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Detection of Unintended Stress Effects Based on a Metabonomic Study in Tomato Fruits after Treatment with Carbofuran Pesticide. Capabilities of MCR-ALS Applied to LC-MS Three-Way Data Arrays

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A chemometric strategy based on multivariate curve resolution and alternating least-squares (MCR-ALS) applied to LC-MS three-way data arrays has been developed to perform a metabonomic study in tomato (*Lycopersicon esculentum*) fruits (cultivar Rambo) following treatment with carbofuran. This methodology has proved to be adequate for the detection of unintended stress effects due to the previous treatment with this pesticide. MCR-ALS was performed on augmented matrices built with the LC-MS three-way data obtained from treated and nontreated samples through the sampling time. The strategy allowed us to obtain the concentration and spectra profiles of the main components (previously estimated with the SVD algorithm) from samples treated with pesticide as well as from blank samples, showing how they vary with time after plants treatment with the pesticide. In addition, a simple resolved mass spectrum was obtained corresponding to the peaks of a particular component in all matrices, thus avoiding ambiguity in the compound identity assignment. Different time profiles were found for some metabolites in treated and nontreated samples, which demonstrate that the presence of pesticide causes changes thorough time in the behavior of certain endogenous tomato metabolites as a result of physiological stress.

In recent years, new "omics" disciplines (metabolomics and metabonomics) have been increasingly applied in diverse fields (e.g., functional genomics, toxicology, pharmacology, disease diagnosis, food and nutrition science, and environmental science). Thus, metabolomics and metabonomics techniques have been used for multiple and diverse project studies, and the number of reports on these studies in peer-reviewed journals has risen steadily every year to well over 600 in 2008.

Metabonomics has been defined as "quantitative measurement of time-related multiparametric responses of multicellular systems to pathophysiological stimuli or genetic modifications", whereas metabolomics has been defined as "the measurement of metabolite concentrations and fluxes in isolated (and usually identical) cell systems or cell complexes".[1] Despite these two definitions, some authors use both terms indistinctly[2] and six strategies (metabolomics, metabolite profiling, metabolic fingerprint, metabolite target analysis, and metabonomics) have been proposed for metabolomic analysis.[3] In addition, some authors refer to "metabolite profiling" or "metabolomics approaches".[4] Obviously, any of the above-mentioned approaches involves measuring a set of compounds with wide variations in chemical (molecular weight, polarity, solubility) and physical (volatility) properties, which also extend over an estimated 7−9 fold magnitude of concentration (picomole to millimole).[5]

Specifically, metabonomics depends on the possibility of determining changes in low molecular weight organic metabolites in complex biological samples. Analytical strategies in metabonomics are high-field proton nuclear magnetic resonance spectroscopy (^{1}H NMR), direct injection into a mass spectrometer, Fourier transform infrared (FT-IR) spectroscopy, and separation-based techniques such as gas or liquid chromatography or capillary electrophoresis with mass spectrometry detection (GC-MS, LC-MS, and CE-MS).[6] Nowadays, emerging developments in analytical technologies such as fast high-resolution separation systems (e.g., ultraperformance liquid chromatography, UPLC) or high-mass accuracy and large dynamic-range MS instruments such as time-of-flight-MS (TOF-MS), quadrupole-time-of-flight-MS (Q-TOF-MS), Fourier transform cyclotron resonance-MS (FT-ICR-MS), and Fourier transform-Orbitrap-

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MS (FT-Orbitrap-MS) can provide more information from the experimental data generated, leading to assignment of metabolites.[7]

Complete chromatographic separation of the components of complex biological samples is often difficult to achieve because of the existence of overlapping or embedded peaks, even under optimal separation conditions, mainly when using conventional separation systems. Nowadays, the hyphenation of chromatographic with spectroscopic techniques (e.g., GC-MS or LC-MS) yields second-order data that combine instrumental signals built from both spectral and time domains. The response is arranged as a data matrix where each column corresponds to a m/z ratio and each row corresponds to a different time. Using this type of data in combination with multivariate resolution methods allows us to obtain mass and concentration profiles (mass spectra and chromatograms, respectively) for the different sample components.

Resolution of overlapping signals may be carried out by chemometric approaches, including noniterative and iterative methods, as well as those based on pure variable selection. Current noniterative chemometric approaches in curve resolution based on the natural evolution of the data are evolving factor analysis (EFA),[8,9] window factor analysis (WFA),[10,11] heuristic evolving latent projections (HELP),[12,13] orthogonal projection resolution (OPR),[14] evolving window orthogonal projections (EWOP),[15] and subwindow factor analysis (SFA).[16,17] Among the iterative approaches, iterative target transformation factor analysis (ITTFA)[18] and multivariate curve resolution-alternating least-squares (MCR-ALS)[19] may be cited. Pure variable selection methods are the most simple to use and include self-modeling mixture analysis (SIMPLISMA),[20] the orthogonal projection approach (OPA),[21,22] iterative key set factor analysis (IKSFA),[23] and simplified Borgen method (SBM).[24]

The HELP method coupled with GC-MS data was used to determine chemical components of essential oils in *Cortex cinnamomi* from four different production areas resulting in the separation of 88−93 components and the determination of 58−64 of them, representing about 90% of the total relative content.[25] The same algorithm was applied in combination with GC-MS data to analyze volatile components in a traditional Chinese medicinal preparation. Ninety-three components were separated, and 65 of them were qualitatively and quantitatively analyzed which represented about 90.28% of the total content.[26] Further, in the characterization of essential oil components of Iranian geranium oil, a total of 61 components accounting for 91.51% were identified using similarity searches between the mass spectra and the MS database, and this number was extended to 85 compounds using HELP for solving overlapping peak clusters, after determining the number of components, pure variables, zero concentration, and selective regions by application of different chemometric approaches.[27] GC-MS combined with OPR and DS-MCR-ALS (distance-selection multivariate curve resolution-alternting least-squares) was used to characterize the essential oil components of Iranian cumin and caraway. A total of 19 and 39 compounds were identified by direct similarity searches for cumin and caraway oils, respectively, and these numbers were extended to 49 and 98 components, respectively, when applying chemometric techniques.[28]

In the environmental field, MCR-ALS was applied to LC-ESI-MS data in the investigation of main microcontaminant sources of endocrine disruptors in coastal and harbor waters and sediments,[29] and the same approach was further applied to analyze wastewaters and sediments by using fused data from LC-DAD and LC-ESI-MS.[30]

The main aim of the present work was to use MCR-ALS for detecting changes in the concentration of tomato metabolites as a result of stress after treatment with carbofuran, specifically with Botrán 20, an insecticide belonging to the carbamate family.

The Rambo tomato is a good size fruit (size G-GG), firm, spherically shaped, and slightly ribbed. The skin is relatively thin and has an attractive red color with green streaks. The good taste, both in early and advanced states of maturation, qualify it as an excellent ingredient for salads. In addition, it is very resistant to tomato mosaic virus, fusarium races 1 and 2, verticillium, and *Stemphylium radicis* but only has moderate resistance to nematodes.[31]

Carbofuran is a systemic insecticide with nematicide, insecticide, and miticide activity, which acts by surface contact and through ingestion by interfering with the transmission of nerve impulses by inhibiting cholinesterase.[31] So, it causes reversible acetylcholinesterase carbamylation, allowing the accumulation of acetylcholine.

THEORY

Data Pretreatment: Use of Wavelet Transform (WT) To Compress Matrices. Since 1996, the number of papers involving wavelet transform (WT) treatments, devoted to compressing and denoising signals, has considerably increased. Some of these

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papers involve high performance liquid chromatography with diode array detection (LC-DAD),[32−34] as well as liquid and gas chromatography with mass spectrometry detection (LC-MS and GC-MS).[35,36] In addition, WT has been applied as a signal pretreatment before chemometrics approaches such as curve resolution[34] and other chemometric techniques.[37,38]

Wavelets are a family of basis function having a compact support, which means that they differ from zero only in a limited time domain. This property makes the wavelet very appropriate to represent the different features of a signal, especially sharp signals and discontinuities.[39] The WT decomposes the signal onto a set of basis functions called the wavelet basis, each member being obtained by dilation and contractions (scaling) as well as by shifts (translations) of a single prototype called the mother wavelet Ψ.

$$\Psi_{a,b} = a^{-1/2}\Psi\left(\frac{t-b}{a}\right) \quad (1)$$

where a, $b \in R$ and $a \neq 0$; a is the scaling variable, and b is a translation variable.

To achieve computation, dyadic (power of 2) dilations and translations of the mother wavelet are used

$$\Psi_{(s,l)} = 2^{-s/2}\Psi(2^{-s}t - l)$$

where s is the scale index, and l the location index.

WT techniques can be classified in two categories: the continuous wavelet transform (CWT) and the discrete wavelet transform (DWT). The latter retains sufficient spectral information and can be implemented much faster than CWT, the signal decomposition is unique, and the problem of the basis selection does not exist.[40]

The DWT can be represented in a vector matrix form

$$\mathbf{w} = \mathbf{W}f$$

where f is the signal of interest, $\mathbf{w}$ is the vector of the wavelet transform coefficients, and $\mathbf{W}$ is an orthogonal matrix consisting of the wavelet basis functions.

Each basis vector is characterized by a set of coefficients (c_0, c_1, c_2, etc.) which are organized in the matrix $\mathbf{W}$ into a low-pass and a high-pass filter, depending on the pattern in which they are ordered. The low-pass filter, constructed with the coefficients (c_0, c_1, c_2, ...c_N) acts as a smoothing filter, whereas the high-pass filter, with coefficients ($c_N - c_{N-1}$, ...$c_1 - c_0$), can be considered a difference filter.[41]

According to the fast decomposition algorithm proposed by Mallat,[42] the full length vector describing the original signal is passed through the low-pass and the high-pass filters and outputs are split in approximations and details (or wavelet coefficients). Approximation coefficients represent a smooth version of the signal at half resolution and detail coefficients contain details of the signal at that level of decomposition. Approximation coefficients are then used as new input for the matrix $\mathbf{W}$ to obtain a new vector of approximation coefficients and new details of the signal. The process can continue until one approximation coefficient remains, but usually it is finished when the optimal decomposition level is achieved. Then, reconstruction of the signal is carried out by the inverse wavelet transform, whose transform matrix is the transpose of WT matrix ($\mathbf{W}^T$).

Among the different types of wavelets, the simplest one is the Haar wavelet, which is also the first member of Daubechies family of orthonormal wavelets, characterized by the two coefficients c_0 and c_1.[43] The Haar wavelet is the only wavelet which keeps the non-negativity property in the approximations (low-frequency) of the signal, allowing the application of ordinary multivariate curve resolution methods with non-negativity constraints.[30]

Compression can be in one dimension (1D) or two dimensions (2D). There are two ways to generalize the one-dimensional wavelet transform to two dimensions, known as the standard and nonstandard approaches.[41] Standard decomposition of an image is appealing because it simply requires performing one-dimensional transforms on all rows and then on all columns. The nonstandard decomposition alternates between operations on rows and columns. The choice of an approach depends on the application to be carried out.

Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS). The main function of resolution methods is the mathematical decomposition of a global mixed instrumental response into the pure contributions due to each of the components in the system. MCR-ALS has become a popular chemometric tool that has been used successfully to resolve multiple component responses in unknown unresolved mixtures.[44−46] So, this technique has been shown to be a powerful tool for resolving two- and three-way data arrays, the main advantage being easy adaptation to data sets of different complexity and structure (trilinear or nontrilinear), providing optimal least-squares solutions.[47] Also, its versatility allows application to any multicomponent system, giving as a result data tables or data matrices that can be described by a bilinear model, i.e., processes such as chemical reactions, chromatographic elutions, environmental data, and others, monitored by diverse multivariate responses, such as spectroscopic measurements, electrochemical signals, or composition profiles.[46]

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MCR-ALS is an iterative resolution algorithm that is used to recover the process contributions, expressed as the concentration profile and the pure spectrum of each of the compounds involved.[48,49] This algorithm is based on a bilinear model (eq 2) that decomposes the data matrix **D**, containing the raw information about all the components present in the data set, into the product of two matrices **C** and $\mathbf{S}^T$, containing the pure response profiles associated with the variation of each contribution in the row (matrix **C**) and the column directions (matrix $\mathbf{S}^T$).

$$\mathbf{D} = \mathbf{C}\mathbf{S}^T + \mathbf{E} \tag{2}$$

where $\mathbf{D}(J \times K)$ is the original data matrix, $\mathbf{C}(J \times N)$ and $\mathbf{S}^T(N \times K)$ are the matrices that contain the concentration profiles of each component and the pure spectra, respectively. **E** is the error matrix, i.e., the residual variation of the data set that is not related to any chemical contribution. Parameters J and K are the number of rows and the number of columns of the original data matrix, respectively, and N is the number of chemical components or principal components in the mixture or process.

C and $\mathbf{S}^T$ matrices are responsible for the observed data variance. Usually, the column profiles of matrix **C** are associated with the concentration profiles, and the row profiles of $\mathbf{S}^T$ are associated with pure spectra profiles of the resolved components. The superscript "T" means the transpose of matrix **S**, where pure spectra are column profiles.[46]

Singular Value Decomposition (SVD). MCR-ALS solves iteratively eq 2 by an alternating least squares algorithm which calculates concentration **C** and pure spectra $\mathbf{S}^T$ matrices optimally fitting the experimental data matrix **D**. This optimization is carried out for a proposed number of components, whose estimate is the first step in MCR-ALS. The estimation of the principal components may be done using principal components analysis (PCA), one of the most basic and widely used chemometric tools devoted to finding the number and direction of the relevant sources of variation in a bilinear data set[50] or singular value decomposition (SVD). Both techniques consist of a statistical summary which involves a reduction in the size of the information.

Implementation of Constraints in ALS Optimization. Besides the estimation of the principal components of the experimental matrix **D**, the optimization of eq 2 requires an initial estimate of either the spectra in $\mathbf{S}^T$ or the concentration profiles associated with these spectra, which can be obtained using SIMPLISMA-derived methods.[51,52] The main goal of this approach is to find the purest or most representative contributions to the data matrix using real variables. Most of resolution methods start with initial estimates of **C** or **S** and work by optimizing iteratively the concentration or response profiles, introducing the available information about the system through the implementation of constraints.[53] A constraint can be defined as any mathematical or chemical property systematically fulfilled by the whole system or by some of its pure contributions. So, constraints force the iterative optimization process to model the profiles respecting the conditions desired.[54] Also, during the optimization, the constraints modify the least-squares pseudoinverse estimations, and the constrained solutions are not truly least-squares solutions. In any iterative method, the appropriate application of constraints is crucial to drive the optimization to the right solution.

The constraints that can be imposed commonly on the algorithm are intended to decrease the possibility of rotational ambiguities and to provide a physically reliable optimization path. The two most generally used constraints in ALS process are nonnegativity[55] and unimodality.[56] The former is the most used in resolution methods and prevents the presence of negative values in profiles. It can be applied to concentration profiles and to various types of spectra because their intensities are always positive. On the other hand, the unimodality constraint guarantees and forces only the presence of one maximum per concentration profile. This is applied to the concentration profiles related to chromatographic elution processes.

Augmented Matrix Arrangements: Column-Wise Data Matrices. MCR-ALS can be applied to a single data matrix (two-way data sets) or to three-way data sets, i.e., row-wise or column-wise augmented data matrices. In both cases, multiexperiment data arrangements still follow the same bilinear model as a single data matrix.[57] Working with column-wise augmented data matrices requires that these matrices, belonging to different processes, are appended one on top of each other so that the spectral direction is common and the data matrix lengthens in the process direction. Constraints mentioned above can be applied to one or more species within an experiment.

Figures of Merit. The quality of the MCR-ALS model is assessed by different indicators or figures of merit linked to the correct reproduction of the original data set through the use of the resolved MCR-ALS model, i.e., the $\mathbf{C}\mathbf{S}^T$ product. One of these figures of merit is the "lack of fit", that is defined as the difference between the input data **D** and the data reproduced from the $\mathbf{C}\mathbf{S}^T$ product obtained by MCR-ALS. This value is estimated according to

$$\text{lack of fit (\%)} = \sqrt{\frac{\sum (d_{ij}^* - d_{ij})}{\sum d_{ij}^2}} \times 100 \tag{3}$$

where d_{ij} is an element of the experimental matrix **D** and d^*_{ij} the element of the MCR-ALS reproduced matrix **D***. The other two figures of merit are percent of variance explained (r^2) (eq 4) and standard deviation of residuals with respect to experimental data (eq 5) calculated as[57]

$$r^2\ (\%) = \frac{\sum d_{ij}^{*2}}{\sum d_{ij}^2} \times 100 \tag{4}$$

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$$\sigma = \sqrt{\frac{\sum (d_{ij}^{*} - d_{ij})^2}{n}} \quad (5)$$

where n is the number of elements in the data set ($n_{\text{rows}} \times n_{\text{columns}}$).

EXPERIMENTAL SECTION

Chemicals and Solvents. Acetonitrile (ACN) of HPLC grade was obtained from J.T. Baker (Holland). Acetic acid glacial (AcOH, 99.7%) was obtained from Panreac (Spain). Magnesium sulfate anhydrous ($MgSO_4$) and sodium acetate 3-hydrate ($AcONa \cdot 3H_2O$) were obtained from Merck (Germany). Ultrapure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA). Mobile phases were filtered through a 0.45 μm cellulose acetate (water) or polytetrafluoroethylene (PTFE) (organic solvents) and degassed with helium prior to and during use. All the extracts were filtered through a Millipore membrane of cellulose acetate (0.45 μm particle size) before pumping it into the chromatographic system. Finally a concentrated suspension of Botrán 20 (carbofuran 20% w/v) was obtained from Tragusa (Sevilla, Spain).

Instrumentation and Software. LC separation was carried out with a Hewlett-Packard (H−P) series 1100 system (Hewlett-Packard, Wilmington, DE) with an H−P Chem Station for MS control and spectral processing. The HPLC system consisted of a model G 1311 gradient pump and Rheodyne six-port injection valve (model 7725i) with a 20 μL loop. The analytical separation was performed with a 150 mm × 4.6 mm i.d. Agilent Zorbax EclipseXDB C_8 column (5 μm particle size). An H−P G 1948 A Platform benchtop single quadrupole mass spectrometer with an ESI interface was used to detect the target compounds in the LC column effluent.

Also a 230 V, 50 Hz crusher (0.5 kW maximum power) from Sammic S.L. (Azpeitia, Spain) and a polytron PT1035 from Kinematica AG (Switzerland) were used. A rotary evaporator (R-114) with a B-480 thermostatted water bath was purchased from Buchi (Flawil, Switzerland). A Sigma 4-15 centrifuge with a Sigma 11150, 143/F, 5100/min rotor incorporated was used during the extraction step. Finally, crushed and homogenized samples were stored in a −86 °C ultralow freezer. For MCR-ALS application, a graphical user interface was used, which additionally provides detailed information about the implementation of this algorithm.[46]

Plant Samples and Pesticide Treatment. Four different cultivars of tomato (Rambo, Raf, Zayno, and RZ 74-668) were planted in a 1 ha greenhouse located at the Experimental Farm UAL-ANECOOP foundation involved in the Cooperative Society of grade 2 ANECOOP and the University of Almería, on March 6, 2007.

The four cultivars were arranged according to an experimental design ensuring uniformity and symmetry in order to minimize possible errors due to the spatial distribution of plants. The cultivable surface was divided into two plots, each of which, in turn, was divided into two sectors. Thus, the total area was composed of four sectors (A, B, C, D), each of them containing 22 lines arranged in pairs to form rows. Each tomato cultivar was arranged in each sector as a plot containing 30 plants by double rows (60 plants/sector).

The cultivar under study was Rambo, which was developed by Syngenta Seeds Company, and the other three cultivars will be examined in subsequent work. Average conditions of humidity and temperature in the greenhouse were 20.1 °C and 74.4%, respectively.

Table 1. Sampling Plan of Rambo Tomatoes after Treatment with Carbofuran

sampling number	number of days after carbofuran treatment	name of the treated sample	name of the blank sample
1	1	CA1	CA1b
2	3	CA2	CA2b
3	7	CA3	CA3b
4	9	CA4	CA4b
5	11	CA5	CA5b
6	14	CA6	CA6b
7	18	CA7	CA7b
8	21	CA8	CA8b

The plants were arranged in the greenhouse according to a planting design to ensure uniformity and symmetry and in order to avoid possible contamination of nontreated plants (blank samples) during plant treatment with carbofuran. Plants, receiving routine horticultural treatment, were treated with Botrán 20 on May 21, 2007, at the recommended doses (4 L/ha). The treatment was applied to tomato plants, leaving a block of nontreated plants to be used as blank samples. Security zones were established to prevent contamination between treated and nontreated samples.

Sampling Procedure and Storage. Sampling was performed 8 times (nonconsecutive days) over a period of 21 days after treatment with the pesticide. Each day, three replicate samples of tomato fruits, each of them consisting of three fruits in turn taken from different heights of the plants (low, medium, and upper), were collected. Table 1 shows in detail the sampling plan. The main objective was to obtain representative samples throughout the 21 days of sampling.

After sampling, each replicate sample was put into a polyethylene bag properly labeled and transported immediately to the laboratory. Each analytical sample was obtained by mixing the three replicate samples, which were cut, thoroughly mixed with a crusher, and homogenized with a polytron. Finally, 200 g of the mixture was selected as a representative sample and was kept deep-frozen in a −84 °C freezer until analysis to avoid problems of stability of the pesticide in the vegetable matrix during the storage stage.

Rambo tomato blanks were collected following the same sampling scheme as that used for samples treated with carbofuran. Thus, on each sampling day the same number of treated samples and blank samples were collected.

Samples Extraction and Preparation. Extracts were prepared by using the original QuEChERS method.[58−60] The steps in the extraction process are as follows: (1) weigh 15 g of thoroughly homogenized sample into a 50 mL Teflon centrifuge tube; (2) add 15 mL of ACN acidified with 1% AcOH; (3) add 6 g of anhydrous $MgSO_4$ and 2.5 g of $AcONa \cdot 3H_2O$; (4) shake vigorously for 3 min by hand; (5) centrifuge the tube at 3700 rpm for 5 min. On the other hand, a preconcentration step was carried out by evaporating to dryness aliquots of 10 mL of

(58) Anastassiades, M.; Lehotay, S. J. *J. AOAC Int.* **2003**, *86* (2), 412–431.
(59) Lehotay, S. J.; Mastovská, K.; Lightfield, A. R. *J. AOAC Int.* **2005**, *88* (2), 615–629.
(60) www.quechers.com (official web site of the method).

supernatant in a rotatory evaporator, which were reconstituted with 1 mL of ACN. Finally, the extracts were filtered through Millipore membrane Teflon filters (0.45 μm particle size) before injection into the chromatographic system.

The dispersive solid-phase extraction (SPE) cleanup using PSA (primary−secondary amine), included in the original Quechers method, was not performed after the ACN extraction step because an exhaustive extraction method is the best choice.

LC-ESI-MS Analysis. The chromatographic separation step was carried out with a solvent gradient consisting of solvent A (ACN) and solvent B (ammonium formate 50 mM acidified at pH 3.5 by adding formic acid) into a EclipseXDB C_8 column. Also, a Phenomenex C_8 precolumn was used. The gradient program was as follows: initially 3 min with 75% B, 15 min linear gradient to 40% B, 7 min linear gradient to 100% A, 1 min at 100% A, and finally 4 min of linear gradient back to initial conditions (75% B) for 1 min with 75% B. The mobile phase was adjusted to a flow rate of 1 mL min^{-1}. The temperature of the column was set at 25 °C, and the injection volume was 20 μL. The chromatogram was run under the established gradient program over 30 min. The MS detector was used in positive ion mode with a fragmentation voltage of 60 V. The desolvation was optimized in order to obtain the highest analytical response for carbofuran. The source temperature of ESI desolvation was selected at 325 °C, and the fragment ions were generated using highly pure nitrogen as a drying gas at a flow rate of 9 L·min^{-1} and a nebulizing gas at pressure of 40 psig. LC chromatograms were obtained by operating in the time scheduled in full scan acquisition mode in the m/z range 50−750 amu.

Data Analysis. Each LC-MS run recorded for every sample corresponded to a two-way matrix of size 507 × 2951, where the first value refers to the number of retention time points in each chromatographic run and the second is the number of m/z points in each spectrum. These data files were provided in *cdf* format by the HP Chem Station Software and then were converted to asci format (by using a cdf2ascii software) and finally to txt to be processed with MATLAB 7.6.0 (R2008a).

Before applying MCR-ALS, these data files were subjected to a reduction in dimensionality. Thus, zero values were first removed from column 710, and multilevel 2-D discrete wavelet transform (DWT) was then applied to compress them to a quarter of their original size without losing relevant chemical information.

Before detailed analysis of the data set was performed, singular value decomposition (SVD) was carried out on each augmented data matrix constituted by the two-way data corresponding to the eight treated samples (picked through the eight sampling days) and to the eight nontreated samples picked on the same days. Finally, MCR-ALS was applied to resolve the column-wise augmented matrices into individual concentration and spectral profiles using non-negativity (spectra and concentrations) and unimodality (concentration) constraints.

RESULTS AND DISCUSSION

Sampling and Extraction. The recommended dose for carbofuran in tomato crops is 4 L/ha with a preharvest interval of 45 days.[61] The latter is an agronomic parameter that relates to the minimum time that must elapse between application of pesticide and crop harvesting. After that time, the concentration of pesticide in the fruit is expected to be lower than its MRL (maximum residues limit). The MRL has been defined by the Codex Alimentarius Commission as the maximum concentration of a pesticide residue (expressed in mg kg^{-1}) which is legally permissible for use in the surface or the inside of food for human consumption and animal feed. MRLs are based on GAPs (good agriculture practices) data and aim to ensure that foods derived from products commonly used, that comply with the respective MRLs, are toxicologically acceptable.[62] The sampling was carried out according to a protocol proposed by the European Union.[63]

The extraction of the samples was carried out with the QuEChERS method, which is widely used in the determination of pesticides residues in food. This method is characterized as quick, easy, inexpensive, effective, rugged, and safe (QuEChERS), hence its name, and by its wide scope of extraction capability for compounds of different polarities.

Data Treatment: Reduction of Dimensionality. When complex samples are analyzed with LC-MS detection in scan mode, data sets of large size are obtained, and as a result their processing becomes extremely difficult. In these cases, data compression methods are advantageous because they reduce the size and computational burden of the data without losing important chemical information. Consequently, each original matrix of 507 × 2951 was subjected to pretreatment. Because of the large m/z range (50−750) selected during the MS scan spectra acquisition, zero values were found in the matrices, from column number 707 in most cases. Therefore, all these columns were removed from all data matrices, resulting in matrices whose dimensions were 507 × 710. Then, the DWT technique was used essentially to perform data compression with the aim of facilitating further chemometric data treatments. Obviously, compression is inherently associated with signal denoising because small coefficients (details) are assumed to represent the noise component of the signal.[41]

Among the numerous different filters belonging to different families (Daubechies, Coiflet, Symmlet), the Haar wavelet was chosen because of reasons mentioned in the Theory section. DWT was applied to both dimensions of each individual matrix by using the standard approach. In this procedure, matrices were decomposed to level 2 in the wavelet coefficients domain, as a compromise between compression and resolution, in such a way that the computer worked fast enough without losses of important MS spectral data. Finally, the wavelet approximation coefficients corresponding to the optimal decomposition level were used to reconstruct the final reduced matrices in its own signal domain. Compression to the above indicated level reduced the MS spectra from 710 to 178 m/z values, whereas in the other dimension the number of rows (retention times) was reduced from 507 to 127. As a result, the data matrix was reduced to 25% of the original size in both dimensions.

This procedure was applied to the eight individual data matrices, corresponding to the eight sampling days of the Rambo

(61) Registro de Productos fitosanitarios. no. registro: 23.092; nombre comercial: Botran 20 SC.

(62) *Comission of Codex Alimentarius: Manual de procedimiento*, 10th ed.; Food and Agriculture Organization of the United Nations (FAO): Rome.

(63) Dirección General de Agricultura (Comisión de la Comunidad Europea) Anexo 1.

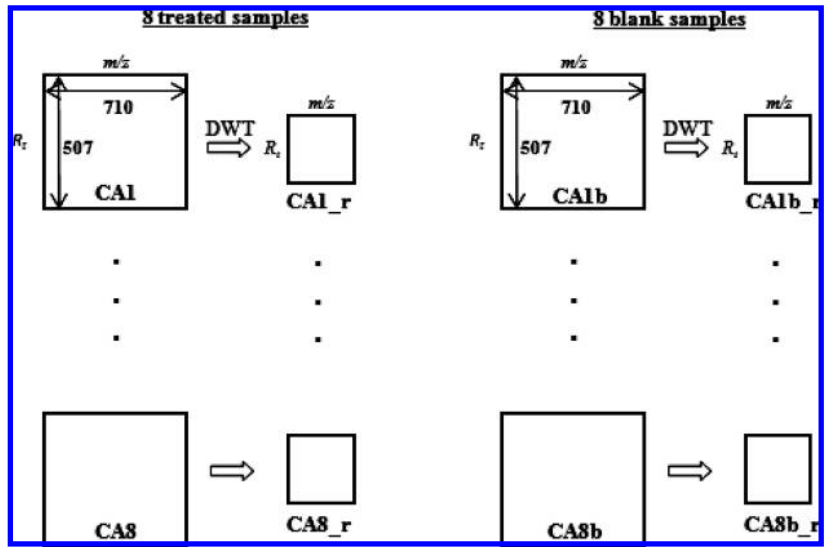

Figure 1. Sets of data matrices obtained during the analysis of samples treated and nontreated with pesticide. Both kinds of samples (treated and blank) were picked in 8 nonconsecutive days after the treatment of "Rambo" tomato plants with carbofuran. All these matrices were compressed by applying the DWT technique.

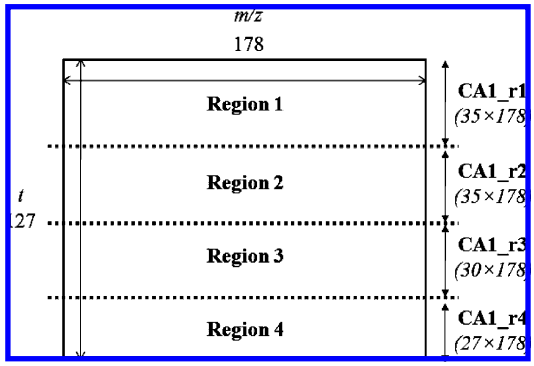

Figure 2. The four submatrices defined by each individual matrix.

tomatoes treated with carbofuran (**CA1** to **CA8**), as well as to the eight matrices corresponding to the nontreated samples (blank samples, from **CA1b** to **CA8b**). Figure 1 shows a complete scheme of the data treatment with DWT.

MCR-ALS Analysis of Augmented Data Matrices. Data obtained in the LC-MS chromatographic analysis of a tomato sample in scan mode provided an array of numbers which are ordered in a data table or data matrix with a number of rows equal to the number of elution times (30 min with 507 points) and with a number of columns equal to the number of mass (50−750 amu with 2951 m/z values). As previously mentioned, after data treatment, every individual data matrix (both treated and blank samples) showed 127 rows (retention times) and 178 columns (m/z). These experimental data are described by a bilinear model such as given by eq 1.

Because of the complexity of the data set, each individual matrix was divided into four regions as schematically shown in Figure 2.

Three-way resolution is more effective than resolution of a single matrix because it always introduces a significant improvement in the recovery of the true response profiles, adding the additional benefit of providing quantitative potential capability. Among the family of three-way resolution algorithms, the iterative ones focus on the optimization of initial estimates by using suitable data structure and chemical constraints.[64] Thus, simultaneous MCR-ALS analysis of multiple independent experiments run under different experimental conditions is a useful and powerful strategy in resolution. As mentioned in the Theory section, eq 2 can be extended to allow for the MCR-ALS simultaneous analysis of several experiments using the same detection technique.

Individual data submatrices corresponding to a selected region of the matrices (both treated and blank samples) can be arranged in a three-way data array structure, which can also be unfolded in an augmented column-wise two-way data matrix. This data arrangement gives rise to a column-wise augmented matrix, where the resolved pure mass spectra are common to all experiments and the concentration profiles can be different from experiment to experiment.

In this way, MCR-ALS was applied in order to obtain the resolved concentration and mass spectral profiles corresponding to the components or endogenous metabolites, over the sampling period. The first step consisted of building up augmented column-wise matrices **D** (one matrix **D** per region of the original compressed matrices), using both treated and blank samples.

So, the different matrices **D** were built from individual data matrices by setting one on top of the other and keeping the column vector space in common. As an example, Figure 3 illustrates how the augmented data matrix **D** corresponding to the first region of the individual matrices was built from the eight treated and their respective nontreated samples.

A similar procedure was carried out for the other three regions, finally obtaining four augmented data matrices corresponding to the four regions or subdivisions in each individual matrix. The dimensions of these four augmented data matrices depended on the dimensions of each region. In this way, augmented data matrices built from regions 1 and 2 had dimensions of 560×178, whereas augmented data matrices corresponding to regions 3 and 4 had dimensions of 480×178 and 432×178, respectively.

The complete resolution of an augmented data matrix depends mostly on the presence of pure variables or selectivity and on the

(64) Smilde, A. K.; Tauler, R.; Henshaw, J. M.; Burgess, L. W.; Kowalski, B. R. *Anal. Chem.* **1994**, *66*, 3345–3351.

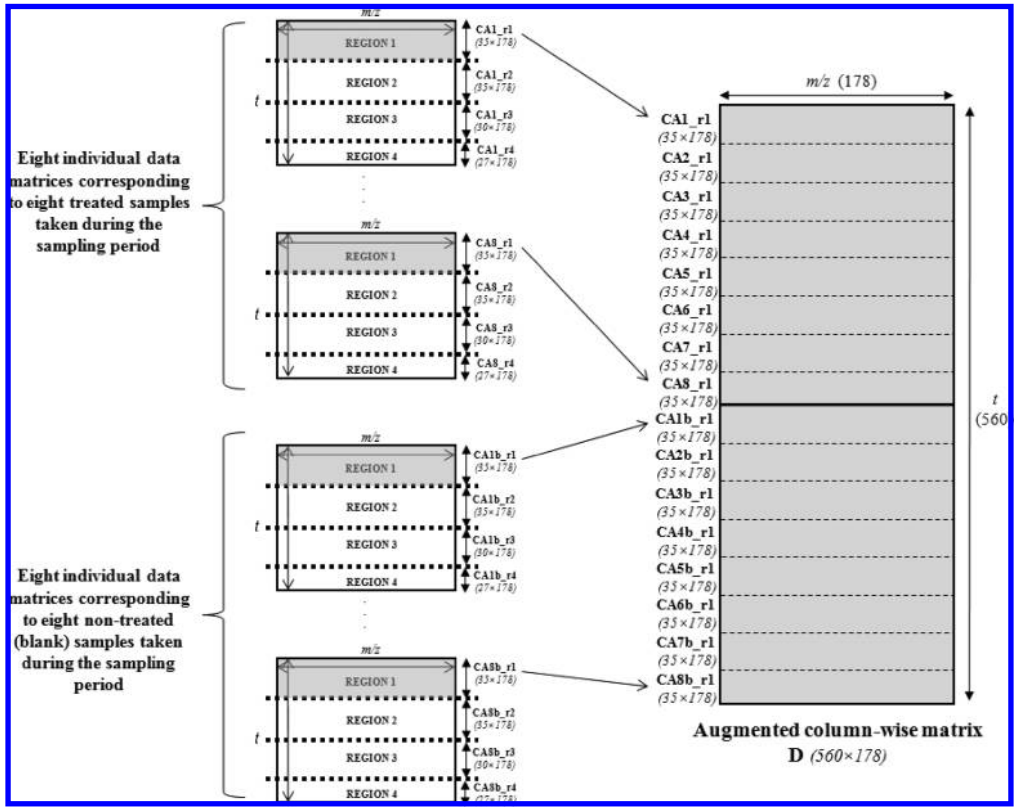

Figure 3. Construction of the augmented column-wise data matrix **D** using the region 1 of the eight data matrices (treated and blank samples).

local rank structure of the data matrix, which is given by the number of principal components of the system. So, a step previous to MCR-ALS analysis was to determine the number of components, explaining an acceptable value of the total variance of each augmented data matrix. In this work, a 90% of total variance explained was chosen as a compromise between the number of components and computational time. The number of principal components in each augmented matrix **D**, explaining a 90% of total variance, was optimized throughout SVD algorithm. This estimation gave a total of 109 principal components in region 1, 88 in region 2, 60 in region 3, and 55 in region 4.

To estimate the matrices **C** and $\mathbf{S}^T$, from each augmented data matrix, an ALS procedure is used, starting with the implementation of initial estimates to spectral profiles. Because of the high complexity of the samples, no pure component spectra were available. Therefore, initial estimates for mass spectra profiles were carried out by applying the SIMPLISMA algorithm, fixing the noise level at 0.1. Also, during the iterative optimization, two constraints were applied to obtain chemically meaningful solutions. Thus, at each cycle, the MCR-ALS algorithm calculates new matrices **C** and $\mathbf{S}^T$ and incorporates a set of constraints arising from chemical knowledge of the system in the study so that the value of **E** is a minimum. These constraints were non-negativity (applied to concentrations and spectra) and unimodality (applied to concentrations). The application of the non-negative constraint was carried out according to "fast non-negative least squares" algorithm. The unimodality constraint was implemented through the "average" option. In this way, secondary maxima are corrected, taking averages, similar to that in unimodal least-squares algorithms. Also, a constraint tolerance can be selected to allow for some local departures of the unimodality condition. In our case, a value 1.0 was chosen for tolerance, which means that no departures from the unimodal condition are allowed. Finally, the optimization ends when a convergence criterion is reached. Convergence is achieved when, in two consecutive iterative cycles, relative differences in standard deviations of the residuals between experimental and ALS calculated data values (σ) are less than a previously selected value, usually chosen as 0.1%. This value may be modified depending on the stage of the optimization.

Table 2. Figures of Merit of MCR-ALS Analysis for Each Region

region	lack of fit (%)	r^2 (%)
1	16.48	97.28
2	13.18	98.26
3	18.91	96.42
4	11.84	98.60

After the optimization procedure, MCR-ALS provides information structured as four variables that consist of two matrices related to the resolved pure concentration (**C**) and spectral profiles ($\mathbf{S}^T$) and the figures of merit related to the optimization procedure. These figures of merit are (i) the lack of fit between the resolution results and the original matrix (eq 3), (ii) the percentage of variance explained (r^2) (eq 4), and (iii) the standard deviation (eq 5) which represents a vector that contains the optimal percent of lack of fit in relative standard deviation units.

In this way, concentration and mass spectra profiles of the corresponding components of each augmented matrix were obtained. Each column of the matrix **C** provided the concentration

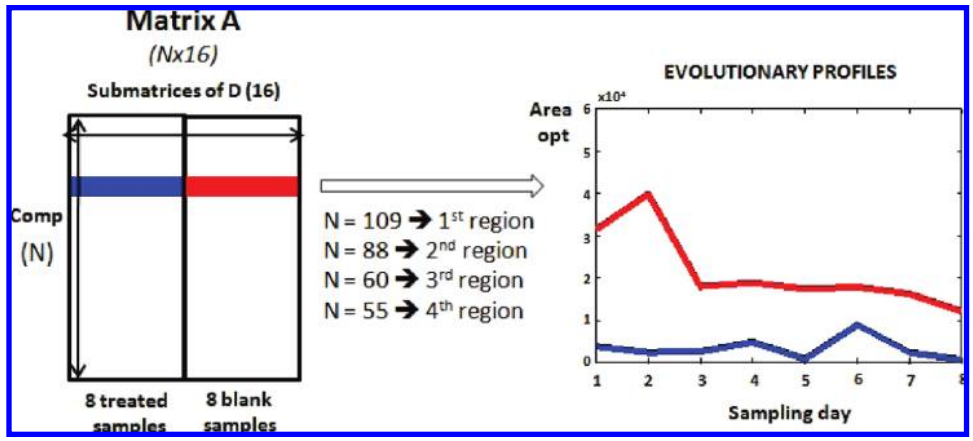

Figure 4. Evolutionary profiles of a component through the eight sampling days in treated (blue) and nontreated (red) samples, extracted from matrix **A**.

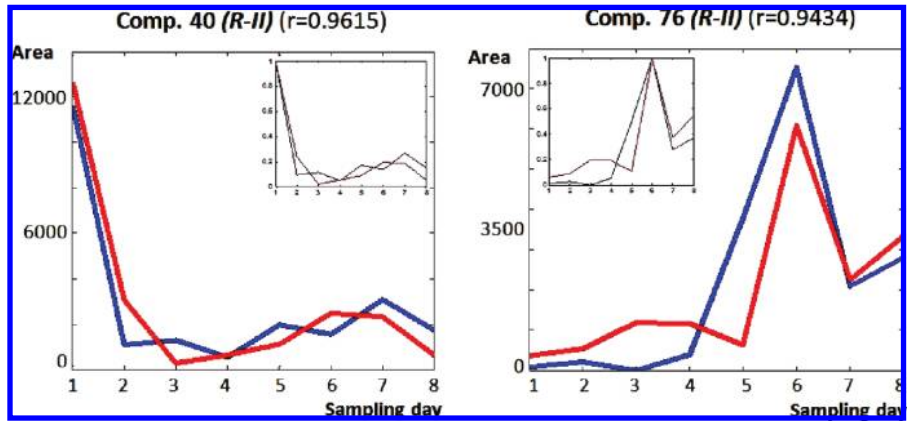

Figure 5. Evolutionary profiles of the 40th and 76th components over eight sampling days in treated (blue) and in nontreated (red) samples, extracted from region 2 (R-II). The inserted plots correspond to the profiles scaled by its maximum values.

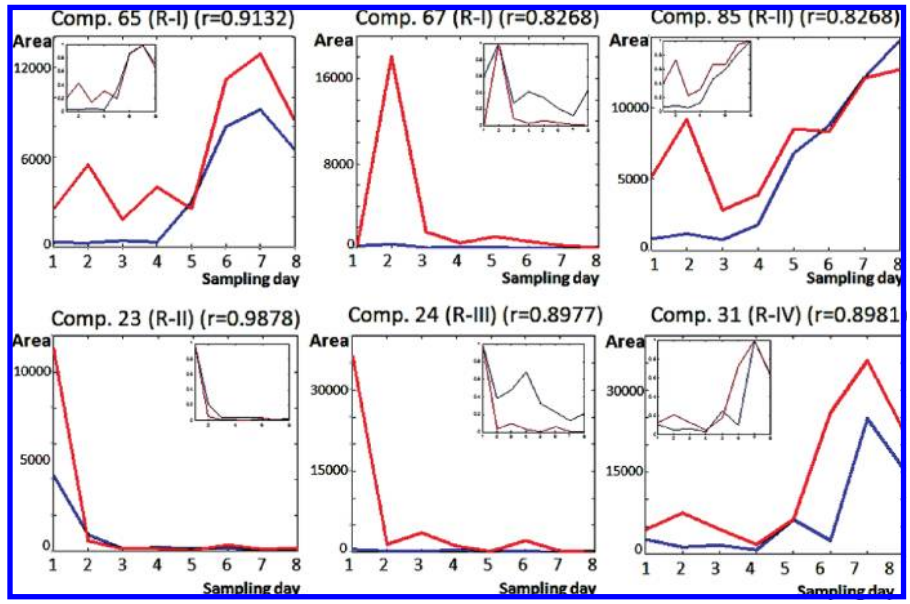

Figure 6. Evolutionary profiles of some components through the eight sampling days in treated (blue) and nontreated (red) samples, extracted from the four regions. The kinetic curves are similar, but the concentration level of each component in treated samples is lower than in blank samples.

profiles corresponding to each sampling day (for the eight treated samples and their respective blank samples), while each row from matrix $\mathbf{S}^T$ gave the mass spectrum profile of each component, which is the same in all **D** submatrices; that is to say, only one unique mass spectral profile was obtained per component in treated and blank samples.

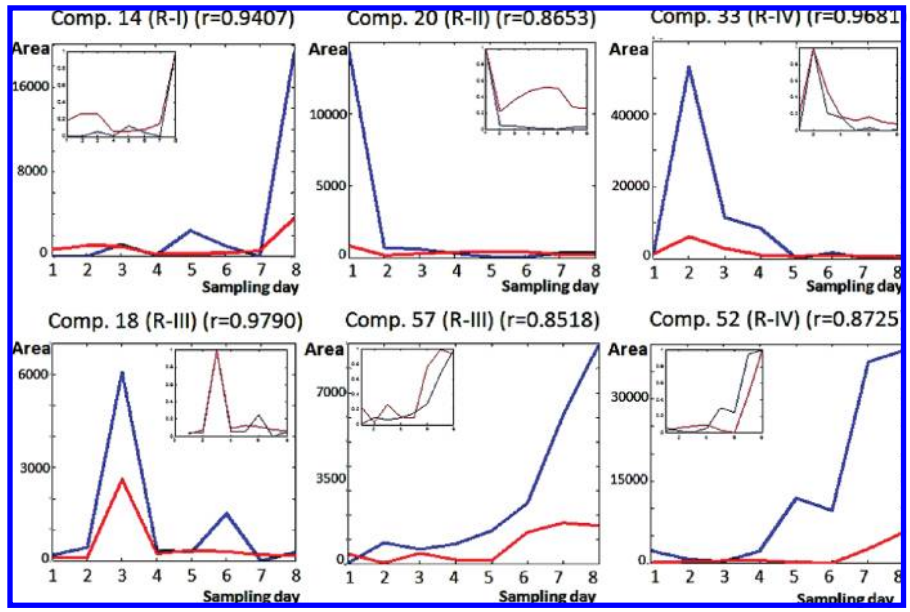

Figure 7. Evolutionary profiles of some components through the eight sampling days in treated (blue) and nontreated (red) samples, extracted from the four regions. The kinetic curves are similar, but the concentration level of the component in blank samples is lower than that in treated samples.

Comparison of Evolutionary Profiles in Treated and Nontreated Samples. After ALS optimization, output information consisting of the above-mentioned figures of merit was obtained (Table 2). As can be seen, the r^2 values (ranged between 96 and 99%) and the lack of fit (ranged between 12 and 16%) can be considered aceptable if it is taken into account that only 90% of the explained varianece is being gathered with the number of components selected for each region, as mentioned above.

Also a matrix **A**, containing the areas under the concentration profiles for each component at a sampling date, was obtained. It is to say, each value, a_{ij}, of this matrix corresponds to the area of the ith component in the jth submatrix (jth sampling day). Each row of **A** corresponds to a component, i.e., the matrix **A** contains a total of 109, 88, 60, or 55 rows, depending on the region being considered. On the other hand, each column corresponds to the area under the concentration profile obtained by MCR-ALS for each of these components (for treated and nontreated samples). Therefore, each row of **A** represents the evolutionary profile of each component over time, which shows how the concentration of each component varies through the eight sampling days in treated and blank samples. As the augmented data matrices were built with eight treated samples and their eight blank samples, the first half of the columns of matrix **A** corresponds to treated samples and the other half to nontreated samples. These evolutionary profiles were obtained from matrix **A**, as depicted in Figure 4.

The study of the behavior of the different components in the tomatoes subjected to carbofuran was first attempted with a multivariate analysis by principal component analysis (PCA), carried out on the peak areas obtained in matrix **A**, with vs confusing results. Therefore, an exhaustive comparison of the individual evolutionary profiles of all the components which are present in treated samples vs the evolutionary profiles of the same components in blank samples was carried out for each region, by obtaining a Pearson's coefficient (r) for each pair of evolutionary profiles. This parameter provided the correlation between these profiles, allowing a qualitative classification of the different behaviors of each compnent in treated and nontreated samples to be established. Since blank and treated samples were picked in the same way and in a similar state of maturation, any behavior can be interpreted as an effect due to the presence of pesticide.

Thus, r values close to 1 indicate a high degree of similarity in both evolutionary profiles, that is, a similar behavior over time for those components in blanks and treated samples. In this way, it can be assumed that some of the tomato components are not affected in their natural metabolism by the presence of carbofuran. In Figure 5 some examples are presented. Profiles scaled by its maximum values show the similarity between the kinetic behavior corresponding to both metabolites (components 40 and 76).

However, in some cases, despite obtaining good correlations in the evolutionary profiles in treated and nontreated samples (i.e., similar kinetics of evolution), the profiles appear at different levels of concentration. Thus, good correlations were obtained for the evolutionary profiles of some components, for which the evolution curve of a treated sample is below the curve of the blank sample. This can be interpreted assuming that the pesticide did not modify the kinetic evolution of the component in the tomato but did alter its concentration level, their appearance being inhibited in the fruit. Figure 6 shows examples of this behavior.

Again, profiles scaled by its maximum values show the similarity between the kinetic behavior followed by the metabolites in both kinds of samples.

Inverse behavior was also observed for other components, that is, metabolites with evolutionary profiles showing high correlations, the profile for the blank sample being below the profile corresponding to the sample treated with pesticide. This behavior seems to indicate that the pesticide favors the formation of this metabolite but without affecting its kinetic profile. Some examples are shown in Figure 7.

On the other hand, some evolutionary profiles showed very low r values, which usually indicate a very poor correlation between the profiles, that is, a different behavior due to the

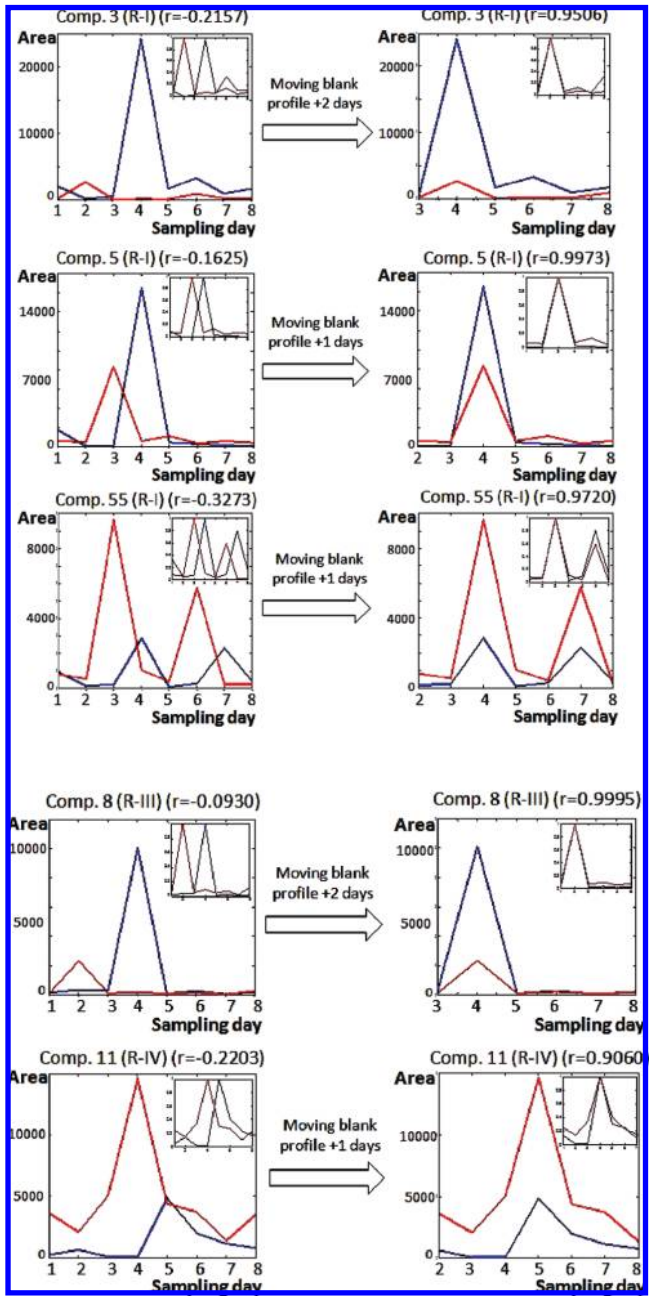

Figure 8. Moved evolutionary profiles of some components through the eight sampling days in treated (blue) and nontreated (red) samples.

presence of pesticide. However, when viewing these profiles, one became aware that this poor correlation was due to different evolution curves which were similar when moving one with respect to another over time. Therefore, a study, which consisted of moving over time the metabolite profiles of the treated samples with respect to the corresponding metabolite profiles of the blank sample (or vice versa) and calculating the corresponding Pearson coefficients, was carried out. In this procedure, a homemade Matlab algorithm based on a mobile window strategy was used. The result of this strategy was to obtain high r values when moving the evolutionary profile corresponding to the blank sample 1 or 2 days with respect to the profile of the treated sample. One possible explanation may be that the presence of pesticide in the treated samples can delay or advance in time the evolutionary

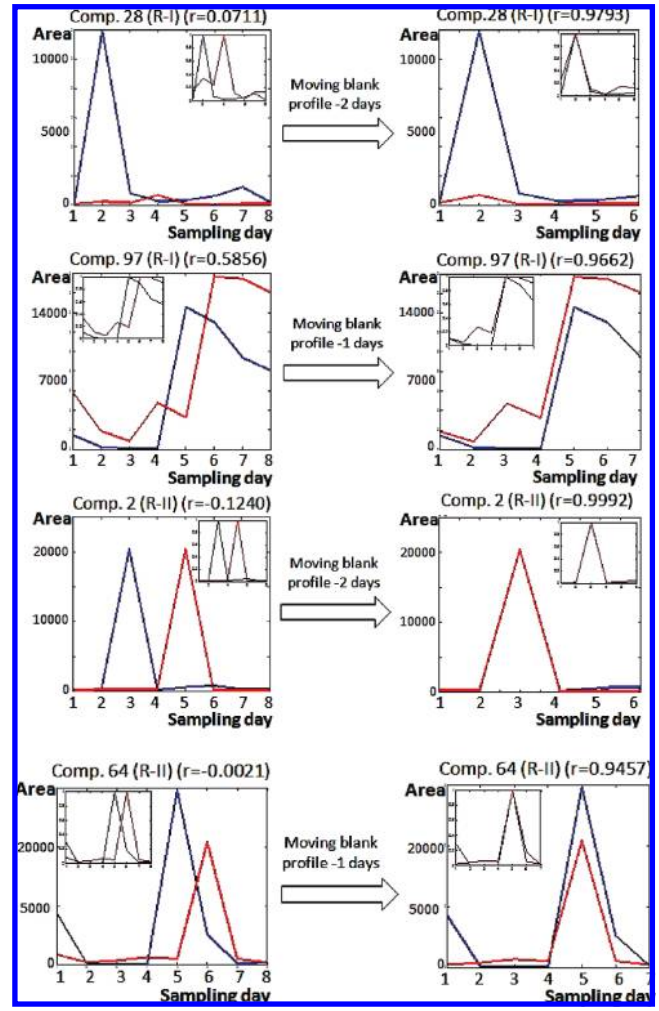

Figure 9. Moved evolutionary profiles of some components through the eight sampling days in treated samples (blue) and in nontreated samples (red).

profile of a given metabolite with respect to the profile that occurs in the corresponding blank sample, not affecting its kinetic profile.

Figure 8 shows some examples where the metabolism of the component has been delayed in a treated sample in comparison with the metabolism in the untreated sample. As can be seen, a forward movement of the metabolite profile in blank samples with respect to the metabolite profile in treated samples illustrates this behavior.

On the other hand, Figure 9 shows some examples illustrating a contrary behavior. A movement of the blank profile to the left with respect to the profile in the treated sample shows how the metabolism of the component is moved forward due to the presence of pesticide. In both cases, it the influence of the pesticide in the concentration of the metabolites can also be observed.

Finally, the evolutionary profiles with a r value close to -1 indicates an inverse correlation between them, i.e., a contrary behavior of the components in treated and nontreated samples. Figure 10 shows some examples of this behavior.

CONCLUSIONS

The use of a chemometric strategy based on the MCR-ALS algorithm applied to LC-MS three-way data arrays has been shown

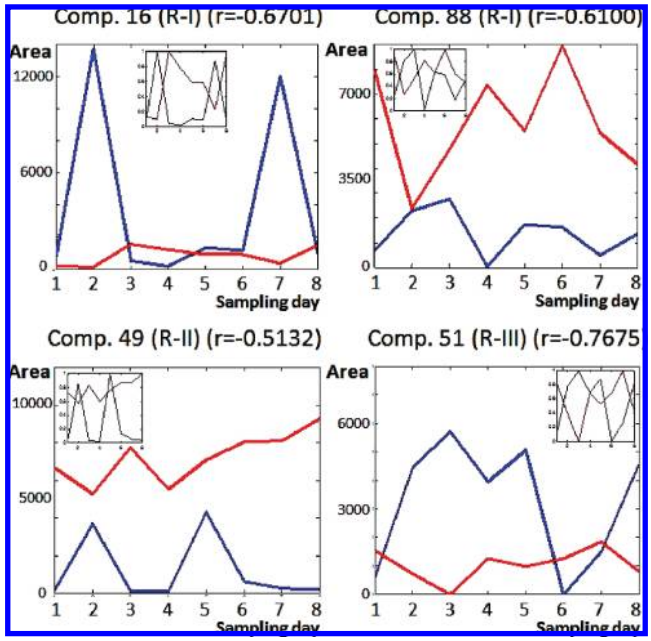

Figure 10. Evolutionary profiles of some components through the eight sampling days in treated (blue) and nontreated (red) samples with inverse behaviors through the time

to be adequate to perform a metabonomic study in tomato (*Lycopersicon esculentum*) fruits (cultivar Rambo) after treatment with carbofuran. Following this strategy, the evolutionary profiles of endogenous compounds can be observed in samples nontreated and treated with carbofuran, allowing their behavior to be studied. Interestingly, several metabolites present different evolutionary profiles over eight sampling days, depending on the presence of the pesticide. Also, a few components do not present any variation in their profiles. These findings suggest that the presence of pesticide causes changes over time in the behavior of certain endogenous tomato metabolites as the result of physiological stress.

After this qualitative study, future research in this field will be to identify the nature of the metabolites affected by the presence of pesticide. For this purpose, other sophisticated techniques involving the use of accurate mass measurements such as time-of-flight (TOF) or quadrupole-time-of-flight (q-TOF) detection and/or tandem MS (MS/MS) must be used.

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