Anal. Chem. **2001,** *73,* 185−191

A Method for Quantification from Composite Spectra: Application to the Determination of Isomeric DNA Photoproducts by Tandem Mass Spectrometry

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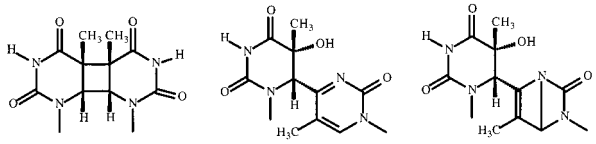
Quantification of mixture components from their composite optical or mass spectra is a common need in analytical chemistry. We encountered the need when applying a combination of enzymatic digestion with nuclease P1 and tandem mass spectrometry to a mixture of isomeric photomodified oligodeoxynucleotides. In the procedure, we collisionally activated the $[M - H]^-$ or $[M + Na - 2H]^-$ ion of trinucleotide triphosphates, which were extricated enzymatically from the larger, damaged oligodeoxynucleotides, and we measured the relative abundances of characteristic fragment ions. The results sometimes yield curved calibrations for plots of the relative fragment ion abundances in the product ion spectra of isomers versus their relative amounts. We developed a normalized linear model, which brings understanding to the nonlinear plots and allows quantification of the mixture components from their composite spectra. The outcome demonstrates a general quantification procedure and shows that different yields for generating fragment ions from different constituents of the mixture cause the curved calibration lines.

Often isomers and other closely related substances cannot be completely separated by chromatographic methods. In those cases, it is desirable to quantify the isomers by a spectroscopic or, more commonly, a mass spectrometric method provided there are some differences in the spectra of the individual components. For simple mixtures, the quantification can be achieved rapidly without prior chromatographic separation, but when the mixtures are complex, coupling the separation method with a spectroscopic or mass spectrometric determination is required. In both cases, however, a data analysis strategy is necessary to extract from the composite spectrum the concentration of the individual components. We report here the development of a data analysis method for extracting quantitative information from composite spectra. We also demonstrate the utility of the data analysis by applying it to a tandem mass spectrometric quantification of isomeric photoproducts (e.g., structures **1**−**3**) formed in UV damage[1] of model oligodeoxynucleotides.



1 T[c, s]T **2** T[6, 4]T **3** T[Dewar]T

Early efforts aimed at resolving composite spectra in mass spectrometry began with McLafferty and co-workers' use of the simple superposition principle to determine from collisionally activated decomposition (CAD) spectra the structural composition of mixtures of gas-phase ions.[2−6] Bass and Bowers[7] questioned this principle and pointed out the importance of using the whole spectrum. Cook and co-workers[8] also addressed the problem when resolving composite electron ionization (EI) mass spectra of isomeric hydrocarbons in process monitoring.

More recently, a number of workers have turned their attention to quantification of mixtures of biologically interesting molecules. Desaire and Leary[9] derivatized diastereomeric hexosamine monosaccharides with metal−ligand complexes, fragmented the complexes, and quantified the isomers, using the relative abundances of product ions from pure standards for the calibration. Schnier and Williams[10] used global analysis data reduction to determine the abundances of two isomeric bradykinins from their dissociation spectra caused by blackbody infrared radiative dissociation at multiple temperatures. They overcame the problems of fitting a double-exponential function to a single data set by using all the temperature-dependent data. For these two methods to be valid, the ionization efficiencies of the isomers and the detection efficiencies of the product ions should be the same.

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10.1021/ac000821m CCC: $20.00
Published on Web 12/08/2000

Krishna et al.[11] quantified a mixture of *N*-benzoylleucine and -isoleucine from the CAD spectra of an $[M - COOH]^{+\bullet}$ precursor by using linked scanning at constant B/E. They obtained a linear correlation between the relative intensities of the characteristic fragment ions and the fraction of one isomer in the mixture. Similarly, Seymour and Turecek[12] quantified leucine−isoleucine isomers and lysine−glutamine isobars by empirically fitting calibration data from tandem mass spectrometry of copper(II)−diimine complexes.

The need for further developments became obvious to us in our study of the consequences of DNA photodamage, where we seek to identify and quantify the amounts of the photoproducts. An enzymatic digestion/tandem mass spectrometric assay[13] is the basis for determining the structure of isomeric photoproducts from damaged oligodeoxynucleotides. A photodamaged oligodeoxynucleotide (ODN) yields, under the conditions of nuclease P1 digestion, a trinucleotide triphosphate that contains the damaged site. The product ion spectra of the $[M - H]^-$ ions are remarkably distinctive, permitting identification of the damage that remains preserved in the trinucleotide triphosphate.

Quantification of isomers is the next step. Cadet et al.[14] employed HPLC separation and multiple-reaction monitoring with a triple-quadruple mass spectrometer to quantify the amounts of different photoproducts formed at a TT site after enzymatically digesting a photomodified DNA to give photoproduct-containing dinucleotides. We instead chose to develop the quantification method around the nuclease P1 digestion, which gives the trinucleotide triphosphate, because the CAD spectra are distinctive. Furthermore, we hoped to dispense with the slow HPLC step, which does not afford rapid separation of the isomeric trinucleotide triphosphates that contain the photoproduct.

Our first efforts to quantify the relative amount of isomeric DNA photoproducts by ESI-MS/MS gave curved calibrations when we plotted ion intensity ratios of product ions versus mole fractions of one isomer in mixtures. Interestingly, Seymour and Turecek[12] obtained a similar curve for lysine−glutamine isobars. They adjusted an exponential function to fit the curve. We seek to understand the cause of the curved correlation and to find a nonempirical way to fit the correlation. To this end, we developed a normalized linear model for fitting the calibration curve and applied to the determination of photoproducts formed from oligodeoxynucleotides.

EXPERIMENTAL SECTION

Preparation of ODNs. All the ODNs used in this study were obtained from Integrated DNA Technologies, Inc. (Coraville, IA) and used without further purification. Pure *cis,syn*-cyclobutane pyrimidine dimer (CPD)-, and pyrimidine (6,4)pyrimidone photoproduct (6-4PP)-containing ODNs were obtained by UVC irradiation followed by separation with reversed-phase HPLC, as described previously.[15] Briefly, ODNs were dissolved in doubly distilled water, degassed with N_2 for 5 min, dispersed in a Petri dish, which was introduced to a N_2-filled zip-lock bag, and irradiated on ice for 2 h. A 4.6-mm-i.d. Dynamax C18 column (Varian Inc.) was used for separation by employing a linear gradient of 6−12% acetonitrile in 50 mM triethylammonium acetate at a flow rate of 1 mL/min. The HPLC profile for the separation of a UVC−photolysis mixture of d(GTTTGA) shows that d(GT-[c,s]TTGA), d(GTT[c,s]TGA), d(GT[6−4]TTGA), d(GTT[6−4]-TGA), and the undamaged d(GTTTGA) elute at 20.2, 21.5, 22.0, 22.8, and 29.1 min, respectively. The concentrations of photomodified ODNs were determined by measuring their UV absorbances at 260 nm.[16]

Nuclease P1 Digestion. Nuclease P1 was procured from Boehringer-Mannheim (Mannheim, Germany) and used without further purification. For calibration, *cis,syn*-CPD and 6−4 PP or *cis,syn*-CPD and Dewar photoproduct-containing ODNs were mixed in molar ratios of 1:0, 9:1, 8:2, ..., 1:9, and 0:1 while keeping the total concentrations constant at 10 μM. The digestions were carried out at 37 °C for 5 min by keeping the concentration of nuclease P1 at 2.5 munits/μL. No additional buffer was used except that contained in the commercial enzyme preparation. After digestion, the sample was injected directly to mass spectrometer without purification.

ESI-MS. Electrospray ionization-mass spectrometry (ESI-MS) experiments were carried out with an LCQ ion trap mass spectrometer (Finnigan, San Jose, CA). A solution of 50/50/0.5 (v/v) $CH_3CN/H_2O/NH_3$ was used as the carrier solvent for electrospray, and the flow rate was held at 5−10 μL/min by using a syringe injector. A 5-μL aliquot of the digestion solution was injected in each run without further dilution. The spray voltage was 4.6 kV, and the capillary temperature was maintained at 200 °C. Tandem mass spectrometry experiments were done by selecting the deprotonated molecule, $[M - H]^-$, or the $[M + Na - 2H]^-$ ions for collisional activation. The mass width for precursor selection was set at m/z 3, and the collision gas was helium at a pressure of 1×10^{-4} Torr, as measured on the remote ion gauge that is a standard component of the instrument. The resonance excitation voltage was ∼35% of the maximum available voltage, which was 5 V peak to peak. The q value for activation was 0.25, and the activation time was 30 ms. The product ions were allowed to cool for at least 2 ms before the detection period.

Ten scans were signal-averaged to obtain a product ion mass spectrum. Peak heights of product ions were used for calibration. Exponential curve fitting and calculations with the normalized linear model were done by using KaleidaGraph 3.0 (Synergy Software, Reading, PA) and MathCad 8 (Mathsoft, Inc., Cambridge, MA), respectively. The experimental and fitted data were imported to a Microsoft Excel spreadsheet (Microsoft Inc.), the means and standard deviations were calculated, and the calibration curves were plotted thereafter.

THEORY

Normalized Linear Model for Curve Fitting. The premise for the fitting model is that the response of ESI-MS is linear; that is, the signal is proportional to the amount of the analyte and additive for mixtures of analytes. In general, any linear operation

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is one that is additive and homogeneous.[17] Taking the product ion spectra of the $[M - H]^-$ ion of a mixture of d(GT[c,s]TTGA) and d(GT[6−4]TTGA) as a specific example of a general case of a composite spectrum, we represent the spectral intensity contributions from pure d(pT[c,s]TT) and d(pT[6−4]TT) as vector quantities $[a_0,b_0]^T$ and $[c_0,d_0]^T$ where a_0 and c_0 correspond to the contributions of fragment ion at m/z 803 from d(pT[c,s]TT) and d(pT[6−4]TT), respectively, and b_0 and d_0 are the corresponding contributions of the fragment ion of m/z 816. The units for a_0, b_0, c_0, and d_0 are arbitrary units per mole. The vector representation of the two peaks in the spectrum of a mixture is

$$\mathbf{S} = \begin{bmatrix} a_0 \\ b_0 \end{bmatrix} m_1 + \begin{bmatrix} c_0 \\ d_0 \end{bmatrix} m_2 \tag{1}$$

where m_1 and m_2 are the molar amounts of d(pT[c,s]TT) and d(pT[6−4]TT), respectively. The first step toward normalization reflects how experimental data from the spectral peaks are processed, giving us eq 2 from eq 1:

$$\mathbf{S} = \begin{bmatrix} \dfrac{a_0}{a_0 + b_0} \\ \dfrac{b_0}{a_0 + b_0} \end{bmatrix} (a_0 + b_0) m_1 + \begin{bmatrix} \dfrac{c_0}{c_0 + d_0} \\ \dfrac{d_0}{c_0 + d_0} \end{bmatrix} (c_0 + d_0) m_2$$

$$= \begin{bmatrix} \dfrac{a_0}{a_0 + b_0} & \dfrac{c_0}{c_0 + d_0} \\ \dfrac{b_0}{a_0 + b_0} & \dfrac{d_0}{c_0 + d_0} \end{bmatrix} \begin{bmatrix} a_0 + b_0 & 0 \\ 0 & c_0 + d_0 \end{bmatrix} \begin{bmatrix} m_1 \\ m_2 \end{bmatrix} \tag{2}$$

If $a = a_0/(a_0 + b_0)$, then $b_0/(a_0 + b_0) = 1 - a$. Similarly, if $c = c_0/(c_0 + d_0)$, then $d_0/(c_0 + d_0) = 1 - c$. The terms a and c are the ion intensity ratios $I_{803}/(I_{803} + I_{816})$ taken from the product ion spectrum of pure d(pT[c,s]TT) and d(pT[6−4]TT), respectively. The terms $(1 - a)$ and $(1 - c)$ are the corresponding ion intensity ratios $I_{816}/(I_{816} + I_{803})$. Note that the ratio of the signal intensities at m/z 803 and 816 for d(pT[c,s]TT) and d(pT[6−4]TT) is

$$\frac{a_0 + b_0}{c_0 + d_0} = \frac{(a_0 + b_0)/k}{(c_0 + d_0)/k} = \frac{\gamma}{1 - \gamma} \tag{3}$$

where

$$k = a_0 + b_0 + c_0 + d_0 \tag{4}$$

is the normalization factor,

$$\gamma = (a_0 + b_0)/k \tag{5}$$

is the normalized yield for producing product ions of m/z 803 and 816 for d(pT[c,s]TT), and $(1 - \gamma)$ is the corresponding yield for d(pT[6−4]TT). Using eq 3, we rewrite eq 2 to give eq 6:

$$\mathbf{S} = \begin{bmatrix} a & c \\ 1 - a & 1 - c \end{bmatrix} \begin{bmatrix} \gamma & 0 \\ 0 & 1 - \gamma \end{bmatrix} \begin{bmatrix} k(1 - \lambda) \\ k\lambda \end{bmatrix} m \tag{6}$$

where λ and m (which is equal to $m_1 + m_2$) are the mole fraction of d(pT[c,s]TT) in the mixture and the total molar amount detected from the mixture, respectively. From eq 6, we find that the signal intensity ratio $I_{803}/(I_{803} + I_{816})$ from the spectrum of a mixture to be

$$\frac{s_1}{s_1 + s_2} = \frac{[c - (a + c)\gamma]\lambda + \alpha\gamma}{(1 - 2\gamma)\lambda + \gamma} \tag{7}$$

Rearranging eq 7, we obtain eq 8

$$\lambda = \frac{\left(a - \dfrac{s_1}{s_1 + s_2}\right)\gamma}{\dfrac{s_1}{s_1 + s_2}(1 - 2\gamma) - [c - (a + c)\gamma]} \tag{8}$$

which forms the basis for computing the fractions of an unknown mixture from the measured peak intensity ratios and the yield γ.

The quantities a, c, and γ are properties of the isomers and the MS/MS analysis. To the extent that the MS/MS is reproducible, these quantities should be constants. Thus $s_1/(s_1 + s_2) \sim (I_{803}/(I_{803} + I_{816}))$ can be viewed as a function of λ. In the exceptional cases of $(a = c)$, $\gamma = 1$, or 0, this function is constant and provides no information about λ. Otherwise, eq 7 establishes a relation between $I_{803}/(I_{803} + I_{816})$ and λ, which may be used to infer λ from $I_{803}/(I_{803} + I_{816})$.

The quantities of a, c, and γ can be established in a calibration procedure, using mixtures of the isomers that are prepared by varying λ from 0 to 1. This gives an experimental evaluation of the λ function $s_1/(s_1 + s_2)$. The quantities a_0 and c_0 are found directly when $\lambda = 0$ and $\lambda = 1$, respectively. The λ function $s_1/(s_1 + s_2)$ can only have a straight-line graph when $\gamma = 0.5$:

$$\frac{s_1}{s_1 + s_2} = (c - a)\lambda + a \tag{9}$$

When $\gamma \neq 0.5$, the function describes a curve that has a shape that depends on γ. Because the curve shape depends on γ, γ can be found by a least-squares fitting of the λ function given by eq 7 to the function $I_{803}/(I_{803} + I_{816})$ as experimentally determined in the calibration procedure. The desired yield minimizes the distance between the two functions as found in a search with γ starting with a value of 0.5.

In the above discussion, we considered spectral contributions from two peaks, but we can extend the method to include spectral contributions from many peaks or even all the peaks. The following treatment is useful for two isomers, one of which produces a dominant fragment ion, and the other gives many fragment ions with similar intensities.

The spectral contributions from the two isomers as vector quantities are

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$$\begin{bmatrix} a_0 \\ . \\ . \\ . \\ a_n \end{bmatrix} \quad \text{and} \quad \begin{bmatrix} c_0 \\ . \\ . \\ . \\ c_n \end{bmatrix}$$

where a_0, ..., a_n and c_0, ..., c_n are fragment ion intensities of the two isomers. The vector representation of the spectrum of a mixture is

$$\mathbf{S} = \begin{bmatrix} a_0 \\ . \\ . \\ . \\ a_n \end{bmatrix} m_1 + \begin{bmatrix} c_0 \\ . \\ . \\ . \\ c_n \end{bmatrix} m_2 \tag{10}$$

where, by analogy to eq 1, m_1 and m_2 are the molar amounts of the two isomers. If we represent $b_0 = \sum_{i=1}^{n} a_i$ and $d_0 = \sum_{i=1}^{n} c_i$, Equation 10 becomes eq 1. Following a similar normalization procedure, we obtain eq 6, except that the definitions of k and λ now become

$$k = \sum_{i=0}^{n} a_i + \sum_{i=0}^{n} c_i \tag{11}$$

$$\gamma = \sum_{i=0}^{n} a_i / k \tag{12}$$

Because we assume that the response in ESI is linear and we choose several normalization steps in the modeling, we call the approach a "normalized linear model".

Extension to Isomers Whose Spectra Are Similar. We admit that the experimental systems we use here are not the most demanding. The most-demanding application could involve isomers whose product-ion spectra produce the same fragment ions but with only moderate differences in relative abundances. The extension to that situation is not difficult as shown in the following.

The spectral contribution from two pure compounds are

$$\mathbf{A} = \begin{bmatrix} a_1 \\ . \\ . \\ . \\ a_n \end{bmatrix} \quad \text{and} \quad \mathbf{C} = \begin{bmatrix} c_1 \\ . \\ . \\ . \\ c_n \end{bmatrix} \tag{13}$$

If we represent the molar amounts of the two isomers as m_1 and m_2, we can write the spectrum of a mixture as

$$\mathbf{S}' = \begin{bmatrix} a_1 \\ . \\ . \\ . \\ a_n \end{bmatrix} m_1 + \begin{bmatrix} c_1 \\ . \\ . \\ . \\ c_n \end{bmatrix} m_2 = [\mathbf{A} \;\; \mathbf{C}] \begin{bmatrix} m_1 \\ m_2 \end{bmatrix} \tag{14}$$

Reducing eq 14 to two dimensions gives eq 15:

$$\mathbf{S} = \begin{bmatrix} \mathbf{A}\cdot\mathbf{S}' \\ \mathbf{C}\cdot\mathbf{S}' \end{bmatrix} = \begin{bmatrix} \mathbf{A}\cdot\mathbf{A} & \mathbf{A}\cdot\mathbf{C} \\ \mathbf{C}\cdot\mathbf{A} & \mathbf{C}\cdot\mathbf{C} \end{bmatrix} \begin{bmatrix} m_1 \\ m_2 \end{bmatrix} \tag{15}$$

Similar to the treatment described in the Experimental Section, normalization will give the five-term product:

$$\mathbf{S} = \begin{bmatrix} \dfrac{\mathbf{A}\cdot\mathbf{A}}{(\mathbf{A}+\mathbf{C})\cdot\mathbf{A}} & \dfrac{\mathbf{A}\cdot\mathbf{C}}{(\mathbf{A}+\mathbf{C})\cdot\mathbf{C}} \\ \dfrac{\mathbf{C}\cdot\mathbf{A}}{(\mathbf{A}+\mathbf{C})\cdot\mathbf{A}} & \dfrac{\mathbf{C}\cdot\mathbf{C}}{(\mathbf{A}+\mathbf{C})\cdot\mathbf{C}} \end{bmatrix} \begin{bmatrix} \dfrac{(\mathbf{A}+\mathbf{C})\cdot\mathbf{A}}{|\mathbf{A}+\mathbf{C}|^2} & 0 \\ 0 & \dfrac{(\mathbf{A}+\mathbf{C})\cdot\mathbf{C}}{|\mathbf{A}+\mathbf{C}|^2} \end{bmatrix} |\mathbf{A}+\mathbf{C}|^2 \begin{bmatrix} \lambda \\ 1-\lambda \end{bmatrix} m \tag{16}$$

If we put $a = \mathbf{A}\cdot\mathbf{A}/(\mathbf{A}+\mathbf{C})\cdot\mathbf{A}$, $c = \mathbf{A}\cdot\mathbf{C}/(\mathbf{A}+\mathbf{C})\cdot\mathbf{C}$, $\gamma = (\mathbf{A}+\mathbf{C})\cdot\mathbf{A}/|\mathbf{A}+\mathbf{C}|^2$, and $k = |\mathbf{A}+\mathbf{C}|^2$, we will get eq 6. The rest of the fitting process is the same as that described in the Experimental Section.

RESULTS AND DISCUSSION

We recently demonstrated that nuclease P1 digestion of an ODN containing a photoproduct gives a trinucleotide triphosphate with a normal base 3′ to the site of the photomodification.[13] We also demonstrated that product ion spectra of the trinucleotide triphosphate are distinctive for *cis,syn*-CPD, 6−4 PP, and its Dewar isomer (**1**−**3**) formed in a TTA sequence context.[13] The product ion spectra of d(pT[c,s]TT) and d(pT[6−4]TT) are additional evidence that the product ion spectra are distinctive, as seen in Figure 1a and c. The many fragment ions in the product ion spectrum of the $[M - H]^-$ ion of d(pT[c,s]TT) contrast with that of d(pT[6−4]TT), which shows a single dominant fragment ion at m/z 816. The latter ion is formed by the loss of a neutral $C_4H_3NO_3$ from the 5′-pyrimidine of the photoproduct.[13]

Because the separation of the trinucleotide triphosphates is time-consuming, we developed the "normalized linear model" to extract the relative amounts of isomeric photoproducts from the product ion spectra. To this end, we mixed authentic d(GT[c,s]TTGA) and d(GT[6−4]TTGA) at different mole ratios (0:1−1:0; see Experimental Section), digested the mixtures with nuclease P1, fragmented the $[M - H]^-$ ion of m/z 929, and measured the signal intensities corresponding to characteristic product ions. We chose the $[M - T]$ ion of m/z 803 for d(pT[c,s]TT) and the $[M - 113]$ ion of m/z 816 for d(pT[6−4]TT). The reason we chose the two ions for quantification is that both are produced abundantly in the fragmentation of *cis,syn*-CPD and 6−4 PP isomers independent of the nature of the bases 3′ to the photomodification sites. An example product ion spectrum of the $[M - H]^-$ ion of a 50:50 (molar ratio) mixture of d(pT[c,s]TT) and d(pT[6−4]TT) shows that both ions are readily seen in admixture with other product ions (Figure 1b). Unexpectedly, we obtained a curved correlation when we plotted the ion intensity ratio $I_{816}/(I_{816} + I_{803})$ versus the fraction of 6−4 PP in the mixture (Figure 2). Interestingly, Seymour and Turecek[12] obtained a similar curved calibration for the quantitation of lysine−glutamine isobars, but they did not seek a general solution.

We used the equation $y = a(1 + e^{bx})$ of Turecek[12] to fit our experimental data (Figure 2). The fit is acceptable but not as good as that obtained using our normalized linear model. If we modify

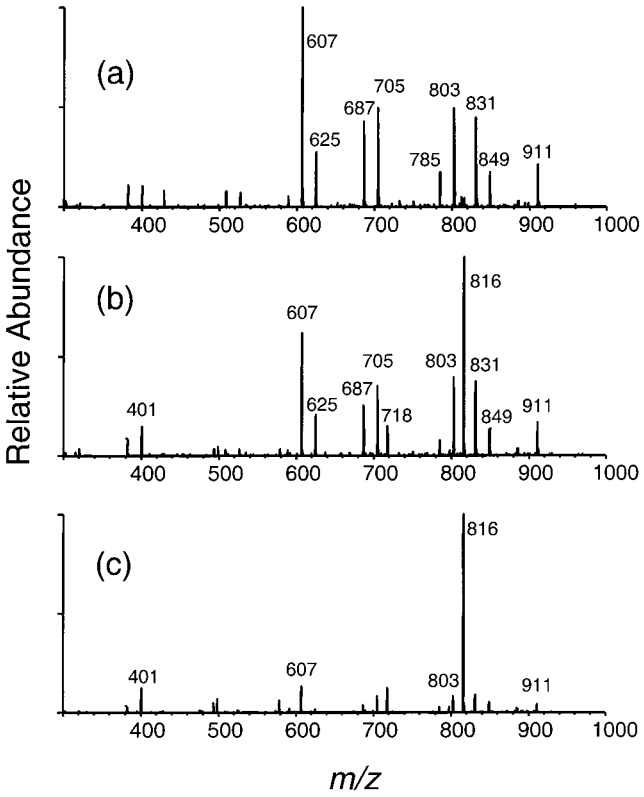

Figure 1. Product ion spectra of ESI-produced $[M - H]^-$ ions of d(pT[ ]TT) obtained after nuclease P1 digestions of (a) d(GT[c,s]TTA), (b) 50:50 d(GT[c,s]TTGA) and d(GT[6−4]TTGA), and (c) d(GT[6−4]TTGA).

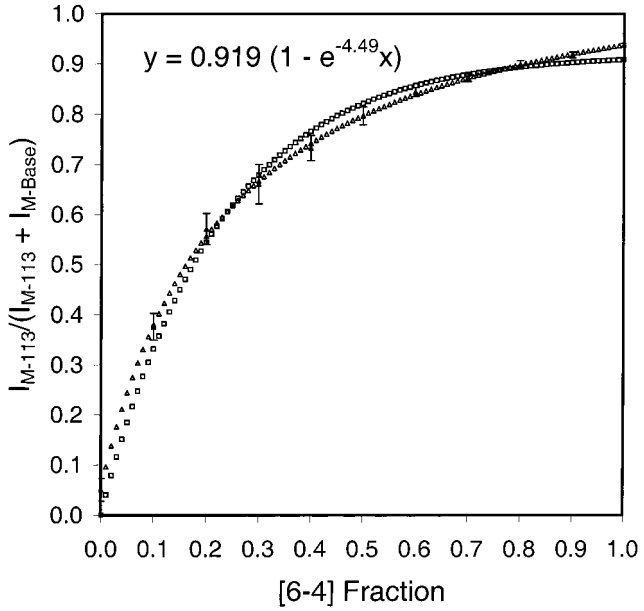

Figure 2. Relation of ion intensity ratios $I_{816}/(I_{816} + I_{803})$ and the molar fractions of d(pT[6−4]TT) in a mixture of d(pT[c,s]TT) and d(pT-[6−4]TT). The solid triangles are experimentally measured ion intensity ratios. The open triangles and open squares are the curves obtained by using the normalized linear model and by empirical exponential fitting, respectively. Error bars are standard deviations based on three independent measurements.

the empirical equation by adding another parameter to give $y = a + be^{cx}$, we obtain a fit that is nearly as good as that by employing the normalized linear model ($a = 0.933$, $b = -0.864$, $c = -3.980$,

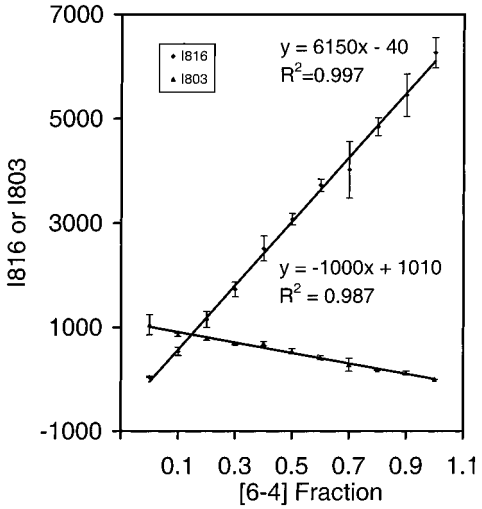

Figure 3. Relation of ion intensities at *m*/*z* 803 and 816 and the mole fractions of *cis,syn*-CPD and mole fractions of [6−4] PP in mixtures of d(pT[c,s]TT) and d(pT[6−4]TT), respectively. Error bars are standard deviations based on three independent measurements.

$R^2 = 0.996$; curve not shown). The advantages of an empirical method are that it is simple and no mathematical derivation is necessary. The disadvantages are that empirical model must be modified for new experimental conditions, and more importantly, it provides no insight or understanding of the origin of the problem.

We could fit well the calibration data by using the normalized linear model. After fitting, the normalized yields for ions of m/z 803 and 816 (γ) were 0.151 and 0.849 for d(pT[c,s]TT) and d(pT-[6−4]TT), respectively. Thus, the cause of the curvature becomes clear: the yield for the ion of m/z 816 from d(pT[6−4]TT) is different from that for the m/z 803 ion from d(pT[c,s]TT). Interestingly, McLafferty and Proctor[18] also obtained evidence that yield is important. They pointed out that different yields could give erroneous calibration when using the superposition principle. The curvature of the calibration line (Figure 2) can be further understood by considering the data-processing steps in the model: the nonlinearity is introduced in eq 7 when the peak ratios are computed. The relation between $s_1/(s_1 + s_2) \sim (I_{803}/(I_{803} + I_{816}))$ and λ does not depend on k or m. This makes the search simpler and tells us that no information about k or m can be expected from $I_{803}/(I_{803} + I_{816})$. The value of λ comes from $I_{803}/(I_{803} + I_{816})$ in eq 8.

In the model, we assume there is a linear response to the analyte concentration in an electrospray ion trap mass spectrometer; that is, the signal is proportional to the concentration of the analyte. To test the assumption, we plotted both the intensity of the m/z 803 ion signal versus the fraction of the d(pT[c,s]TT) and the intensity of m/z 816 ion signal versus the fraction of d(pT-[6−4]TT) in a series of mixtures of the two photoproducts. The correlation lines are linear with reasonable R^2 values (Figure 3). This result shows that, under ESI conditions, the responses of d(pT[c,s]TT) and d(pT[6−4]TT) are independent of the presence of the other compound and that the curvature we observed in Figure 2 is not due to differential ESI of the various analytes that comprise the mixture. The slope of the response of m/z 803 ion

(18) McLafferty, F. W.; Proctor, C. J. *Org. Mass Spectrom.* **1983**, *18*, 272.

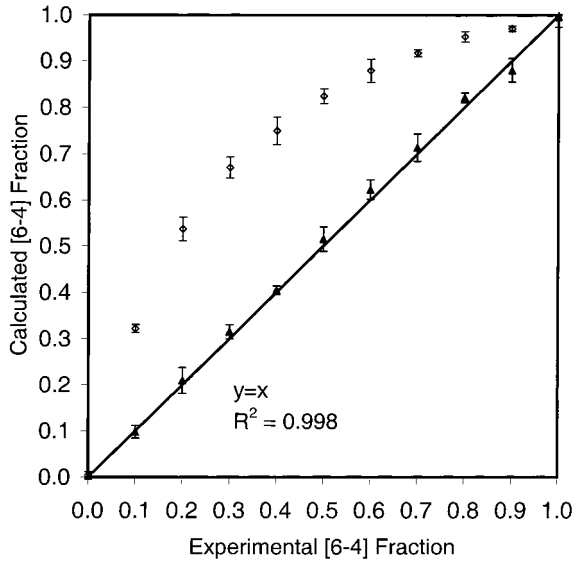

Figure 4. Relation of the back-calculated 6−4 PP fractions from the normalized linear model (filled triangle) or from the spectra of pure d(pT[c,s]TT) and d(pT[6−4]TT) (open diamond) plotted against the actual fractions. Error bars are standard deviations calculated from three independent experiments (line $y = x$ is shown for reference).

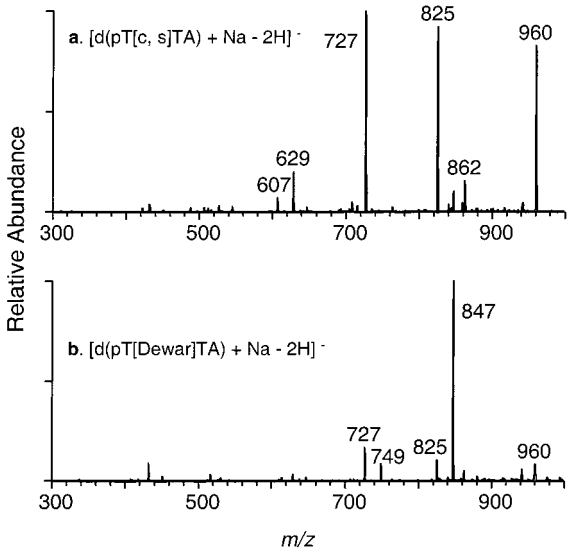

Figure 5. Product ion spectra of the $[M + Na - 2H]^-$ ions of d(pT-[c,s]TA) (a) and d(pT[Dewar]TA) (b).

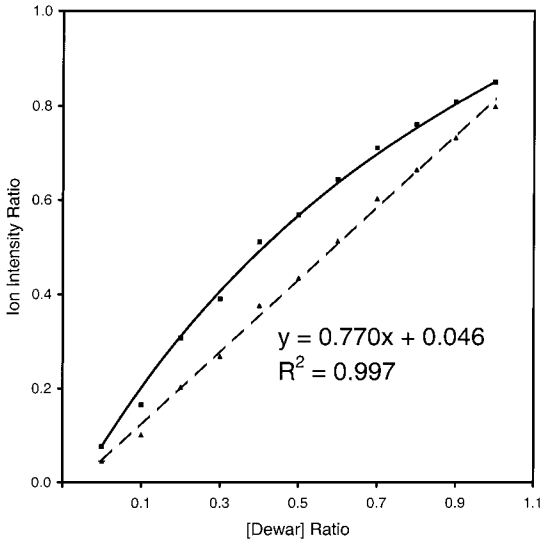

Figure 6. Relation of the ion intensity ratios $I_{847}/(I_{847} + I_{727})$ (solid square) or $I_{847}/(I_{847} + I_{825} + I_{727})$ (solid triangle) and the mole fractions of the Dewar photoproduct in mixtures of d(pT[c,s]TA) and d(pT-[Dewar]TA) that were formed in the nuclease P1 digestion of d(GTAT-[c,s]TAT) and d(GTAT[Dewar]TAT). Solid and dashed lines are fit calibration curves for $I_{847}/(I_{847} + I_{825})$ and $I_{847}/(I_{847} + I_{825} + I_{727})$.

for d(pT[c,s]TT) and that of m/z 816 ion for (pT[6−4]TT) are 1000 and 6150, respectively. We can now calculate the value of γ by using those two slopes: $\gamma = 1000/(1000 + 6150) = 0.140$, a value that agrees well with 0.151, which was obtained by curve fitting based on normalized linear model.

To evaluate the effectiveness of the fitting, we back-calculated the fractions of d(pT[6−4]TT) by using the normalized yields obtained by curve fitting and the measured signal intensities. The fractions that we calculated match very well with those corresponding to the sample preparations (Figure 4). If we use only the relative ion signal intensities for pure d(pT[c,s]TT) and d(pT-[6−4]TT), as Desaire and Leary[9] did for a set of three isomers, we find that the values from the back-calculated fractions deviate considerably from the true values (Figure 4).

We now wish to understand why d(pT[c,s]TT) and d(pT[6−4]TT) have different yields in producing fragment ions of m/z 803 and 816. The fragment ion of m/z 816 for d(pT[6−4]TT) (Figure 1c) is clearly dominant, whereas there are many fragment ions with abundances similar to that of the ion of m/z 803 in the spectrum of d(pT[c,s]TT). Similarly, in the report of the study of copper(II)−diimine complexes,[12] the fragment ion of m/z 277 of $[Cu(Gln - H)bpy]^+$ is considerably more abundant than the other product ion, whereas the fragment of m/z of 235 for $[Cu(Lys - H)bpy]^+$ is less abundant. This suggests that a plot of the sum of the signal intensities corresponding to at least several fragment ions in a composite spectrum would show less curvature. This is indeed the case for an analysis of mixtures of d(pT[c,s]TT) and d(pT[6−4]TT) in which six signal intensities corresponding to ions of m/z 607, 625, 687, 705, 803, and 831 for d(pT[c,s]TT) are compared with that at m/z 816 for d(pT[6−4]TT).

To use the normalized linear model, we represent b_0 in eq 1 as a sum of intensities of ions of m/z 607, 625, 687, 705, 803, and 831. The rest of the model remains the same (see Experimental Section). The new normalized yield for d(pT[c,s]TT) is 0.385. By linear fitting, we obtain a equation of $y = 0.770x + 0.046$ with a R^2 value of 0.997, where y is the signal intensity corresponding to the m/z 816 ion relative to the sum of the intensities corresponding to the five other ions and that of the m/z 816 ion and x is the mole fraction of d(pT[6−4]TT).

We obtained similar results when we analyzed a mixture of d(pT[c,s]TA) and d(pT[Dewar]TA) by using the product ion spectra of the $[M + Na - 2H]^-$ ion of m/z 960. The $[M + Na - 2H]^-$ ion of d(pT[c,s]TA) shows two abundant fragment ions at m/z 825 and 727 (Figure 5a), which are $[M - A + Na - 2H]^-$ and $[M - A - HPO_3 - H_2O + Na - 2H]^-$ (A is adenine), respectively. The $[M + Na - 2H]^-$ of d(pT[Dewar]TA), however, gives one abundant fragment ion of m/z 847, $[M - C_4H_3NO_3 + Na - 2H]^-$ ion (Figure 5b). The product ion spectra are again isomer-specific. A plot of the ion intensity ratios $I_{847}/(I_{847} + I_{727})$ versus the fractions of d(pT[Dewar]TA) is curved, and the

normalized yield of d(pT[c,s]TA) is 0.366. Plotting ion intensity ratios $I_{847}/(I_{847} + I_{825} + I_{727})$ versus the fractions of d(pT[Dewar]-TA), we obtained a straight line (Figure 6). These outcomes are reasonable if we consider that isomeric molecules should have nearly equal propensities for ionization and total fragmentation. Different yields are presumably a consequence of using only a portion of the product ion spectrum. Unfortunately, many approaches to MS/MS analysis require that only a portion of the spectrum be sampled owing to either instrument limitations or the requirement to obtain high sensitivity.

Sometimes, different ionization efficiencies may be the cause for isomers to generate fragment ions with different yields. Gatlin and Turecek,[19] in their studies of ionization efficiencies of Cu(II)−phenanthroline−amino acid complexes, found that the complexes of some amino acids affect the ionization efficiencies of others. Nevertheless, the normalized linear model is still an appropriate choice to fit the calibration curve and quantify the relative amounts of isomers as long as the ESI response is linear.

(19) Gatlin, C. L.; Turecek, F. *J. Mass Spectrom.* **2000**, *35*, 172−7.

CONCLUSIONS

We developed a normalized linear model for quantification, using composite spectra, and demonstrated its utility for the determination of the isomeric components from photodamage of oligodeoxynucleotides. Curved calibrations can be expected for quantification when the yields for diagnostic fragment ions are different. Different yields occur when the number of fragmentation channels or the ionization efficiencies are isomer-dependent. The method is applicable when only portions of a spectrum are available or when the data processing uses the full spectrum. We suggest the method is generally applicable when quantification must be accomplished from composite spectra.

ACKNOWLEDGMENT

The authors acknowledge NIH (Grants P41RR00954, CA40463) for financial support.

Received for review July 19, 2000. Accepted October 25, 2000.

AC000821M