Differentiation of Isomeric Acylcarnitines Using Tandem Mass Spectrometry

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Tandem mass spectrometry, using a four-sector instrument, has been used to distinguish isomeric acylcarnitines. Collisional activation of MH^+ ions (or of acylcarnitine methyl ester cations) induces fragmentations of the acyl moieties, yielding daughter ions with relative abundances characteristic of individual isomers. Direct analyses of crude urine samples from children with metabolic disorders enable unequivocal identification of individual acylcarnitines, thus contributing to the characterization of the disorder.

Carnitine is essential for the β-oxidation of fatty acids and serves to transport acyl groups across mitochondrial membranes (*1*). Recent discoveries of abnormal acylcarnitine/carnitine status in children with various metabolic diseases (*2*) has prompted the development of definitive methods for the identification and determination of these zwitterionic compounds. Fast atom bombardment mass spectrometry (FAB-MS), in combination with constant magnetic (B) to electric (E) field ratio linked scanning, has been successfully employed to characterize short-chain acylcarnitines in propionic acidemia (*3*), methylmalonic aciduria (*4*), isovaleric acidemia (*5*), and Reye's syndrome (*6*). Assays for acetyl- and propionylcarnitine using isotope dilution and FAB-MS with high-resolution selected-ion detection were developed (*6*). Because isomeric acylcarnitines could not be distinguished by this approach, thermospray high-performance liquid chromatography–mass spectrometry (HPLC–MS) was subsequently applied as an alternative procedure (*7*) and then proven successful in a number of situations where isomer differentiation is important (*8*, *9*). At least one pair of biological isomers, isovaleryl- and (2-methylbutyryl)carnitine, however, were not resolved by HPLC.

The application of tandem mass spectrometry techniques for the recognition and determination of trace components in biological samples has increased dramatically since the commercial availability of appropriate instrumentation (*10*). The goals of the present study were to investigate the potential application of tandem mass spectrometry to distinguish between isomeric short-chain acylcarnitines and to identify them intact in unpurified urine of children with metabolic disorders.

EXPERIMENTAL SECTION

Mass Spectrometry. Mass spectrometric analyses were performed on a VG ZAB-4F four sector instrument of B_1E_1–E_2B_2 configuration (VG Analytical, Manchester, UK). Ion production was by fast atom bombardment, using a xenon primary beam of 8 keV energy, of samples introduced in a glycerol matrix. A typical sample size for the analysis of authentic standards was 1 μg. The resolution of MS1 (B_1E_1) was approximately 1000 (10% valley definition). Helium was introduced into the collision cell mounted in the third field-free region (between the electric sectors); the pressure was adjusted to achieve a 50% diminution of the parent ion beam detected at the final detector (following B_2). For all the experiments reported here, the collision cell was at ground potential, so that the collision energy, in the laboratory frame of reference, was 8 keV. Daughter ion spectra of ions selected by MS1 were recorded by linked field scanning of B_2 and E_2, such that the ratio B_2/E_2 remained constant. The resolution of MS2 (E_2B_2), as judged by scanning across the transmitted parent ion peak, was unit or better. Spectra were output via a UV recorder. An alternative procedure, involving data acquisition via a VG 11/250 data system with spectral calibration using a recently described procedure (*11*), was used in some instances.

Synthetic Standards. Acylcarnitines were prepared and purified according to the procedure of Bohmer and Bremer (*12*), in which the corresponding acyl chloride was prepared in situ from equimolar amounts of the acid and thionyl chloride and condensed with excess L-carnitine (Sigma Tau, Rome, Italy) in trifluoroacetic acid. Adipyl and sebacyl monochlorides were purchased from Sigma Chemical Co. (St. Louis, MO) and condensed with carnitine according to the same procedure. Purity was established by NMR spectroscopy and FAB-MS (*6*).

[$^2H_9$]Carnitine was synthesized by exhaustive methylation of 0.5 g of DL-4-amino-3-hydroxybutyric acid (Sigma) with 5 g of [$^2H_3$]methyl iodide (Sigma) in methanol and water saturated with barium hydroxide (5.5 g), essentially as described by Mazzetti and Lemmon (*13*). The product was characterized by NMR spectroscopy and FAB-MS.

Clinical Samples. Urine from patients with organic acidurias of both known and unknown etiology were included in this study. All patients had been carnitine deficient at the time of clinical presentation, but the urine specimens were collected either during chronic carnitine therapy or during the 6-h period following a single bolus of carnitine (100 mg/kg po). Further details of the individual patients are given in the text where appropriate.

Procedure. Acylcarnitines were analyzed before or after conversion to methyl esters. Aliquots (50 μL) of lyophilized urine or dry authentic standards were dissolved in 50 μL of 0.5 N methanolic HCl (Supelco, Bellefonte, PA) and heated for 15 min at 80 °C. Portions of the reaction mixture (1–2 μL) were analyzed directly by mass spectrometry.

RESULTS AND DISCUSSION

The acylcarnitines possess a zwitterionic structure; previous work has established that methylation of the carboxyl moiety produces a substantial improvement in ion yield during FAB (*14*). For the present study, both unprotected and methylated acylcarnitines were analyzed. With the tandem instrument, parent ions corresponding to each intact acylcarnitine (as MH^+), or its methyl ester, were selected by MS1 and subjected to collisionally activated decomposition (CAD). Daughter ion spectra, recorded by scanning MS2 are summarized in Tables I and II for the acylcarnitines and their methyl esters, respectively. Methylation had little affect on the modes of fragmentation. Under the experimental conditions used, the abundances of the individual daughter ions did not exceed 1% of the abundance of the transmitted parent. Fragment

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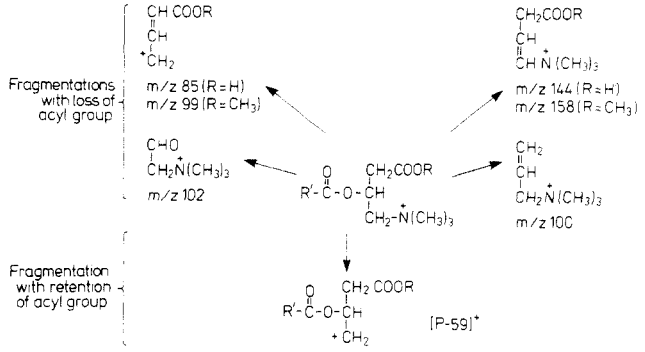

Figure 1. Collisonally activated decompositions of the carnitine moiety in protonated acylcarnitine and acylcarnitine methyl ester cations. "P" indicates the parent ion selected for collisional activation (MH^+ for acylcarnitines, or an acylcarnitine methyl ester cation).

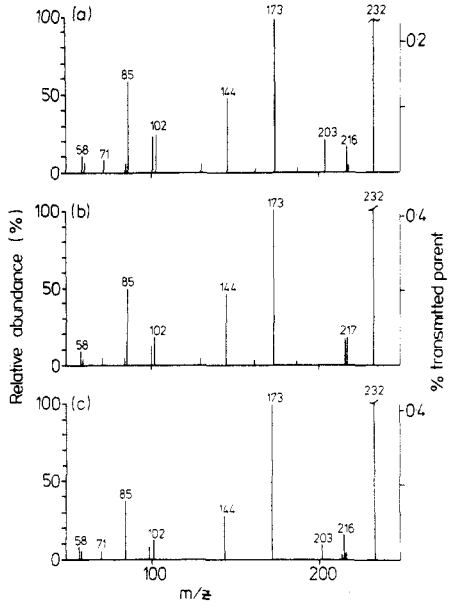

Figure 2. Daughter ion spectra of protonated C_4-acylcarnitines (m/z 232): (a) *n*-butyrylcarnitine; (b) isobutyrylcarnitine; (c) untreated urine of patient A.

ions were observed which were characteristic both of the carnitine moiety and of the acyl substituents. Daughter ions associated with fragmentation of the carnitine moiety are summarized in Figure 1. Ions of m/z 85, 100 and 144 (or 158 for the methyl esters) have also been observed in conventional mass spectra of ion-source-derived ions (*6*); in this instance, high-resolution determinations of the exact masses supported the structures shown. Daughter ion spectra recorded for isovaleryl[$^2H_9$]carnitine (and its methyl ester) and [$^2H_9$]isovalerylcarnitine methyl ester (Tables I and II) are consistent with the proposed assignments.

The mass of the acyl constituent was indicated by fragment ions corresponding to loss of 59 u, which again have been observed in the conventional spectra (*6*). Neither the conventional spectra nor spectra of daughter ions formed by unimolecular decomposition in the first field-free region (recorded by linked field scanning), however, permitted the distinction of isomeric acylcarnitines (*8*). Similarly, metastable decomposition observed in the present work to occur in the field free region between MS1 and MS2 did not include fragmentations of the acyl substituents. Collisionally activated decomposition of MH^+ ions from acylcarnitines, or acylcarnitine methyl ester cations, however, included fragmentations of the acyl moiety with loss of alkyl or alkane groups. Only the former was observed when fragmentation involved cleavage of the C2–C3 bond in an acylcarnitine without

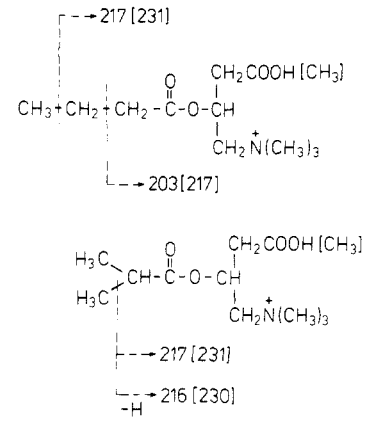

Figure 3. Fragmentations of the acyl moiety following collisionally activated decompositions of MH^+ ions from acylcarnitines and of acylcarnitine methyl ester cations.

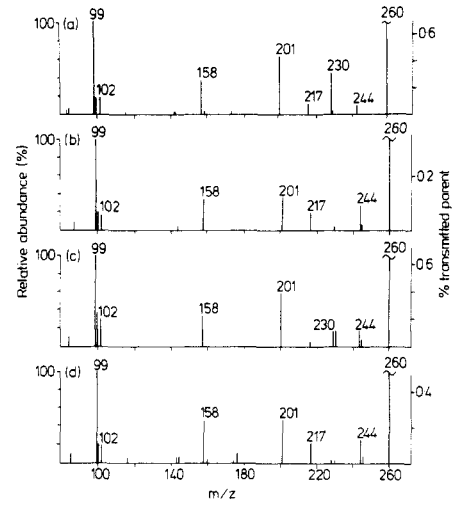

Figure 4. Daughter ion spectra of C_5-acylcarnitine methyl ester cations (m/z 260): (a) *n*-valerylcarnitine methyl ester; (b) isovalerylcarnitine methyl ester; (c) (2-methylbutyryl)carnitine methyl ester; (d) methylated urine of patient C.

branching at C2 (e.g., m/z 203 in the spectrum of the *n*-butyrylcarnitine, Table I). There was no evidence for loss of alkene fragments which would be expected for a charge or radical site-triggered McLafferty-type rearrangement associated with cleavage at the C2–C3 acyl bond.

Figure 2 indicates the differentiation of butyryl- and isobutyrylcarnitine on the basis of the CAD daughter spectra of their MH^+ ions. Loss of $C_2H_5^\bullet$ by a single bond cleavage in the *n*-acyl isomer has no equivalent in the branched analogue (Figure 3); m/z 203 is accordingly not observed in the daughter ion spectrum of isobutyrylcarnitine. The isomers are also readily distinguished by the CAD daughter ion spectra recorded for the methyl ester cations (Table II).

Diagnostic ions associated with cleavage of the acyl substituent were similarly observed in the CAD daughter ion spectra of isomeric C_5-acylcarnitines and their methyl esters (Tables I and II; Figure 4). Thus, valeryl-, isovaleryl-, and (2-methylbutyryl)carnitine methyl esters were distinguished by the relative abundances of daughter ions associated with losses of C_1, C_2, and C_3 fragments. Losses of C_2 and C_3 units by single C–C bond cleavages are not possible for isovaleryl- and (2-methylbutyryl)carnitines, respectively, so the corresponding daughter ions are observed at much reduced abundance. Their presence, however, albeit at low abundance, suggests that sequential fragmentation or rearrangment processes are occurring. Observations of this kind are presently the subject of further study.

Table I. Daughter Ion Spectra Obtained by CAD of Protonated Acylcarnitines

acyl substituent (mass of parent)	daughter ion, m/z (relative abundance[a])										
	with loss of acyl group				with retention of acyl group	with fragmentation of acyl group					
n-butyryl (232)	85 (59)	100 (23)	102 (25)	144 (48)	173 (100)	203 (21)	216 (16) 217 (4)				
isobutyryl (232)	85 (50)	100 (13)	102 (18)	144 (45)	173 (100)		216 (17) 217 (19)				
n-valeryl (246)	85 (17)	100 (11)	102 (9)	144 (38)	187 (100)	203 (23)	216 (53) 217 (3)	230 (12) 231 (2)			
isovaleryl (246)	85 (19)	100 (12)	102 (14)	144 (55)	187 (100)	203 (53)	216 (6) 217 (2)	230 (42) 231 (27)			
isovaleryl[$^2H_2$]carnitine (255)	85 (88)	109 (9)	111 (16)	153 (31)	187 (100)	212 (8)	226 (2)	235 (1) 236 (1) 239 (10) 240 (3)			
2-methylbutyryl (246)	85 (15)	100 (11)	102 (13)	144 (43)	187 (100)	203 (6)	216 (40) 217 (34)	230 (54) 231 (12)			
n-octanoyl (288)	85 (7)	100 (4)	102 (5)	144 (30)	229 (100)	203 (15)	216 (72) 217 (3)	230 (12)	244 (4) 245 (3)	258 (11)	272 (6)
valproyl (288)	85 (4)	100 (2)	102 (2)	144 (8)	229 (100)		216 (43)	230 (3)	244 (11) 245 (9)	258 (15)	272 (2)

[a] Abundances are expressed relative to the principal daughter ion (=100%) which represented 0.2–2% of the abundance of the transmitted parent.

Table II. Daughter Ion Spectra Obtained by CAD of Acylcarnitine Methyl Ester Cations

acyl substituent (mass of parent)	daughter ion, m/z (relative abundance[a])										
	with loss of acyl group				with retention of acyl group	with fragmentation of acyl group					
n-butyryl (246)	99 (100)	100 (17)	102 (17)	158 (30)	187 (16)	217 (5)	230 (5)				
isobutyryl (246)	99 (100)	100 (15)	102 (14)	158 (20)	187 (38)		230 (3) 231 (4)				
n-valeryl (260)	99 (100)	100 (18)	102 (16)	158 (36)	201 (63)	217 (15)	230 (45) 231 (4)	244 (9)			
isovaleryl (260)	99 (100)	100 (20)	102 (17)	158 (47)	201 (37)	217 (24)	230 (4)	244 (26) 245 (9)			
[$^2H_9$]isovaleryl (269)	99 (100)	100 (23)	102 (16)	158 (46)	210 (40)	219 (11)	233 (3)	249 (14) 251 (3) 253 (6)			
isovaleryl[$^2H_9$]carnitine (269)	99 (47)	109 (12)	111 (11)	167 (22)	201 (100)	226 (8)	240 (1)	249 (1) 250 (1) 253 (10)			
2-methylbutyryl (260)	99 (100)	100 (19)	102 (15)	158 (36)	201 (57)	217 (4)	230 (18) 231 (18)	244 (16) 245 (8)			
n-octanoyl (302)	99 (100)	100 (17)	102 (13)	158 (520)	243 (29)	217 (17)	230 (79) 231 (2)	244 (12)	258 (4)	272 (9)	286 (5)
valproyl (302)	99 (84)	100 (13)	102 (8)	158 (21)	243 (60)		230 (100) 231 (2)	244 (11) 245 (4)	258 (19) 259 (14)	272 (32) 273 (3)	286 (4)

[a] Abundances are expressed relative to the principal daughter ion (=100%) which represents 0.2–2% of the abundance of the transmitted parent.

The C_8-acylcarnitines, *n*-octanoyl- and valproyl- (2-propylpentanoyl-) carnitine were readily distinguished by the presence or absence of daughter ions of m/z 203 and 217 in the CAD daughter ion spectra of the unmethylated and methylated species, respectively (Tables I and II). The observed differences in the relative abundances of ions corresponding to fragmentation at various points in the acyl groups, however, suggest possible difficulty in the differentiation of other branched-isomers, at least in the absence of authentic standards.

Analyses of Clinical Samples. Direct analysis was performed of an aliquot (1 μL) of urine from a child (patient A) suffering from the rare metabolic disorder, ethylmalonic–adipic aciduria, reflecting a short-chain fatty acyl coA dehydrogenase deficiency. The urine sample was obtained from this patient (as from the other patients discussed below) following a carnitine load or while receiving carnitine therapy. The daughter ion spectrum obtained by CAD of m/z 232 is shown in Figure 2c; the prominent daughter ion of m/z 203 and the close agreement with the equivalent spectrum obtained from the standard (Figure 2b) provide clear evidence that *n*-butyrylcarnitine is the major isomer, consistent with a metabolic block at butyryl coA dehydrogenase.

While the direct analysis of an untreated urine aliquot is an attractively simple procedure, improved sensitivity of detection is observed following methylation of the acylcarnitines (*14*). In the analysis of urines, the increase in absolute signal intensity is accompanied by a still greater increase in the ratio of signal to background, possibly reflecting the improved surfactant properties of the methylated analytes.

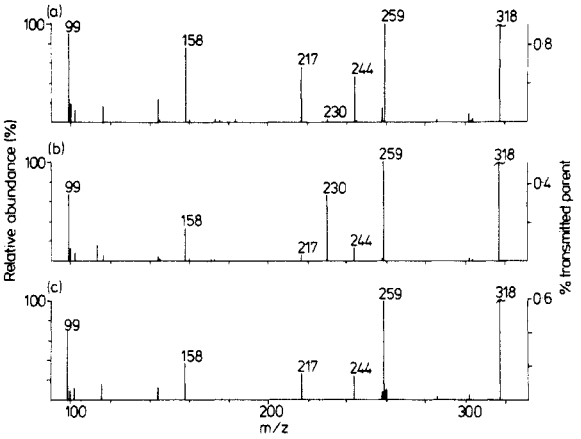

Figure 5. Daughter ion spectra of C_6-carboxyacylcarnitine dimethyl ester cations (m/z 318): (a) (3-methylglutaryl)carnitine dimethyl ester, (b) adipylcarnitine dimethyl ester, (c) methylated urine of patient D.

An aliquot of urine from patient A was lyophilized, methylated, and analyzed by FAB-MS. The daughter ion spectrum of m/z 246, following CAD, confirmed the characterization of n-butyrylcarnitine, made on the basis of the equivalent analysis of the underivatized acylcarnitine. In contrast, similar analysis of the methylated urine of a second patient (B) showed excellent agreement with authentic isobutyrylcarnitine methyl ester. Thus, the m/z 230, 231 doublet was prominent in the CAD daughter ion spectrum of m/z 246 but m/z 217 was not detected. For both patients A and B, the identification of the major acyl substituents was consistent with gas chromatography–mass spectrometry (GC–MS) analyses of methyl esters of short chain acids liberated by mild alkaline hydrolysis of the acylcarnitine fractions (*15*). In common with patient A, B exhibited ethylmalonic–adipic aciduria and the acylcarnitine profiles of the two patients were very similar (with the exception of the difference in the structure of the C_4-acylcarnitine). This observation suggests the existence of a new variant of this disorder in patient B and further study will be necessary to elucidate the primary biochemical lesion.

Figure 4d shows the analysis of a methylated urine from patient C; the excellent agreement with the CAD daughter ion spectrum obtained for standard isovalerylcarnitine methyl ester cation (Figure 4b) established the identity of the major acylcarnitine in the urine of this patient. This observation was consistent with the diagnosis of isovaleric acidemia, made on the basis of grossly elevated urinary isovaleryl glycine and the detection of isovaleryl carnitine by other methods (*5*).

The reproducibility of the relative abundances of daughter ions critical to the differentiation of isomeric acylcarnitines was assessed by the recording of multiple consecutive scans. In the analysis of n-butyrylcarnitine methyl ester in a urine sample after methylation, for example, the ratio of the abundances of m/z 217:230 was 0.82 ± 0.07 (coefficient of variation, 9%). Similarly, no significant variation in relative abundances was observed during the course of a day. For definitive identification of acylcarnitines in biological samples, standard spectra were recorded on the same day as the analyses of urines.

Carnitine Esters of Dicarboxylic Acids. Carnitine monoesters of dicarboxylic acids have recently been identified in human urine (*15*). Tandem mass spectrometry analyses with CAD have been performed to assess the possibility of differentiation of isomers analogous to that achieved for the simple acylcarnitines. Figure 5a shows the daughter ion spectrum obtained by CAD of m/z 318, the parent cation derived from 3-methylglutarylcarnitine dimethyl ester. The origins of the major diagnostic fragments are shown in Figure 6. The branching of the acyl substituent is indicated by the

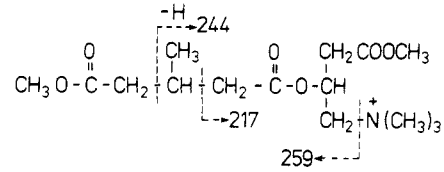

Figure 6. Collisionally activated decompositions of m/z 318 derived from (3-methylglutaryl)carnitine dimethyl ester (see also Figure 1). m/z 259 may also be derived by loss of $CH_3OCO^{\bullet}$.

very low relative abundance of m/z 230. In contrast, the corresponding spectrum obtained for the straight-chain isomer, adipylcarnitine dimethyl ester (Figure 5b), includes an abundant m/z 230, indicating the possibility for isomer differentiation. Figure 5c shows the daughter ion spectrum obtained by CAD of m/z 318, derived from the methylated urine of patient D; the good agreement with the standard spectrum of (3-methylglutaryl)carnitine dimethyl ester permits the identification of the urine component. This agrees with the characterization of 3-methylglutaric acid by GC–MS analysis of the trimethylsilyl esters of acids released by hydrolysis of an acylcarnitine fraction. A diagnosis of (3-hydroxy-3-methylglutaryl)-coA lyase deficiency was confirmed by organic acid analysis. It is noteworthy that two other diagnostic acids excreted in high concentration, 3-methylglutaconic and 3-hydroxy-3-methylglularic, do not apparently form acylcarnitines (*15*).

CONCLUSIONS

Analyses by tandem mass spectrometry with collisionally activated decomposition of parent ions produced by FAB permit detailed structural characterization of acylcarnitines, including the differentiation of species containing isomeric acyl moieties. The sensitivity of analysis is improved by prior conversion to the methyl esters. The distinction of isomeric compounds is based on the relative abundances of daughter ions derived by fragmentation of the acyl substituents. The procedure may be applied to the analysis of acylcarnitines in unpurified urine obtained from patients following carnitine challenge or therapy for metabolic disorders. For definitive identification of acylcarnitines, the method is preferable to the alternative procedure of isolation of an acylcarnitine fraction, hydrolysis, and GC–MS of the resultant acids as their methyl esters. LC–MS analyses of intact acylcarnitines provide a further important approach, though the differentiation of at least one pair of clinically important isomers has proved difficult. Earlier attempts (*6*) to distinguish isomeric acylcarnitines, by linked field scanning of the products of decomposition (metastable or collision induced) in the first field-free region of a double-focusing mass spectrometer, were unsuccessful. The reasons are not presently clear but may relate, in part, to the generally poorer signal/noise ratios observed in these experiments. The tandem mass spectrometry method reported in the present paper provides unequivocal identification of isomeric acylcarnitines; the expense and complexity of the instrumentation, however, suggest that its future use in this context will be to provide rapid confirmation of evidence from simpler procedures or to attempt analyses where alternative methods have failed.

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Registry No. (*R*)-$^-O_2CCH_2CH(OCOPr)CH_2NMe_3^+$, 25576-40-3; (*R*)-$^-O_2CCH_2CH(OCOPr\text{-}i)CH_2NMe_3^+$, 25518-49-4; (*R*)-$^-O_2CCH_2CH(OCOBu)CH_2NMe_3^+$, 40225-14-7; (*R*)-$^-O_2CCH_2CH(OCOCH_2Pr\text{-}i)CH_2NMe_3^+$, 31023-24-2; (±)-$^-O_2CCH_2CH(OCOCH_2Pr\text{-}i)CH_2N(CD_3)_3^+$, 104015-03-4; (*R*)-$^-O_2CCH_2CH(OCOBu\text{-}s)CH_2NMe_3^+$, 31023-25-3; (*R*)-$^-O_2CCH_2CH(OCO(CH_2)_6Me)CH_2NMe_3^+$, 25243-95-2; (*R*)-$^-O_2CCH_2CH$-

$(OCOCHPr_2)CH_2NMe_3^+$, 95782-09-5; (*R*)-$MeO_2CCH_2CH(OCOPr)CH_2NMe_3^+$, 104015-04-5; (*R*)-$MeO_2CCH_2CH(OCOPr\text{-}i)CH_2NMe_3^+$, 104015-05-6; (*R*)-$MeO_2CCH_2CH(OCOBu)CH_2NMe_3^+$, 104015-06-7; (*R*)-$MeO_2CCH_2CH(OCOCH_2Pr\text{-}i)CH_2NMe_3^+$, 104015-07-8; (*R*)-$MeO_2CCH_2CH(OCOCD_2CD(CD_3)_2)CH_2NMe_3^+$, 104034-09-5; (±)-$MeO_2CCH_2CH(OCOCH_2Pr\text{-}i)CH_2N(CD_3)_3^+$, 104015-08-9; (*R*)-$MeO_2CCH_2CH(OCOBu\text{-}s)CH_2NMe_3^+$, 104015-09-0; (*R*)-$MeO_2CCH_2CH(OCO(CH_2)_6Me)CH_2NMe_3^+$, 104015-10-3; (*R*)-$MeO_2CCH_2CH(OCOCHPr_2)CH_2NMe_3^+$, 104015-11-4; $MeO_2CCH_2CH(Me)CH_2CO_2CH(CH_2CO_2Me)CH_2NMe_3^+$, 104015-12-5; $MeO_2C(CH_2)_4CO_2CH(CH_2CO_2Me)CH_2NMe_3^+$, 104034-10-8.

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Rank Annihilation with Incomplete Information

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Rank annihilation is a technique for quantitating a known component in the presence of a variable background of unknowns. It requires a multidimensional data set that can be modeled as the linear summation of the individual components, whether knowns or unknowns. Furthermore, each of the components is assumed to be represented as the outer product of vectors. Each vector corresponds to one of the dimensions (e.g., spectral, spatial, chromatographic). This paper extends the concept of two-dimensional rank annihilation to the case where only one of the vectors describing a particular component is known. It is shown that specification of one of the vectors of a component defines a set of possible linear combinations of the eigenvectors for the other dimension that will decrease the rank of the residual matrix by one. Two methods for further constraining the component distribution are proposed: one requires a minimum fraction of negative values in the residual matrix; the other, a minimum overlap between the residual matrix and the derived component. The general theory is illustrated with a simulated two-component mixture, and a real three-component mixture is successfully analyzed by using data obtained from a multichannel imaging spectrophotometer.

In considering the simultanous analysis of multicomponent, multidimensional data sets, we have previously considered three analytical situations. In the first, all of the components are knowns, for which calibration data are available. Here, the method of maximum likelihood or least squares is appropriate for finding the quantity of each component (*1*, *2*). In the past, this approach has required excellent reproducibility between calibration standards and the data obtained for the mixture. However, analyses of hyphenated chromatographic/spectral data sets are often plagued by retention time and peak shape irreproducibilities. Recently, we have modified the technique to correct for such problems (*3*) and applied it to data from multiwavelength thin-layer chromatography.

In the second analytical situation, none of the components are knowns, and therefore only qualitative analysis is possible. Here, the method of factor analysis can be applied (*4*-*6*). This method provides (a) a lower bound to the number of linearly independent components present in the mixture and (b) estimates for the spectral and retention vectors when low numbers of components are present. Factor analysis approaches have the advantage that no assumptions are made regarding the shapes of the spectral and retention vectors, other than that they all be positive. Unfortunately, with these minimal constraints, there is frequently a great deal of ambiguity in the recovered vectors. Harris and co-workers (*7*, *8*) have shown that, when assumptions can be made regarding the form of the retention vectors, an alternative to factor analysis can be developed that reduces the degree of ambiguity in the recovered spectral and chromatographic vectors. Also, Vandeginste and colleagues have shown that additional constraints can be added to conventional factor analysis algorithms to yield better estimates of the component vectors. In one case, solutions are derived that are all positive, contain only one maximum, and have a minimum overlap (*9*). In a second case, these same constraints are enforced by iterative selection until the vectors match a hypothetically correct target vector (*10*). A similar approach has been tested by Gemperline (*11*).

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