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Multivariate Curve Resolution of Spectrophotometric Data for the Determination of Artificial Food Colors

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In the analysis of food additives, past emphasis was put on the development of chromatographic techniques to separate target components from a complex matrix. Especially in the case of artificial food colors, direct spectrophotometric measurement was seen to lack in specificity due to a high spectral overlap between different components. Multivariate curve resolution (MCR) may be used to overcome this limitation. MCR is able to (i) extract from a complex spectral feature the number of involved components, (ii) attribute the resulting spectra to chemical compounds, and (iii) quantify the individual spectral contributions with or without a priori knowledge. We have evaluated MCR for the routine analysis of yellow and blue food colors in absinthe spirits. Using calibration standards, we were able to show that MCR equally performs as compared to partial least-squares regression but with much improved chemical information contained in the predicted spectra. MCR was then applied to an authentic collective of different absinthes. As confirmed by reference analytics, the food colors were correctly assigned with a sensitivity of 0.93 and a specificity of 0.85. Besides the artificial colors, the algorithm detected a further component in some samples that could be assigned to natural coloring from chlorophyll.

KEYWORDS: Multivariate curve resolution; MCR; PLS; spectrophotometry; food colors; alcoholic beverages; absinthe

INTRODUCTION

Chemometric methods such as principal component analysis (PCA) and partial least-squares (PLS) regression have been used successfully in many applications over the years (*1*–*3*). All of these applications use the multivariate methods to reduce the multidimensional data sets to fewer dimensions and to point out intercorrelations and interdependencies in the data. The majority of applications in chemistry are within the field of spectroscopy. The disadvantage of a PCA or PLS approach is that the obtained principal components are abstract mathematical factors, so-called "latent variables", with usually little or no physical or chemical meaning. The regression coefficient of the PLS sometimes provides hints to attribute defined features in the spectra to the response variable. However, many users, especially in an industrial environment, want to obtain information that is as close as possible to their real life experience in spectroscopy.

Much research has been done to solve the mixture analysis problem and to extract real spectra and concentration profiles from overlapping spectral data without any a priori assumptions about the composition of the system. Several mixture analysis methods are known such as evolving factor analysis (EFA) (*4*), fixed-size moving window evolving factor analysis (FSMWEFA) (*5*), target factor analysis (TFA) (*6*), classical curve resolution (CCR) (*7*), weighted curve resolution (WCR) (*8*), multivariate curve resolution (MCR) (*9*–*12*), and to a certain extent also techniques such as parallel factor analysis (PARAFAC) (*13*).

In food chemistry and especially the analysis of food additives, emphasis in the past has been on the development of separation methods to analyze the target compounds as selectively as possible. Food colors are regularly analyzed using thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) (*14*–*24*). The direct spectroscopy of the original food matrix without separation was not possible in the past, because the resulting spectra are difficult to interpret and often lack specificity. This disadvantage can be solved using MCR, as it implies the following objectives:

1. Resolve the number of chemical compounds simultaneously present in the mixture from a complex spectral signature.
2. Identify these species by transforming mathematical solutions to real spectra, thus increasing specificity by applying mathematical and chemical constraints.
3. Quantify each component without any prior assumption or knowledge of the chemical model involved.

Unlike deconvolution, MCR provides spectra of pure compounds and not only resolution of single bands, which are difficult to attribute to chemical compounds in a complex

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mixture. The algorithm for MCR has been previously described in detail (*9, 10, 12, 25, 26*). The following is meant as a short summary.

The goal of MCR is to decompose spectra of mixtures into the n pure contributions of each component involved in the system studied. The spectral data can be arranged in a data matrix $\mathbf{D}$ ($r \times c$), with the spectra as the r rows and the c columns, which are the measured responses at each wavelength. The MCR decomposition of matrix $\mathbf{D}$ is carried out, according to the following equation:

$$\mathbf{D} = \mathbf{C}\mathbf{S}^{\mathrm{T}} + \mathbf{E} \quad (1)$$

The matrix $\mathbf{C}$ ($r \times n$) describes the individual contributions (concentration profiles) of the n species involved in the given spectra. The matrix $\mathbf{S}^{\mathrm{T}}$ ($n \times c$) is then the spectral contribution of these n species in the c columns of the data matrix (pure spectra profiles). $\mathbf{E}$ ($r \times c$) is the residual matrix, which contains the data variance unexplained by the product $\mathbf{C}\mathbf{S}^{\mathrm{T}}$.

One important and frequently used iterative approach to solve eq 1 is MCR by alternating least squares (MCR-ALS). The optimization process starts from initial guesses of $\mathbf{C}$ and $\mathbf{S}^{\mathrm{T}}$ that are then refined to yield profiles with chemical meaning.

Critical aspects in the application of MCR-ALS are the determination of the number of factors or components that cause the variability in the data set and the rotational ambiguity of the solution. The number of factors is often determined by prior PCA or by using a priori knowledge of the components involved. The rotational ambiguity of the solution can usually be reduced by introducing constraints to the solution. The constraints are derived from previous knowledge of the system and guided by the physical and chemical nature of the system under study. In the case of pure components describing spectral responses, a common constraint is to allow only positive values for $\mathbf{S}^{\mathrm{T}}$ and $\mathbf{C}$. For reaction-based systems, concentration profiles are often unimodal, and closure or mass balance equations should be fulfilled. If chemical information about the spectra or concentration profiles is available, the so-called equality constraint can be applied.

MCR-ALS has been applied successfully in many chemical fields. A summary is given in refs *26–28*. In this study, we demonstrate the possibilities and power but also the restrictions of this method for the analysis of food colors in spirits. The objective was to demonstrate the ability of MCR to resolve real life spectra from the original food matrix without perturbation by a chemical extraction or separation procedure.

MATERIALS AND METHODS

Apparatus. Spectrophotometric measurements were performed on a Perkin-Elmer Lambda 12 dual beam spectrometer equipped with automatic cell changer. The spectrometer was operated with the UV WinLab software (version 2.80.03). The spectra were acquired in a range between 350 and 700 nm at a scanning speed of 60 nm/min with a data interval of 1.0 nm. All measurements were made against ethanol (60% vol).

Reagents and Materials. All reagents were of analytical grade. The color standards tartrazine (E102), quinoline yellow (E104), patent blue V (E131), and brilliant blue FCF (E133) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Ethanol (absolute, 99.9%) was from Merck (Darmstadt, Germany). All standard solutions were prepared in ethanol diluted to 60% vol.

Data Sets and Multivariate Analysis. A first data set containing different standard solutions was prepared. To benchmark the MCR algorithm, the data set was prepared to contain a mixture of all four colors in any case (i.e., no spectra of pure components were included). The concentrations were simultaneously varied in a randomized fashion using the Software Package Design Expert V7 (Stat-Ease Inc., Minneapolis, MN) for all components between 0.2 and 4 mg/L in 0.2 mg/L steps ($n = 40$). This data set was used for calibration. For possible use as "initial guess" in the MCR algorithm, the spectra of pure components (4 mg/L) were measured as well.

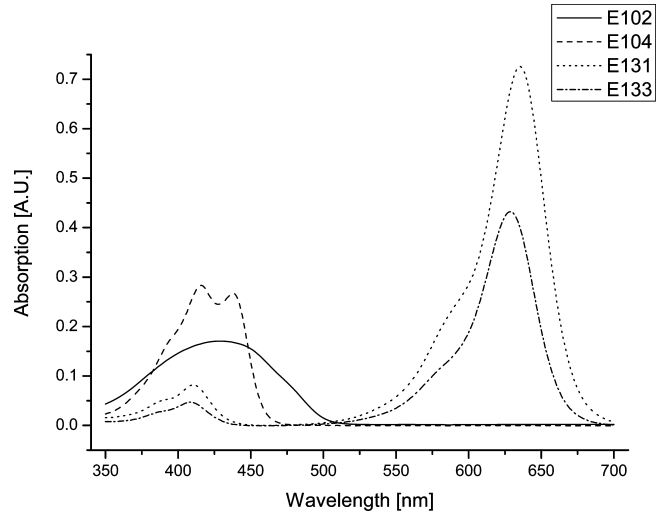

Figure 1. Absorption spectra of the studied analytes (E102, E104, E131, and E133; each at a concentration of 4 mg/L).

The second data set was prepared from freshly prepared standard solutions of the same color standards (different day and different operator). This data set was set up as a mixture design in the concentration range between 0.2 and 1.4 mg/L and contained 30 different samples including five replicated samples. This data set was used for validating the calibration models calculated from data set 1.

The third data set comprised only authentic absinthes that were sampled in the context of official food control by governmental food inspectors in the German Federal State Baden-Württemberg ($n = 52$). The samples were diluted 1:2 with ethanol 60% vol prior to measurement.

For MCR, the spectra were exported to the software PLS Toolbox Version 4.0 for use with Matlab (Eigenvector Research Inc., Wenatchee, United States). For comparison, PLS regression models were calculated to predict the concentration of the food colors in the liquids (used software, Unscrambler v9.7 CAMO Process AS, Oslo, Norway). Again, data set 1 was used for calibration, and data set 2 was used for validation. With data set 3, it was possible to compare the routine application of the PLS and the MCR method with the results received by TLC as a reference procedure.

RESULTS AND DISCUSSION

MCR of Standard Calibration Samples (Data Sets 1 and 2). For this first evaluation of the MCR algorithm for the determination of artificial colors in spirit samples, we chose two yellow (E102 and E104) and two blue (E131 and E133) colors that are regularly used in combination to achieve the green color in absinthes (*29*). The absorption spectra of the pure components with a concentration of 4 mg/L are shown in **Figure 1**. It can be seen that E131 and E133 have very similar peak shapes with a complete overlap and only a slight difference in the absorbance maximum at 636 and 628 nm, respectively. E102 has a very broad peak, which completely overlaps that of E104, which exhibits two maxima. In addition, the two blue colors also incorporate absorption bands around 400 nm within the range of the yellow colors. Therefore, we think it is a nontrivial challenge for any multivariate algorithm to correctly separate components with such similar spectra when they are combined in mixtures.

In a first step, sample set 1 is analyzed using the classical PLS approach. A PLS calibration model is calculated for each of the colors. The coefficient of determination (squared correlation coefficient R^2), which describes how much of the

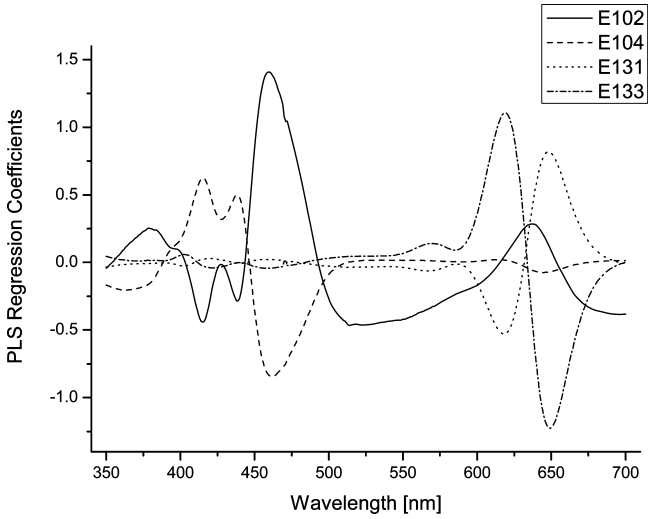

Figure 2. PLS regression coefficients calculated for calibration data set 1.

original variability is explained by the chosen regression model, is shown in **Table 1**. Furthermore, the root mean squared error (RMSE), which summarizes the overall error of the regression to a single number, is calculated by applying full cross-validation to the PLS calibration. It is also given in **Table 1**. The corresponding PLS regression coefficients for the optimal model (five PLS components for E102 and E104 and four PLS components for E131 and E133) are visualized in **Figure 2**. We clearly see that the PLS coefficients put most emphasis on those wavelengths, which separate the colors. This may lead to problems if real samples are measured, which have a significantly different background matrix because of additional color compounds, which were possibly not included in the calibration. To test the quality of the PLS calibration, the model is applied to the data set 2. **Table 2** shows the coefficient of determination (R^2) and the RMSE for the validation. It is worth mentioning that the RMSE of the validation is smaller than the RMSE of the calibration. This is indeed an indication of a correct PLS model, as the validation data set 2 only spans a subspace of about the first quarter of the calibration range. For the first MCR approach, the only constraints that we use in this case are nonnegativity in the spectra and concentration profiles and the number of relevant factors. As an initial guess for the starting point of the MCR algorithm, the approximate range of existence of each component present in the data was determined with the EFA algorithm [EFA is a multivariate technique to detect how many factors are present in a data set and where in the data set the factors first appear and where they disappear (*4*)]. The resolved pure component spectra are shown as normalized spectra in **Figure 3**. It is surprising how well the four pure component spectra are resolved. E131 and E133 are clearly separated at the peaks 636 and 628 nm, respectively; E104 shows the double peak. The only spectrum, which is not so well-resolved, is E102 with a broader peak than expected and two additional peaks at 592 and 647 nm. However, it must be emphasized that the only information gone into this calculation are the mixture spectra and the constraints. No information on any concentration has been supplied to the MCR algorithm, but together with the spectra, MCR also calculates the corresponding concentration profiles. If we look at the correlation coefficients and RMSE values in **Table 1**, the MCR algorithm with only positive constraints and initial guess used from EFA showed inferior results as compared to the PLS algorithm. However, it should be kept in mind that for calculating the PLS model, it is necessary to know the color concentrations for each spectrum. Therefore, it is remarkable that MCR is able to gain correlations, for example, as high as 0.98 in the case of E102 by just using the spectral information. This MCR model is as well applied to the validation data set 2 (**Table 2**).

If we use the pure spectra as an initial guess for the MCR algorithm, the results improve and become comparable to PLS calibration as **Table 1** shows. This approach is of course realistic and permissible in food analysis as generally the possible additives are known and the question is only the qualitative and quantitative verification. The resolved spectra given in **Figure 4** now excellently correspond to those of the pure components in **Figure 1**. This approach of using spectra as initial guess is very advantageous because no quantitative reference analysis is needed in this case.

The MCR results obtained using EFA as compared to pure component spectra as initial estimates yield an equally well fitting of the raw data in both cases. The explained variance using EFA is 99.96%, and it is 99.97% using pure component spectra. This means that the differences in the resolved spectra are due to nonresolved rotational ambiguities. Using the pure spectra as an initial guess helps the algorithm to select a solution closer to the "truth".

From these results, the following procedure for easy analysis of foods for artificial colors can be derived. First, a MCR screening of the data set should be done to get first information about spectra of the compounds contained in the food samples. Second, the pure spectra of likely candidates may be used as constraints to determine the MCR model. When this model is computed, the corresponding concentrations for each pure component are calculated simultaneously. With two reference values for each component, it is possible to gain concentration profiles in real concentration units. We use this approach to analyze the third sample set of authentic absinthes from commercial trade described in the following section.

MCR of Authentic Spirit Samples. The previously established PLS and MCR models were used to predict the concentrations of the food colors in 52 authentic absinthe samples. In food control, the qualitative presence of food colors is of primary interest as this leads to a labeling requirement. To test for artificial colors in absinthes is of special importance as previous tests showed that 41% of all absinthe samples under investigation lacked the necessary labeling of the use of color additives (*30*).

Therefore, we have used the multivariate models to parametrically judge the presence or absence of the food colors and compare these results with those from TLC (**Table 3**). The results show satisfactory sensitivities and specificities. The sensitivity is the proportion of spirits that tested positive to contain artificial colors (true positives) divided by the total amount of spirits containing artificial colors (true positives + false negatives). Thus, it describes the probability that the test is positive, when the spirit actually contains artificial colors. The specificity on the contrary describes the probability that the test is negative when the spirit indeed contains no artificial color [true negatives/(true negatives + false negatives)]. See ref *31* for further explanation of terms. The specificity was generally lower than the sensitivity, meaning that more samples were false positively classified to contain colors than false negatively. We judge both PLS and MCR models to be adequate for screening for artificial food colors. Of course, positive results should be confirmed by reference analytics as in the case of every screening procedure.

Table 1. Comparison of Method Performance for PLS Regression and MCR with Different Initial Assumptions for Calibration Data Set 1

	E102		E104		E131		E133	
	R^2	RMSE (mg/L)	R^2	RMSE (mg/L)	R^2	RMSE (mg/L)	R^2	RMSE (mg/L)
PLS calibration	0.999	0.022	0.993	0.08	0.988	0.041	0.996	0.055
MCR (initial guess: EFA profiles)	0.955	0.250	0.981	0.146	0.885	0.396	0.885	0.40
MCR (initial guess: spectra)	0.984	0.141	0.995	0.078	0.999	0.037	0.998	0.049

Table 2. Comparison of Method Performance for PLS Regression and MCR with Different Initial Assumptions for Validation Data Set 2

	E102		E104		E131		E133	
	R^2	RMSE (mg/L)	R^2	RMSE (mg/L)	R^2	RMSE (mg/L)	R^2	RMSE (mg/L)
PLS validation	0.994	0.040	0.998	0.022	0.982	0.051	0.9880	0.054
MCR (initial guess: EFA profiles)	0.794	0.163	0.973	0.058	0.835	0.145	0.824	0.152
MCR (initial guess: spectra)	0.9882	0.119	0.9956	0.067	0.9990	0.038	0.9987	0.045

Table 3. Sensitivity and Specificity[a] of the Detection of Artificial Food Colors in Authentic Absinthe Samples Using PLS and MCR Models as Compared to Qualitative TLC

	E102		E104		E131		E133		artifically colored overall	
	sensitivity	specificity	sensitivity	specificity	sensitivity	specificity	sensitivity	specificity	sensitivity	specificity
PLS	1.00	0.45	1.00	0.97	0.76	1.00	1.00	0.33	0.95	0.76
MCR	0.98	0.36	1.00	1.00	0.71	1.00	1.00	0.59	0.93	0.85

[a] Sensitivity = true positives/(true positives + false negatives). Specificity = true negatives/(false positives + true negatives).

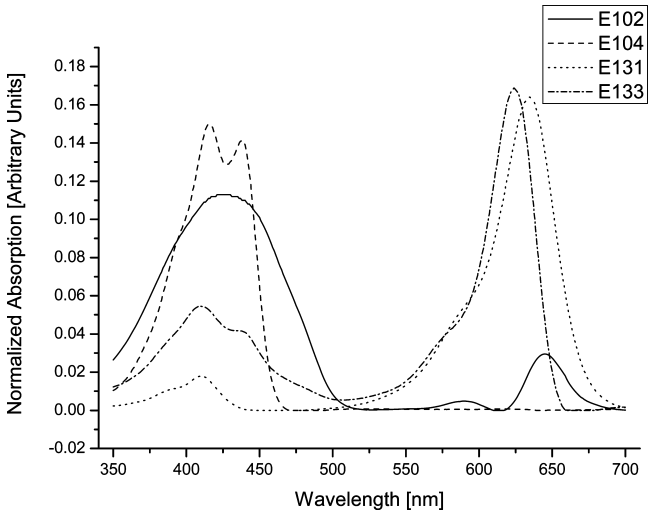

Figure 3. MCR-resolved pure compound spectra of calibration data set 1 (constraints: positive spectra and concentrations; initial guess: EFA results, four components; spectra are normalized to unit length).

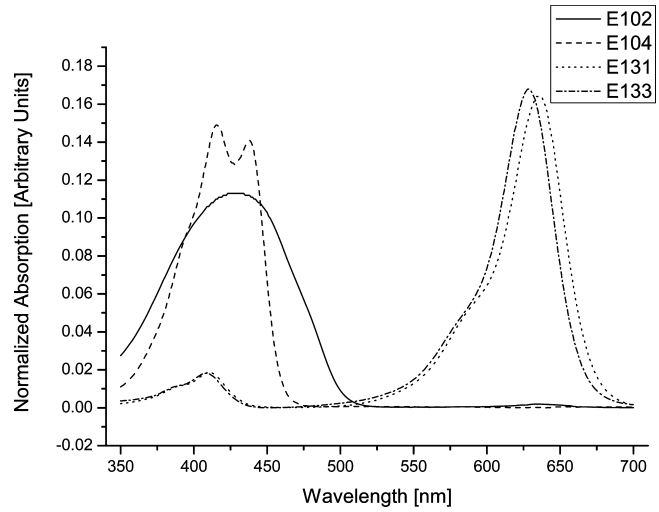

Figure 4. MCR-resolved pure compound spectra of calibration data set 1 (constraints: positive spectra and concentrations; initial guess: pure component spectra, four components; spectra are normalized to unit length).

During the MCR prediction, it was noted that some of the samples contained a significant amount of residual information (i.e., the spectrum after subtracting the absorptions explained by the model). The residual spectra looked very similar, with the highest absorbance at 350 nm and exponentially falling down until it reaches baseline at around 450 nm. This spectrum was predominantly contained in naturally colored absinthes (i.e., absinthes colored with Roman wormwood and other herbs; see ref *32* for details about absinthe coloration). Therefore, we have analyzed a model absinthe that was manufactured according to a historic recipe by ourselves as described in ref *33* and that was exclusively colored with wormwood. This spectrum was used as a fifth reference spectrum besides the artificial colors for a recalculation of the MCR model. The comparison between the spectrum of this absinthe to the spectrum predicted by MCR is shown in **Figure 5**. It is clearly demonstrated that the MCR resolved spectrum is well-matched by the real life spectrum. This proves our theory that the residual spectra are caused by the natural coloring (most probably by the chlorophyll contained in the herbs used for coloring). The MCR concentration profiles show that the natural color is predominantly contained in those absinthes that are exclusively colored by maceration of different herbs according to historical recipes. Some of the absinthes contained both natural and artificial colors. All in all, the MCR model appears to be suitable to determine artificial as well as natural colors in absinthes with the aim to control food labeling requirements.

Possibilities and Restrictions of MCR for Analysis of Food Additives. MCR is to our knowledge the only method that offers a calibration free resolution of chemical compounds from a complex spectral matrix. The spectral information acquired without previous chromatographic separation can be extracted and transferred into meaningful chemical as-

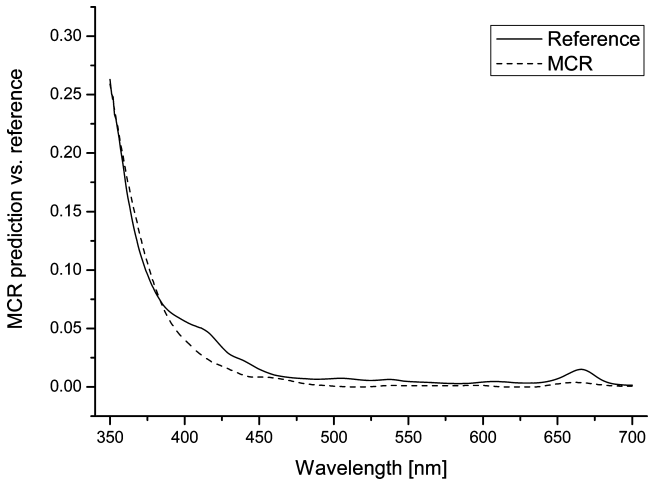

Figure 5. Comparsion between MCR prediction of unknown component and spectra of model absinthe colored purely with wormwood.

signments. MCR is ideally suited for this task providing information on the chemical constituents including semi-quantitative data.

Even a fully quantitative approach is possible with MCR if the reference concentration information is used as a constraint during MCR resolution. This is in addition a possibility to decrease rotational ambiguities. MCR is then used similarly to PLS regression, but like in PLS, quantitative information is necessary during the calibration process (*28*).

The qualitative or semiquantitative approach is very helpful if no or little reference values are available. A typical application is the evaluation of kinetics especially in chemical and biotechnological reactions, when reference values are difficult to obtain (*25, 34*). A comprehensive summary of MCR-ALS applications of spectroscopic data is given in ref *26*. So far, the MCR algorithm is not widely implemented in commercially available analytical software packages. However, there is freeware software for calculating MCR available on the Internet (*35*). Some implementations (e.g., in the Unscrambler software) lack the possibility to predict quantitative information from unknown spectra (i.e., spectra not contained in the original MCR model). To our knowledge, this is only implemented in the PLS Toolbox for Matlab used in this study. Most preferably for the routine use for food control purposes, the MCR algorithm should be included in the standard software that operates the spectrophotometer so that an "online" prediction would be possible similar to the PLS capabilities of many analyzers.

We think that the advantage of MCR lies not necessarily in models with better quantitative prediction possibilities than PLS models but in the largely improved clearness and chemical meaning of the MCR predicted spectra.

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