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Short communication

Application of attenuated total reflectance Fourier transform infrared spectrometry to the determination of cephalosporin C in complex fermentation broths

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Abstract

An analytical procedure has been developed for quantitative determination of cephalosporin C in complex fermentation broths. The method is based on the partial least-square treatment of data obtained by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectrometric method in the wavenumber range of 1227–1257 cm^{-1}. Absorbance spectra were employed for measurement using a set of eleven binary aqueous standard solutions of cep halosporin C and deacetoxycephalosporin C. The method is simple, rapid and accurate (to within $\pm 1.49\%$). The developed method has been used to measure cephalosporin C in aqueous solution of biosynthetic samples only after freeze drying the sample at -30 °C and dissolving it in distilled water.
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1. Introduction:

Cephalosporin C (CPC) is a β-lactam antibiotic and major precursor of semi-synthetic cephalosporins. CPC is produced by the mold acremonium chrysogenum by a batch or fed-batch fermentation technique [1]. Optimization of this process requires accurate, simple and reliable measurements of CPC concentration over along period. Determination of CPC in fermentation liquors usually involves many difficulties due to the presence of related contaminants and various mineral salts [2]. Because of this, traditionally the measurement of CPC in complex broths is performed in two steps of separation and detection. The most frequently used methods are biological high performance liquid chromatography (HPLC), with more precise results of CPC determination in later ones [3–14].

In this present paper a new simple technique for determination of CPC at the presence of related

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contaminants and mineral salts without any separation is described.

2. Experimental

2.1. Apparatus and software

A Bomem (Quebec canada) MB series FTIR spectrometer equipped with a DTGS (DC31B) mid-range detector, a KBr Ge/Sb 253 coated beam splitter and a SiC source, was employed to record the IR spectra using spectra tech (Warrington, UK) in-compartment contact with sampler horizontal attenuated total reflector with a 45° ZnSe through plate. PlS-plus/IQ button to the GRAMS/32 (version5) and higher software (Galactic industries corporation) was used to process the absorbance data. Liquid chromatographic assay was carried out using a HPLC instrument (Knauer) equipped with a UV detector (model K-2500) and Eurochrom 2000 software.

2.2. Reagents

All of the reagents used were of analytical-reagent grade or equivalent. CPC was obtained from Sigma and acetic acid and phosphate salt from Merck. DAOC was supplied by Antibioticos Ltd. (Spain). To optimize and evaluate the prediction capability of the PLS-IR model, two sets of 16 binary and unicomponent standard solutions of CPC and DAOC were made up in phosphate buffer solution (pH 3.5, 0.01 M) containing acetic acid. Binary solutions were prepared from CPC with concentrations of 0.00–4.00 g/100 ml and DAOC with fixed concentration equal to 0.4 g/100 ml. The CPC concentrations of the unicomponent solutions were the same as the binary ones. The weight of the CPC and DAOC were determined to three decimal points. Eleven standards were used to set up the PLS models and five other standards were employed to evaluate these models.

The CPC fermentation broths samples were supplied by Gazvin University of Medical Science. The biosynthesis of CPC was carried out in a stirred-tank reactor by fed-batch operation. The complex fermentation medium contained solid peanut flour and other substrates such as glucose, phosphate buffer and antifoam [2]. The phosphate buffer solution used to induce the fermentation in the pathway of CPC biosynthesis.

2.3. Procedure

For several years, HPLC has been used for separation and determination of CPC in complex fermentation broths [2] Also, in the present investigation in order to evaluate the results obtained by the proposed ATR-FTIR method, same samples were analysed by HPLC technique. The analytical column (250×4.6 mm) was packed with Nuclesil 1005C18 and was thermostated at 25–30 °C. The HPLC system was controlled by the integrator which recorded the chromatograms and calculated the concentrations. The most suitable analytical wavelength for monitoring CPC turned out to be 254 nm. Different eluents were tested and a mobile phase of 0.01 M phosphate buffer solution (pH 3.5) containing acetic acid proved to be optimal for the separation of CPC from its precursors. A constant flow-rate (1ml/min) was maintained during the analysis.

The ATR-FTIR spectra were obtained in the wavenumber range of 1000–2000 cm^{-1} employing cosine appodization and with the use of 25 scans and a nominal resolution of 8 cm^{-1}.

The biosynthetic sample was directly injected into the HPLC column and the peak area was measured and CPC concentration calculated.

A 50 ml portion of complex fermentation broths sample was vacuum distillated at 0 °C and then freeze-dried at −30 °C. The ATR-FTIR spectra was recorded after dissolving this sample in distilled water and diluting it to 5 ml.

3. Results and discussion

3.1. Spectral acquisition

During CPC fermentation, CPC and its precursors deacetylcephalosporin C(DAC), deacetoxycephalosporin C (DAOC) and penicillin N (PENN) are produced in the biosynthesis. The IR spectra of these compounds are similar. All of

these products, PEN N, DAC, DAOC and CPC contain four C=O groups. To distinguish CPC from DAC, DAOC and PEN N in fermentation liquors, there is no other way but monitoring the ester group. On the other hand in biosynthesis of CPC in present work, the phosphate buffer solution was used to induce the fermentation in the pathway of CPC biosynthesis.

However, the ATR-FTIR spectra of CPC in phosphate buffer solution (a), DAOC in buffer solution (b) and buffer solution containing both of these cephalosporins (c) are shown in Fig. 1 These IR spectra show two main bands at 1241 and 1765 cm^{-1} for CPC which are due to the present of ester group in CPC. These two bands are completely covered by phosphate buffer solution and only appear after subtraction.

Further experiments showed that the wavenumber regions of 1227–1257 and 1751–1782 cm^{-1} could be selected for CPC quantitative determination, (see Fig. 2). As we can see in Fig. 2, the binary aqueous solutions of CPC and DAOC with CPC concentrations of 0.00–4.00 and DAOC fixed concentration equal to 0.4 g/100 ml present well defined and intense bands in the above mentioned wavenumber regions. The resulting difference spectra show that the relative changes in absorbance appear to be linearly spaced, with the best variations centered around 1227–1257 cm^{-1}.

3.2. Data treatment

By using multivariate calibrations, the overlapping of C=O band of CPC precursors and also other bands of CPC with ester group of CPC can be calculated and the simultaneous inclusion of multiple spectral intensities can greatly improve the precision and the predictive ability [15]. With the aim of improving the analysis for CPC, a multivariate method, PLS, was applied with the use of absorption spectra.

One of the main advantages of Fourier transformation is the possibility of accumulating a large number of scans, which could provide better limit of detection for IR measurements. An increase in the number of accumulated scans does not affect the absorbances of the analytes but reduces the noise level of the spectra obtained and therefore the S/N is improved [15,16]. The use of higher resolution achieves more data points, but in a longer time. The use of 25 scans and a nominal resolution of 8 cm^{-1} seems to be a good compromise in order to obtain a good LOD for the analyte in the present work. However, these two spectral ranges, were evaluated by performing PLS calibration method. A set of 11 unicomponent CPC standard samples were taken as the calibration samples. Also in order to ensure that these calibration models would provide accurate predictions, five other unicomponent standard samples

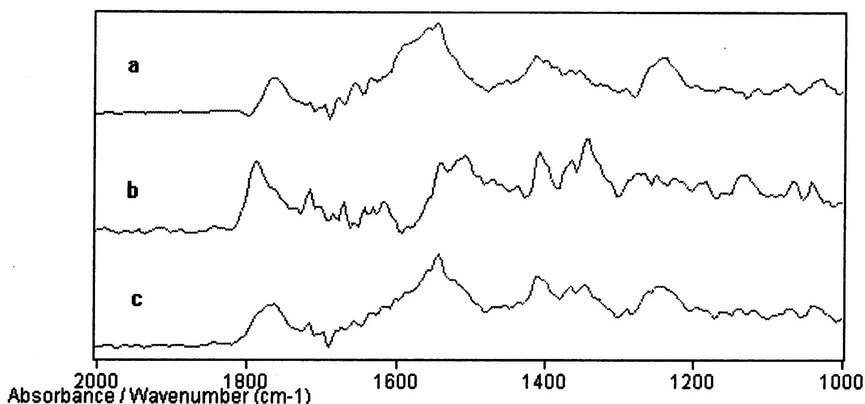

Fig. 1. The FTIR spectra of: (a) CPC in phosphate buffer solution, (b) DAOC in buffer solution and (c) buffer solution containing CPC and DAOC, using buffer solution as reference.

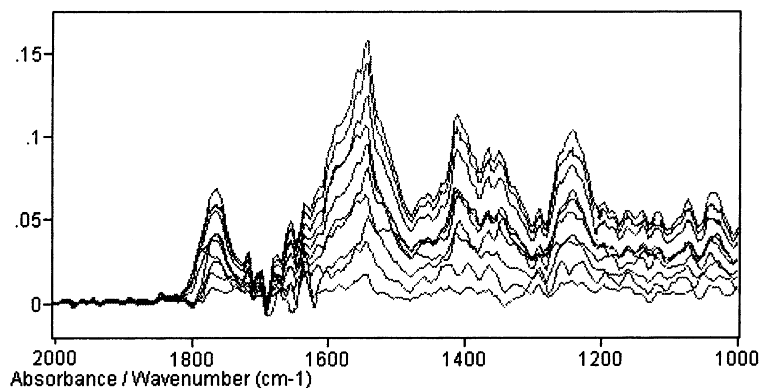

Fig. 2. The FTIR spectra of binary standard solutions of CPC and DAOC, with CPC concentration of 0.00–4.00 g/100 ml and DOAC fixed concentration equal to 0.4 g/100 ml.

were proposed as validation set (samples nos. 12–16). The concentration of CPC in these unicomponent standard samples were varied between 0.00 and 4.00 g/100 ml.

As we can see in Fig. 1, the C=O group of CPC precursors shows some aborbance at 1227–1257 and 1751–1782 cm^{-1} spectral regions. In order to study the effect of these CPC precursors on determination of CPC, another set of 16 binary standard solutions (11 calibration samples and five validation samples) of CPC and DAOC were prepared. The CPC concentration in these 16 binary samples were same as in unicomponent standard samples and the DAOC concentration were kept constant and equal to 0.4 g/100 ml in all 16 binary standards. Four sets of absorption spectra were obtained at two above spectral regions for these two sets of standard samples, following the same procedure as detailed for the ATR-FTIR procedure in the experimental section. For each set of spectra a calibration model was generated. In each experiment, mean centering procedure were carried out for all spectra prior to any other treatment. These four calibration models were tested by varying the number of PLS factors from 1 to 4 in order to optimize the models. To select the optimum number of factors in PLS algorithm, the cross validation method [17], leaving out one sample at a time, was used. The prediction error sum of squares (PRESS) was calculated each time a new factor was added giving rise to different PLS models. One reasonable choice for the optimum number of factors would be that number (h*) which yielded the minimum PRESS. However, the best calibration models were obtained for unicomponent and binary standard solutions with two and three PLS factors, respectively. These four sets of the results are shown in Table 1.

The standard error of estimation (SEE) for calibration was calculated for these four sets of results by using the appropriate expressions [18]. Cross validation (leave one out cross validation) was also performed for evaluating these five calibration models. The standard error of cross validation (SECV), together with relative standard deviation (RSD% with $n = 9$) and the squared correlation coefficient of regression lines (R^2) for these four calibration models are also calculated. The results are shown in Table 2. However considering these SEE, SECV, RSD and (R^2) values, the spectral range of 1227–1257 cm^{-1} was selected as chosen spectral range and binary standard solutions were used as more reliable standards. The calibration curve for this binary standard solutions was found to be linear up to 4 g/100 ml and can be described by the equation $Y = (0.13 \pm 0.03)X + 1.07$ (± 0.04), where Y is the area of the transient peaks and X is the CPC concentration (g/100 ml).

Table 1
Actual and predicted concentrations of CPC in the calibration (1–11) and validation (12–16) unicomponent and binary standard solutions at 1227–1257 and 1751–1782 cm^{-1}

Samples	Actual concentration (g/100 ml)	Predicted concentration (g/100 ml)			
		Binary standard		Unicomponent standard	
		1227–1257 cm^{-1}	1751–1782 cm^{-1}	1227–1257 cm^{-1}	1751–1782 cm^{-1}
1	0.00	0.06	0.04	0.05	0.04
2	0.40	0.40	0.42	0.46	0.45
3	0.80	0.68	1.01	0.77	0.76
4	1.20	1.20	1.17	1.16	1.16
5	1.60	1.61	1.55	1.63	1.64
6	2.00	1.98	1.94	2.15	2.08
7	2.40	2.40	2.36	2.37	2.47
8	2.80	2.70	2.62	2.73	2.73
9	3.20	3.15	3.14	3.25	3.23
10	3.60	3.62	3.72	3.90	3.65
11	4.00	4.01	4.08	3.87	3.91
12	1.80	1.72	1.74	1.75	1.74
13	2.20	2.26	2.12	2.00	2.03
14	2.90	2.85	2.94	3.04	2.95
15	3.40	3.43	3.48	3.42	3.40
16	3.80	3.77	3.72	3.81	3.95

3.3. Analysis of complex fermentation broths

The developed ATR-FTIR procedure was employed to analysis the CPC in biosynthetic samples. During CPC fermentation, CPC and its precursors DAC, DAOC and PEN N are produced as product and by-products respectively. Fig. 3 shows the spectrum of (a) fermentation sample, (b) fermentation sample to which the standard CPC is added and (c) the fermentation sample to which the standard DOAC is added. By comparing Figs. 1 and 3a,b, it can be seen that standard addition of CPC to biosynthetic sample caused a well intense change in one of the above bands (wavenumber region of 1227–1257 cm^{-1}). So this band can be correlated to the ester group of CPC in fermentation sample.

In Fig. 3a and c, it is also shown that addition of standard DAOC to fermentation liquor sample caused no defined and intense change in 1227–1257 cm^{-1} band.

Table 2
Statistical comparison of the results obtained in analysing binary and unicomponent standard solutions at two spectral range of 1227–1257 and 1751–1782 cm^{-1}

Samples parameters	Spectral regions (cm^{-1})			
	Binary standard		Unicomponent standard	
	1227–1257 cm^{-1}	1751–1782 cm^{-1}	1227–1257 cm^{-1}	1751–1782 cm^{-1}
SEE (g/100 ml)	0.067	0.126	0.147	0.074
SECV	0.054	0.070	0.112	0.110
RSD ($n = 9$)[a]	1.49	1.81	2.30	2.12
R^2	0.997	0.997	0.981	0.996

[a] 0.95 g/100ml CPC standard solution.

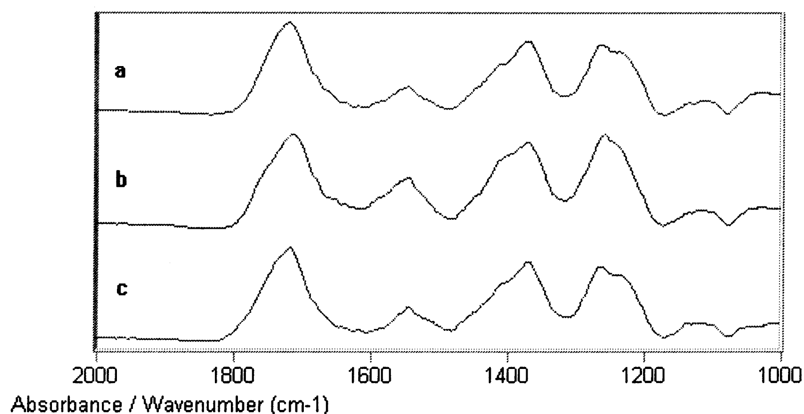

Fig. 3. The FTIR spectra of (a) fermentation sample, (b) fermentation sample to which the standard CPC is added and (c) fermentation sample to which the standard DOAC is added.

Table 3
Determination of the CPC concentration in fermentation samples by HPLC and proposed methods

Sample	HPLC method			Proposed method		
	Mean values[a] (g/100 ml)	S.D.[b]	R.S.D.[b]	Mean values[a] (g/100 ml)	S.D.[b]	R.S.D[b]
1	0.40	0.007	1.75	0.37	0.008	2.10
2	0.12	0.003	2.50	0.12	0.003	2.30
3	0.98	0.014	1.42	0.97	0.018	1.87
4	1.70	0.024	1.41	1.72	0.030	1.69

[a] Mean concentration for five measurements.
[b] For five measurements.

The influence of the concomitant species other than CPC precursors in biosynthetic samples on the determination of CPC was studied. The results showed that these concomitant species cause no severe interference effects.

A recovery study was carried out by measuring the CPC concentration in four biosynthetic samples spiked with CPC. The original CPC concentration of these samples was also determined before spiking. The recovery, established by the above experiment, was 94–97% ($n = 4$).

However, the CPC concentration in four biosynthetic samples were measured by both HPLC and proposed method at 1227–1257 cm^{-1} band, the results are shown in Table 3. Generally good agreement was found between the two sets of the results and it can be seen that the ATR-FTIR method could provide an accurate determination of CPC in biosynthetic samples at the presence of related contaminants. In analysing biosynthetic samples by proposed method, a LOD of 0.02 g/100 ml was obtained. This LOD was calculated as 3 SEE and by taking into account a freeze-dried preconcentration factor of 10.

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