MULTIVARIATE CURVE RESOLUTION IN ON-LINE GRADIENT HPLC-FOURIER TRANSFORM INFRARED SPECTROSCOPY.

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INTRODUCTION

Mid-infrared spectroscopy provides detailed molecular specific information on the chemical composition of almost any sample under investigation. While this feature is of advantage in many analytical applications it makes direct on-line FTIR spectroscopic detection in HPLC difficult due to strong solvent absorptions of the liquid phase. Whereas solvent absorption can be handled in case of isocratic HPLC by recording an appropriate background spectrum advanced techniques of data analysis are required in case of gradient HPLC-FTIR to cope with the changing background during a chromatographic run.

In this contribution we present a novel strategy to correct for changes in solvent composition during gradient HPLC-FTIR. With the help of multivariate curve resolution alternating least squares (MCR-ALS) the changes in background spectra can be efficiently modelled. Using this advanced data analysis technique the elution profiles of the analytes as well as their FTIR spectra can be extracted from the recorded data set. The principle of this novel analytical technique will be shown on the example of the determination of four phenolic acids (gallic acid (GA), 3,4-dihydroxybenzoic acid (dHB), p-hydroxybenzoic acid (pHB) and vanilic acid (VA)) by using a Methanol:H_2O (1% v/v acetic acid) gradient. For on-line recording of FTIR spectra a 25 µm flow-cell made of CaF_2 windows has been used. The calculated spectral matches of the recovered spectra were very satisfactory reassembling the standard spectra up to 98 %. Furthermore quantitative information could also be obtained from the calculated elution profiles covering a typical concentration range from 0.1 to 2 mg ml^{-1}.

CHEMOMETRICAL DATA TREATMENT

Background data treatment

Selection of the number of components and MCR-ALS calculation of the optimized background gradient spectra

Principal Component Analysis:

The first PC explained >99.92 % of the variation in the data while the second PC explained <0.1%, as can be seen in Table 2. So, using a single PC the variation in the eluent absorbance spectra due to the mobile phase gradient can be explained to a greater extent.

PC	Eigenvalue of Cov(X)	%Variance This PC	%Variance Cumulative
1	0.0198	99.92	99.92
2	0.0000160	0.08	100.00
3	0.000000341	0.00	100.00

MCR-ALS:

The calculation of the optimized background gradient spectra was carried out by MCR-ALS analysis of the HPLC-FTIR chromatographic data of a sample solvent injection.

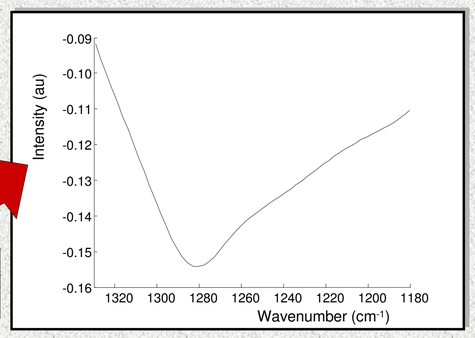
Initial estimation: Evolving Factor Analysis (EFA)

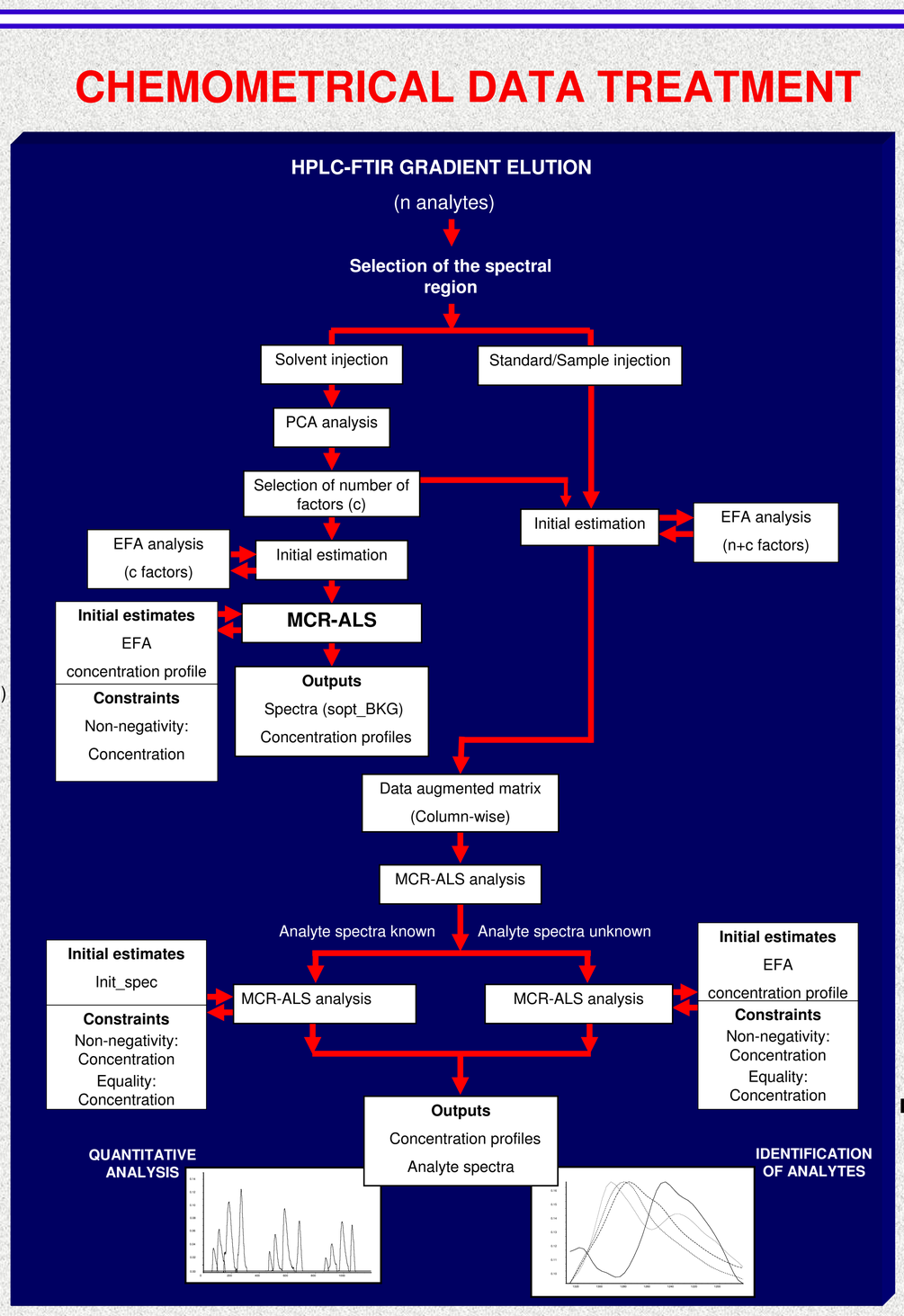
Number of components: Previously calculated by PCA.

Constraints: Non negativity in concentration (fast non-negative least-squares approach)

Convergence limit: 0.1

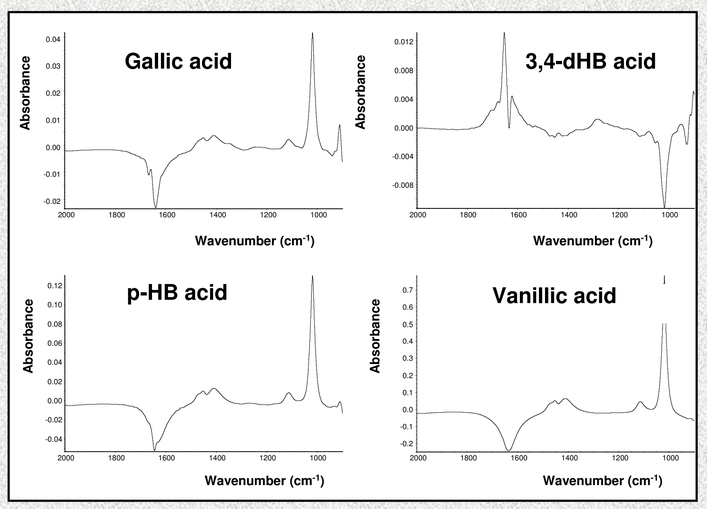
Optimized spectra (sopt_BKG)





Selection of the spectral region

- The analytes should present characteristic absorption bands
- The different components of the eluent do not present intense absorption bands .



Analytes spectra (aprox. 3 mg ml^{-1}) recorded after elution from the HPLC column

MCR-ALS chemometrical analysis of the HPLC-FTIR chromatograms of standards

- The MCR-ALS analysis of the set of standards was carried out on a column-wise augmented data matrix (C-direction) (**Data_augmented**).

Initial estimation:

EFA elution profiles: every chromatogram is analyzed separately by EFA (number of components = n+c) to obtain a first estimation of the elution profiles. After that, a new matrix (EFA_augmented) is built by setting the EFA results matrices one on top of each other as aforementioned. Afterwards, the **EFA_augmented** matrix is used as initial estimate for the MCR-ALS analysis of the matrix containing the whole set of chromatograms (Data_augmented).

Reference spectral matrix: a matrix **Init_spec** (w x k, where k=n+c) is built up using the n previously measured reference spectra and the **sopt_BKG** calculated.

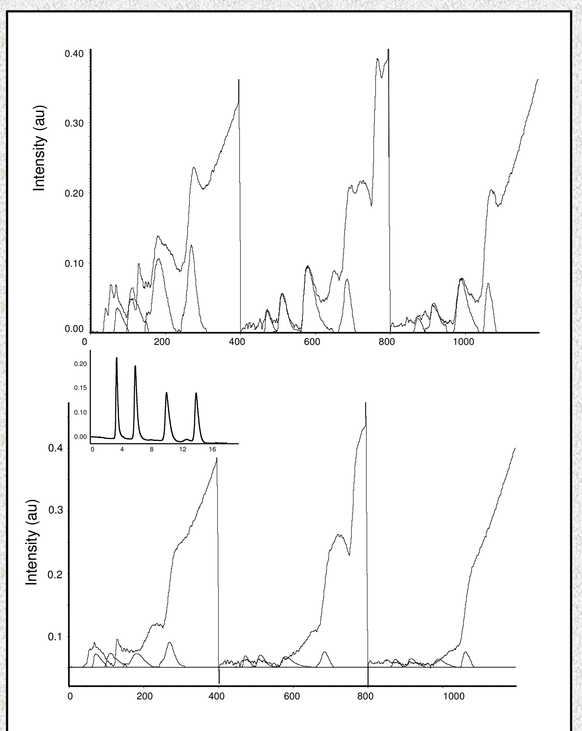
Constraints:

Non-negativity: In the studied HPLC-FTIR system the non-negativity of concentrations is valid for the whole set of components and so it can be applied during the MCR-ALS process. On the contrary, the non-negativity of the spectrums can not be applied because during the gradient the relative concentration of water and acetic acid in the eluent is decreasing, and so, the contribution of both components in the eluent spectrum is negative.

Unimodality: Non applicable because of the possibility of peak splitting.

Selectivity (equality): It was employed a concentration equality constrain.

MCR-ALS output: concentration profiles and analytes spectra



MCR-ALS resolved concentration elution profiles of the four analytes and that of the eluent obtained using as initial estimates EFA elution profiles (top) and a spectral matrix (bottom). Each elution point value corresponds to 3 s. Elution order: GA, dHB, pHB, VA.

Inset: HPLC-UV (254 nm) chromatogram of a standard solution containing 200 µg ml^{-1} of GA, dHB, pHB and VA.

Results obtained from the MCR-ALS analysis of the gradient HPLC-FTIR spectral data using different initial estimates. Constraints employed: non-negativity in concentration (5 components, fast non negative least squares algorithm); low rank concentration regions.

	$A^a = (a \pm s_a) + (b \pm s_b)$ C (ppm) $(10^5)^c$	R^2	$Sx_y{}^d$	USP Tf[e]			As[f]			Spectral match[h]	% Variance explained[i]	Initial estimate[j]
				C1[g]	C2	C3	C1	C2	C3			
GA	(-0.02 ± 0.03) + (15.9 ± 0.8) C	0.9951	0.049	2.35	2.27	1.43	3.77	3.50	1.86	92.46	98.9349	EFA profile
dHB	(-0.05 ± 0.08) + (36 ± 2) C	0.992	0.064	1.96	1.82	1.19	1.66	2.39	1.43	94.58		
pHB	(0.08 ± 0.15) + (82 ± 4) C	0.995	0.052	2.42	1.08	1.73	2.94	1.03	2.55	79.13		
VA	(-0.1 ± 0.2) + (54 ± 5) C	0.995	0.093	1.09	0.91	1.10	1.03	0.82	1.19	79.17		

	$H^b = (a \pm s_a) + (b \pm s_b)$ C (ppm) (10^5)	R^2	Sx_y
GA	(-0.001 ± 0.002) + (0.71 ± 0.07) C	0.983	0.093
dHB	(-0.01 ± 0.1) + (1.56 ± 0.08) C	0.992	0.053
pHB	(0.004 ± 0.007) + (2.3 ± 0.2) C	0.985	0.089
VA	(-0.001 ± 0.007) + (2.5 ± 0.1) C	0.986	0.043

	$A = (a \pm s_a) + (b \pm s_b)$ C (ppm) (10^5)	R^2	Sx_y	USP Tf			As			Spectral match	% Variance explained	Initial estimate
				C1	C2	C3	C1	C2	C3			
GA	(-0.01 ± 0.01) + (9.6 ± 0.4) C	0.9964	0.042	2.31	2.19	0.75	3.75	3.40	2.00	93.63	99.7485	Spectral data
dHB	(-0.02 ± 0.03) + (11.7 ± 0.7) C	0.9930	0.061	1.90	1.67	1.50	2.91	2.21	1.81	97.98		
pHB	(0.02 ± 0.03) + (13.2 ± 0.9) C	0.9904	0.690	2.30	1.40	1.75	2.66	1.85	2.30	85.84		
VA	(-0.00 ± 0.04) + (17 ± 1) C	0.991	0.077	1.22	1.00	1.38	1.10	1.00	1.50	80.16		

	$H = (a \pm s_a) + (b \pm s_b)$ C (ppm) (10^5)	R^2	Sx_y
GA	(0.001 ± 0.002) + (0.44 ± 0.06) C	0.9661	0.132
dHB	(0.001 ± 0.001) + (0.43 ± 0.03) C	0.9905	0.069
pHB	(0.0002 ± 0.04) + (0.442 ± 0.012) C	0.9985	0.028
VA	(0.000 ± 0.004) + (0.79 ± 0.11) C	0.9611	0.142

[a]:Peak Area; [b]: Peak height; [c]: Analytes regression lines obtained using peak area and peak height measurements. Calibration curves obtained for 4 standards of GA, dHB, pHB and VA; [d]: Standard deviation $Sx_0=Sy\ b^{-1}$; [e]: USP tailing factor; [f]: Asymmetry factor; [g]: Standard concentration levels (g ml^{-1}), C1=5.0, C2=3.5, C3=2.5; [h]: % Spectral match compared with reference spectra; [i]: % Variance explained after the MCR-ALS analysis; [j]: MCR-ALS initial estimate.

HPLC measurement conditions:

Column: Nucleosil 100 RP18 (150 x 2.1 mm, 3 µm)

Flow rate: 150 µl min^{-1}

HPLC gradient

Start time (min)	% H_2O	% CH_3OH
0.00	85	15
15.00	60	40
20.00	60	40

FTIR measurement conditions:

Spectrometer: Bruker IFS 55

Resolution: 8 cm^{-1}

Scans: 50

Flow cell: 25 µm pathlength, CaF_2 windows

Mirror velocity: 150 kHz

Detector: MCT

CONCLUSIONS

Multivariate curve resolution based on alternating least squares under constraint has been applied to the analysis of gradient HPLC-FTIR spectral data. Different procedures suitable to the calculation of analyte elution profiles and spectrums have been described. After evaluation of the use of different constraints, it was only used only the non-negativity in concentration and local rank concentration constrains. From results obtained, it can be concluded that MCR-ALS allows accurate extraction of the spectra of the analytes in the presence of a changing spectral background when reduced spectral ranges are selected as well as their elution profiles in order to quantify them. Moreover, the use of a flow cell for the coupling between gradient HPLC and FTIR spectrometry instead of a solvent removal interface permits the use of other common HPLC detectors (UV-Vis, Fluorescence or MS, for example) without additional instrumental requirements.

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