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Elemental Sulfur as a Matrix for Mass Spectrometry of Photosynthetic Pigments and Fullerenes

Daniel C. Brune*

Department of Chemistry and Biochemistry and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, AZ 85287-1604, USA

Elemental sulfur works well as a matrix for laser desorption time-of-flight mass spectrometry of bacteriochlorophylls and carotenoids, the major pigments in photosynthetic bacteria. Because sulfur lacks protons, pigment ionization probably involves electron, rather than proton, transfer. Fragmentation of chlorophylls that are esters of allylic alcohols occurs, but is partially suppressed when sulfur is used as a matrix. Mass spectrometry on sulfur itself shows that the most abundant positive ion is S_5^+, while the most abundant negative ion is S_3^-, indicating that light absorption causes photodissociation of S_8 rings into these products. A similar pattern was observed with red selenium, which also occurs as 8-membered rings. Molecular masses of other hydrophobic analytes, such as fullerene compounds, can also be determined using elemental sulfur as the matrix.

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The ability to determine accurate molecular weights of large, nonvolatile molecules by mass spectrometry has been greatly enhanced through the technique of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.[1] In this technique, gas phase ions of analyte molecules are generated when laser pulses are absorbed by a matrix in which the analyte molecules are embedded. Common matrices are 2,5-dihydroxybenzoic acid, sinapinic acid and α-cyano-4-hydroxycinnamic acid (CHCA), which are thought to generate analyte cations by proton transfer,[2,3] although the possibility of electron transfer has also been considered.[1] While these matrices work well with biological molecules such as proteins, their acidity makes them unsuitable for use with others, such as chlorophylls, which are pheophytinized under acidic conditions. This can also be a problem with 2-hydroxy-1-naphthoic acid, as indicated by partial loss of Zn from its octaethylporphyrin complex when that matrix was used to analyze porphyrins.[4]

In some cases, pure porphyrins can be desorbed and ionized by a laser pulse, and one porphyrin has even been used as a matrix for mass spectrometry.[5] Although chlorophylls can be desorbed and ionized without using a matrix, fragmentation usually occurs. Elemental sulfur is known to absorb ultraviolet (UV) light well, and melting has even been observed on illuminating sulfur particles with high intensity UV light (340–390 nm).[6] In principle, ionization of porphyrins can occur either by proton or electron transfer. Sulfur is well suited to participate in electron transfer reactions, but lacks protons so that, barring the presence of protonated impurities, any ionization observed with sulfur as the matrix should be due only to electron transfer reactions. Electron transfer is also thought to be involved in ion formation with terthiophene and similar aromatic hydrocarbons[7,8] and possibly with C_{60}[9] as matrices. The experiments presented here show that, in the presence of an elemental sulfur matrix, desorption of chlorophylls and other hydrophobic analytes is enhanced and fragmentation is suppressed.

In an application illustrating use of the sulfur matrix, the pigment composition of green photosynthetic bacterial chlorosomes was determined. Chlorosomes are structures containing a mixture of chlorophylls and carotenoids that absorb light and efficiently transfer excitation to the adjacent membrane, where photochemical electron transfer events that initiate photosynthesis in green bacteria occur.[10]

EXPERIMENTAL

Elemental sulfur (Humco laboratory) was purchased from a local pharmacy and dissolved in carbon disulfide (J.T. Baker) to form a 0.6M solution. Bacteriochlorophyll *c* was extracted from the thermophilic green gliding bacterium *Chloroflexus aurantiacus* and purified by reversed-phase high-performance liquid chromatography (HPLC) as described previously.[11] Chlorosomes were isolated from *Cf. aurantiacus* as described by Gerola and Olson[12] and pigments extracted into methanol. Extracted pigments were dried and redissolved in carbon disulfide to form a dark green solution (about 1 mM). Pigments were diluted either 1 to 1 or 1 to 10 with the sulfur matrix solution, resulting in final pigment concentrations between 0.1 and 1 mM and thus matrix to analyte ratios between 600 and 6000 to 1. Aliquots of the mixture were dried on stainless steel sample pins for analysis by mass spectrometry. In some experiments, sulfur without added pigments or pigments without the sulfur matrix were dried on the sample pins for analysis. The fullerene (C_{60}) cyclopropyl adduct was synthesized by Dr. Paul Liddell, who provided the sample for the experiments shown in Fig. 6. Red selenium was prepared by reaction of selenium dioxide with sodium sulfite as described by Bacon and Ingledew.[13]

*Correspondence to: D. C. Brune, Department of Chemistry and Biochemistry and Centre for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, AZ 85287-1604, USA.
E-mail: dbrune@asu.edu

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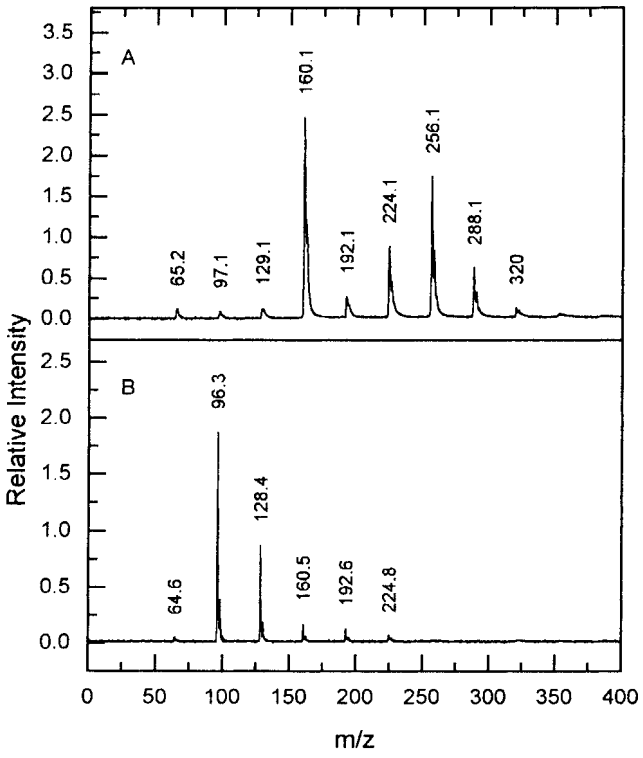

Figure 1. Positive ion (a) and negative ion (b) mass spectra of elemental sulfur. Numbers above the peaks indicate their mass/charge ratios determined by calibration against an external standard. Note that the dominant positive ions are S_5^+ and S_8^+, while the dominant negative ion is S_3^-.

Mass spectra were obtained using a Vestec Lasertec Research mass spectrometer operating in the positive ion mode, unless otherwise specified. This instrument incorporates a nitrogen laser providing 3 ns pulses of 337 nm light of variable intensity. Mass spectral data were collected on a Tectronics TDS 520 digital storage oscilloscope and analyzed on a personal computer using LabCalc software (Galactic Industries Corp.). Each mass spectrum was the average of 128 shots.

RESULTS AND DISCUSSION

The sulfur matrix

When elemental sulfur alone was analyzed in the positive ion mode, the mass spectrum was dominated by ions containing 5, 8, 7, 9, and 6 sulfur atoms, listed in the approximate order of decreasing abundance (Fig. 1(a)). This result implies that absorption of the laser pulses caused fragmentation of the S_8 rings present initially. Shoulders on the high mass sides of the S_n^+ peaks are due to ions containing ^{34}S. In the negative ion mode, the dominant sulfur ions were S_3^- and S_4^- (Fig. 1(b)).

The most direct explanation for these results is that light absorption causes S_8 to photodissociate, primarily into S_5^+ and S_3^-. An alternative mechanism might be electron transfer between two sulfur molecules, initially generating S_8^+ and S_8^- which then decompose primarily into $S_5^+ + S_3$ and $S_3^- + S_5$, respectively. The decomposition of S_8^+, primarily into $S_5^+ + S_3$ and $S_6^+ + S_2$, was observed previously by Fales *et al.*,[14] who also noted the relatively high stability of the S_5^+ product. A comparable study on the

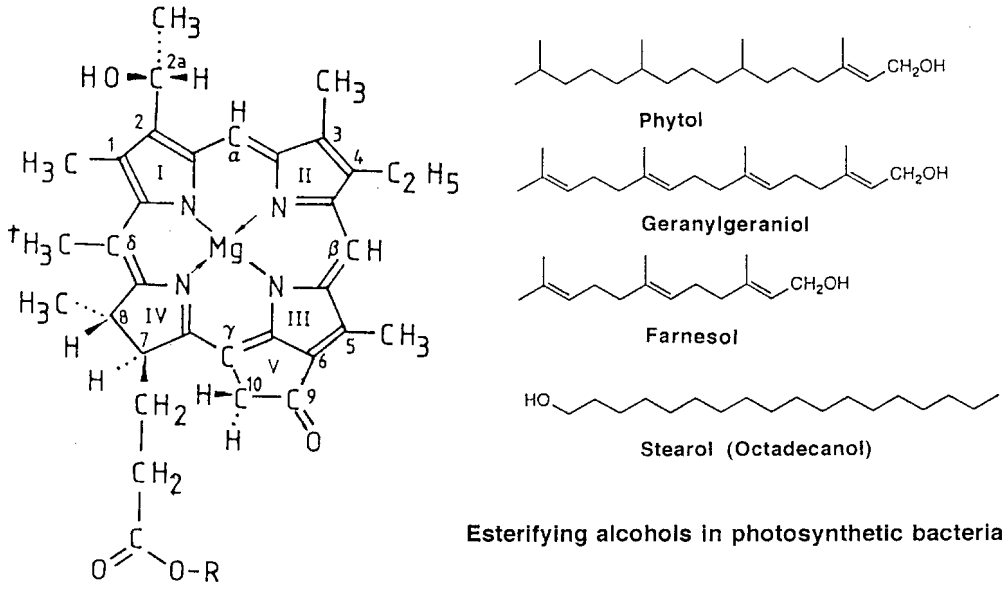

Figure 2. The structure of bacteriochlorophyll *c*. Various forms of the pigment occur, differing mainly in the nature of the alcohol (R) attached to the propionic acid side chain on ring IV. The most abundant form of the pigment in *Chloroflexus aurantiacus* has a stearyl, or octadecyl, ($C_{18}H_{37}$) group, while other forms with phytyl ($C_{20}H_{39}$) and geranylgeranyl ($C_{20}H_{33}$) groups are also present. Farnesol is the predominant esterifying alcohol in green sulfur bacteria. In bacteriochlorophyllide *c* (formed by fragmentation in some experiments), R is a proton. In bacteriochlorophyll *d*, a proton replaces the methyl group marked by the symbol †. Bacteriopheophytin differs from bacteriochlorophyll in that the central Mg is replaced by two protons. Bacteriochlorophyll *a* differs from bacteriochlorophyll *c* by having an acetyl group in place of the hydroxyethyl group at position 2, a single bond between carbons 3 and 4, and a carbomethoxy group in place of one of the hydrogens at position 10.

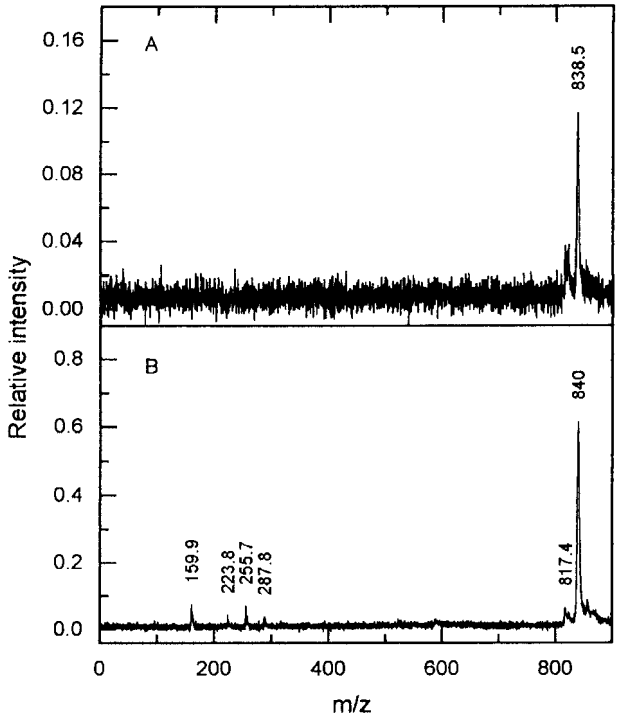

Figure 3. Mass spectra of purified BChl *c* stearyl ester from *Cf. aurantiacus*. The spectrum in (a) was obtained without any matrix, while that in (b) was obtained with sulfur as the matrix.

fragmentation of S_8^- seems not to have been done. Positive ions larger than S_8^+ (i.e. S_9^+ and S_{10}^+) might then be formed by recombination of neutral products of the initial fragmentation reactions (i.e. S_2, S_3, S_4 and S_5) with the cations S_5^+, S_6^+ and S_8^+, although further experiments clearly are necessary to establish the mechanism for sulfur ion formation.

Purified bacteriochlorophylls

Chlorosomes from the green gliding bacterium *Chloroflexus aurantiacus* contain a mixture of homologous bacteriochlorophyll *c* pigments (See Fig. 2 for structures). Figures 3(a) and (b) show mass spectra obtained for the major homologue, BChl *c* stearyl ester without a matrix and with elemental sulfur as the matrix, respectively. Both spectra reveal a single peak with a mass of about 840 Da, but the signal-to-noise ratio was better with the sulfur matrix.

Figures 4(a)–(d) show results obtained for the homologous pigment with phytol as the esterifying alcohol. In this case, even at low laser power, the phytyl group was completely lost in the absence of the sulfur matrix (Fig. 4(a)), giving a peak at *ca*. 587 Da due to bacteriochlorophyllide *c*. When BChl *c* phytyl ester, dissolved in acetone, was mixed with a saturated solution of CHCA (also in acetone) and dried on the sample pin, a major peak at *ca*. 842 Da due to bacteriopheophytin (BPheo) *c* (formed from the chlorophyll by loss of Mg) was observed (Fig. 4(b)). In this case, fragmentation was strongly inhibited; however, experiments in which BPheo *c* was formed simply by adding trifluoroacetic acid to a solution of BChl *c* and irradiated without a matrix (Fig. 4(c)) showed that BPheo *c* is intrinsically much more resistant to fragmentation than BChl *c*. In the presence of sulfur (Fig. 4(d)), the parent BChl *c* peak at 866 Da is clearly seen, although fragmentation was not completely prevented. The asymmetric skewing of the bacteriochlorophyllide peak to higher apparent masses indicates in-source decay (i.e. continued fragmentation as ions are being accelerated in the source prior to reaching the field-free drift region) and has been observed before with other fragile molecules, such as peptide sulfate esters.[15] Experiments on other bacteriochlorophylls indicate that the geranylgeranyl and farnesyl esters fragment similarly to the phytyl ester (data not shown).

A common feature of the esters that fragment is the presence of a double bond in the alcohol adjacent to the

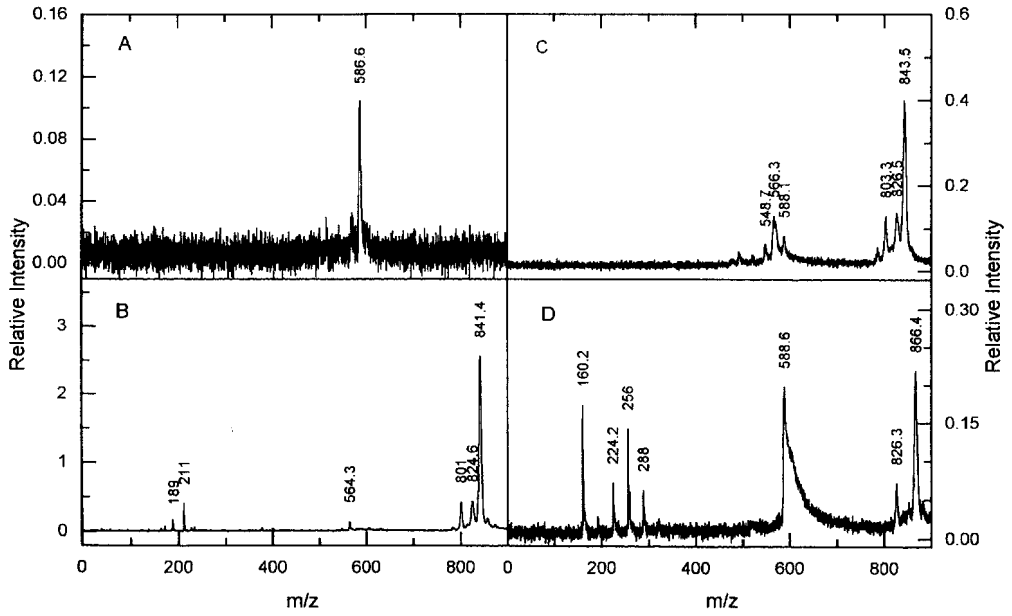

Figure 4. Mass spectrometric analysis of purified BChl *c* phytyl ester from *Cf. aurantiacus*. The spectra shown were obtained without any matrix (a), with α-cyano-4-hydroxycinnamic acid (b), after addition of trifluoroacetic acid and without any matrix (c), and with sulfur (d). Note the complete loss of the phytyl group in (a), and the loss of Mg (indicated by a decrease in mass of about 22 Da) in (b) and (c).

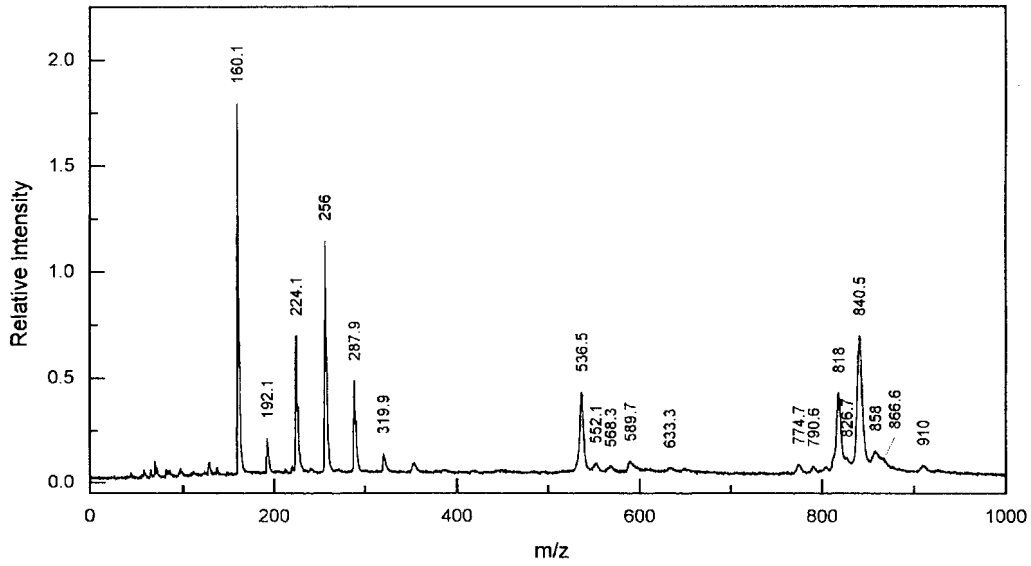

Figure 5. Analysis of *Chloroflexus* chlorosome pigments by mass spectrometry using sulfur as the matrix. Suggested identities of pigment peaks in this mass spectrum, with calculated masses given to the nearest Da in parentheses, are as follows: 536.5, β- + γ-carotene (537 Da); 552.1, hydroxy-γ-carotene (553 Da); 589.7, bacteriochlorophyllide *c* (588 Da); 633.3, bacteriochlorophyllide *a* (630 Da); 818, stearyl bacteriopheophytin *c* (818 Da); 826.7, stearyl BChl *d* (826 Da); 840.5, stearyl BChl *c* (840 Da); 858, geranylgeranyl BChl *c* (860 Da); 866.6, phytyl BChl *c* (866 Da); 910, BChl *a* (910 Da).

carbon bound to oxygen in the ester linkage. A similar fragmentation has been observed in allylic alcohol esters of retinoic acid.[16] In that case, however, there was also partial intramolecular elimination of CO_2 from the ester carboxyl group, yielding ions 44 Da lower in mass than the parent ion. Perhaps this reaction is sterically inhibited in chlorophylls by the proximity of the bulky porphyrin group to the ester linkage. The reason for the greater stability of pheophytins to fragmentation is not known. Possibly the greater flexibility of the porphyrin ring in pheophytins than in chlorophylls facilitates delocalization of vibrational excitation onto the porphyrin ring and away from the fragile ester linkage.

Chloroflexus chlorosome extract

As shown in Fig. 5, the pigment composition of a chlorosome extract can be determined, at least qualitatively, by mass spectrometric analysis. Not all peaks have been assigned; however, those due to the stearyl, geranylgeranyl, and phytyl esters of BChl *c*, respectively, can be seen at 840.5, 858, and 866.6 Da, as can a peak at 589.7 Da from bacteriochlorophyllide *c* formed by fragmentation of the latter two pigments. In addition, a peak due to BChl *a* phytyl ester occurs at 910 Da as does a peak at 633.3 due to its bacteriochlorophyllide. BChl *a* is found in the chlorosome baseplate, where it mediates energy transfer from BChl *c* to the adjacent photosynthetic membrane.[10] A peak at 536.5 Da can be attributed to β- and γ-carotenes (which account for about 80% of the carotene content of chlorosomes; both have calculated masses of 536.85 Da), while the peak at 552.1 Da is probably due to hydroxylated γ-carotene (which accounts for about 10% of the carotene in chlorosomes) (See Blankenship *et al.*[10] for a discussion of chlorosome pigment content). A small peak at 826.7 Da may be due to stearyl BChl *d*. BChl *d* is known to be present in chlorosomes,[11] but whether or not it occurs as the stearyl ester has not been determined previously.

A peak at 818 Da can be assigned to stearyl bacteriopheophytin *c*. This peak is large relative to the amount of bacteriopheophytin present in chlorosome extracts as determined by other methods.[10,11] In general, the relative sizes of peaks from different analytes in a mixture are a function of analyte desorption and ionization efficiencies, which can vary with the matrix used and other experimental conditions, and are not necessarily a measure of their relative concentrations in the mixture.[17,18] BPheo *c* is less polar than BChl *a*, which may result in better incorporation in crystals of the nonpolar sulfur matrix on drying.

Fullerene compounds

During the course of analyzing several synthetic derivatives of fullerene molecules, it was observed that these molecules give good mass spectra using sulfur as a matrix. Representative spectra obtained with a cyclopropyl adduct of C_{60} having the structure shown as an inset in Fig. 6(a) are shown in Figs 6(a)–(c). In the absence of a matrix (Fig. 6(a)), substantial fragmentation occurs, giving rise to the underivatized fullerene molecule, C_{60} (mass = 720 Da). With CHCA as the matrix (Fig. 6(c)), fragmentation to yield C_{60} is inhibited but, in addition to the cyclopropyl compound (mass *ca*. 993 Da), additional peaks are seen, including one at 1016.3 that is probably a Na^+ adduct. It is apparent that sulfur (Fig. 6(c)) yields a cleaner spectrum. Possibly the greater solubility of the fullerene derivative in CS_2, the solvent used for the sulfur matrix, than in acetone, which was used to dissolve CHCA, resulted in better mixture with the sulfur matrix and thus a better spectrum.

This spectrum also illustrates analyte suppression of matrix peaks, a phenomenon that occurs when a high concentration of the analyte is present in the matrix, and was also observed with high concentrations of chlorophylls and other porphyrins. The significance of this phenomenon for the mechanism of ion formation by protonation or alkali metal cationization in MALDI experiments has been

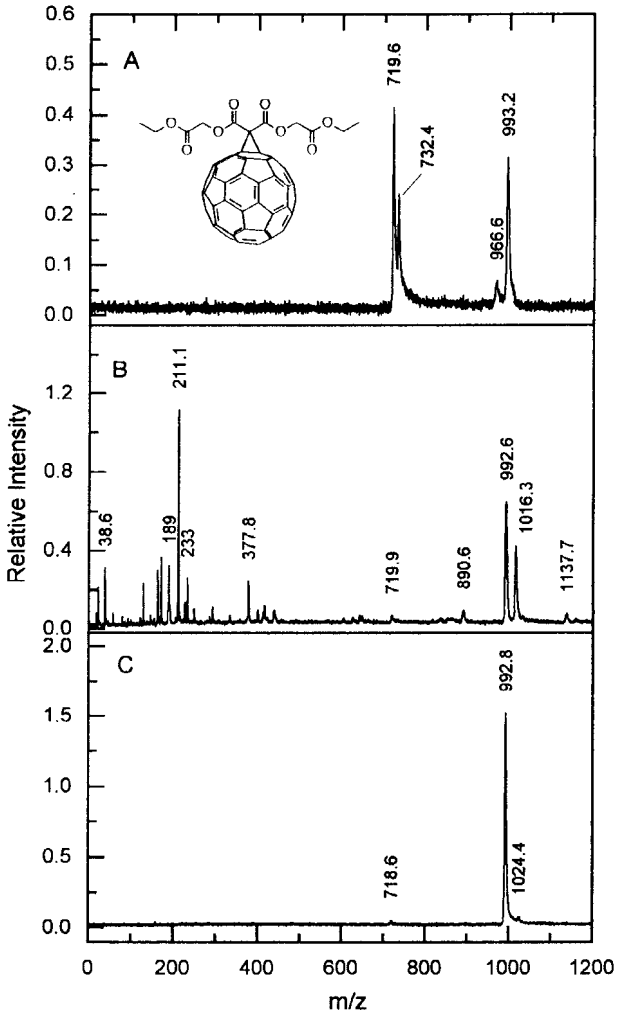

Figure 6. Mass spectra of a cyclopropyl derivative of C_{60} (structure shown in (a)). (a), without matrix; (b), with α-cyano-4-hydroxycinnamic acid; (c), with sulfur as the matrix.

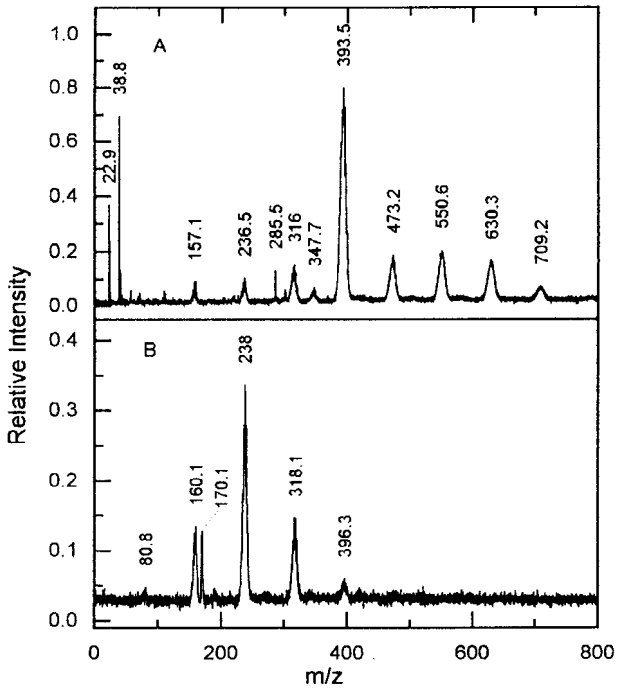

Figure 7. Positive ion (a) and negative ion (b) mass spectra of red selenium. Numbers above the peaks indicate their mass/charge ratios determined by calibration against an external standard. Note that the dominant positive ion is Se_5^+, while the dominant negative ion is Se_3^-.

discussed in detail by Knochenmuss *et al.*[3] By analogy with their proposed mechanism, this implies that any positive sulfur ions formed adjacent to an analyte molecule are quenched by abstracting an electron from the analyte, thus generating the analyte cation. As predicted by this model, formation of sulfur anions (observed in the negative ion mode) was not affected by high analyte concentrations (data not shown).

Red selenium

Selenium, like sulfur, is a group VI element and resembles sulfur in many of its chemical properties. The metastable red allotrope in particular resembles sulfur in that it exists in the form of 8-membered rings and is soluble in CS_2. Therefore, experiments were done to see if a similar pattern of ions was generated by irradiating Se with 337 nm laser pulses. As shown in Fig. 7, the predominant ions formed were Se_5^+ (393.5 Da) and Se_3^- (238 Da). This indicates that Se_8 photodissociates similarly to sulfur in response to 337 nm pulses (cf. Fig. 1). The prominent Na^+ and K^+ peaks in the positive ion spectrum are probably due to impurities incorporated into the selenium during its precipitation from an aqueous SeO_2 solution by reduction with sodium sulfite. The major isotopes of Se are ^{80}Se and ^{78}Se, but significant amounts of ^{76}Se, ^{77}Se and ^{82}Se also occur, resulting in greater complexity of the ion peaks than is the case with sulfur. Desorption and ionization of selenium required higher laser power than did sulfur, and a few experiments with selenium showed it to be considerably less effective than sulfur as a matrix for bacterial pigments.

CONCLUSIONS

Elemental sulfur was found to be useful as a matrix for molecular weight determinations of porphyrins, carotenoids, and fullerene compounds. Because sulfur is an inorganic compound lacking protons, it is most likely that ionization occurs by electron transfer. The initial ionization steps involve dissociation of sulfur molecules, primarily into S_5^+ and S_3^-. Possible mechanisms for sulfur ion formation include direct photodissociation and initial electron transfer between S_8 molecules to generate unstable ions. The red allotrope of selenium yields a similar pattern of ions.

Experiments on different homologous bacteriochlorophylls showed that those that are esters of allylic alcohols fragment at the ester linkage to yield a bacteriochlorophyllide cation. This fragmentation is partially suppressed by the sulfur matrix, a fact that makes sulfur useful for determining pigment compositions in photosynthetic complexes such as green bacterial chlorosomes. BChl *c* stearyl ester, in which the ester-linked alcohol is a fully saturated hydrocarbon, is considerably more stable, as are the demetallated pigments (pheophytins).

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