired during the whole chromatographic run. *Note*: Chromatograms were extracted as described in Table 1.

Table 3
Chromatographic noise values found for PEGs separated in methanol:water using different background correction strategies.

	$Noise_{1640}$	$Noise_{1450}$	$Noise_{1412}$	$Noise_{1115}$
Without correction	652	182	184	147
AR-UBC-RSM	12	4	5	3
CSS-BGC	0.33	0.10	0.13	0.27

Note: Noise values are measured as root mea square (RSM) in the time-window were no analytes elute (between 2.7 and 9.2 min). All noise values are given in mAU.

The best results were obtained using the cubic smoothing splines method which provided a baseline without sloping and a minimum of noise, influencing directly the limit of detection.

Despite the results shown in Fig. 8, it has to be remarked that the quality of the background correction obtained using the UBC-RSM approach also depends strongly on the size of the RSM (in this case it only contained 189 spectra), on the range of mobile phase compositions and on the stability of the instrument, which in this case showed a remarkable slope drift with time (data not shown). Fig. 8b shows the corresponding spectra of the same data set obtained during the whole chromatographic run. PEG spectra can be identified and, once again, the best results were obtained using the cubic smoothing splines method.

Table 3 summarizes the chromatographic noise values found at wave numbers were the eluents show typical absorption bands before and after background correction. Applying both background correction methods, noise values can be reduced drastically. Comparing UBC-RSM and the cubic smoothing splines method, the latter one reduces the noise between 44 and 13 times at the four considered wave numbers, therefore clearly improving the achievable limit of detection.

For the application of the CSS background correction method no reference spectra matrix is needed. Furthermore, using the UBC-RSM approaches, high instrument stability is required, which is also not the case applying CSS. On the contrary, knot selection can be troublesome in the presence of a high number of overlapping peaks. In short, the selection of an appropriate background correction method depends strongly on the instrumental conditions and must be decided case-by-case.

4. Conclusions

The CSS correction method was tested and evaluated by means of simulated as well as real data sets. Applying the new background correction method to simulated data sets it could be shown that it works well for baseline resolved peaks. Background corrected spectra showed high quality and could be used to identify the analytes. Results lead to the conclusion that this background correction might be suitable for many practical applications. Furthermore, this method shows high potential to achieve a high grade of automatization in the background correction process as the peak detection cannot only be realized manually but also automatically. However, the critical step in the background correction process is the selection of the knots. Real data sets were obtained during the separation of PEGs of different molecular weights on a LC reversed phase column employing aqueous mobile phase systems with three different alcoholic organic modifiers (methanol, 2-propanol and ethanol). Obtained results provided analyte specific chromatograms and recovered background corrected spectra suitable for identification and quantification of all four considered analytes. Figures of merit of the method were adequate for quantitative analysis of PEGs showing high linearity (R^2 of above 0.98) and appropriate LODs and LOQs.

Results obtained by the UBC-RSM approach are strongly dependent from quality and size of a previously obtained RSM. Using the proposed CSS approach there is no need to record a RSM and therefore problems caused by poor instrument stability are avoided. Furthermore, the new method minimizes user-interaction as no variable selection prior to background correction is required. This makes the whole process more feasible and requires less user-experience.

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