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Is the incorporation of analytes into matrix crystals a prerequisite for matrix-assisted laser desorption/ionization mass spectrometry? A study of five positional isomers of dihydroxybenzoic acid

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Abstract

The 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-positional isomers of dihydroxybenzoic (DHB) acid were investigated with respect to their function as matrix-assisted laser desorption/ionization (MALDI) matrices. Optical absorption spectra of solid matrix samples, recorded in diffuse reflection from samples revealed peak broadening and a red shift of the peaks relative to the solution spectra. Single crystals of all isomers were grown from solution and analyzed by x-ray crystallography. Single crystals, as well as standard dried droplet and thin layer preparations with added cytochrome *c*, were analyzed by UV- (266, 308, 337, 355 nm) and IR- (2.94 μm) MALDI time-of-flight mass spectrometry (MALDI-TOF-MS). A spectrophotometric measurement of the heme absorption around 400 nm of redissolved single crystals showed a quantitative incorporation of the protein into crystals of 2,5-DHB and a partial protein incorporation with large statistical fluctuations into single crystals of 2,4-DHB. No protein incorporation above the detection limit of 10^{-5} molar analyte-to-matrix ratio was found for the other isomers. Best MALDI spectra from standard preparations were recorded for dried droplet preparations of 2,5-DHB at 337 and 355 nm and for 2,4-DHB at 266 and 308 nm. Both matrices performed well in the IR, too. 2,6-DHB yielded spectra of comparable quality at 337 nm and 2.94 μm, but only when prepared in a thin layer from an acetone solution. The results suggest that protein incorporation into the crystals of solid MALDI matrices is helpful, but not a prerequisite for MALDI. A large surface-to-volume ratio, typical for microcrystalline thin layer preparations supports protein desorption if no measurable incorporation occurs. Undesirable matrix adduct formation to the protein ions was seen for all DHB isomers at the wavelengths of 308 and 266 nm. (Int J Mass Spectrom 185/186/187 (1999) 859–870) © 1999 Elsevier Science B.V.

Keywords: MALDI-MS; Matrix effects; Ion formation

1. Introduction

The proper choice of matrix and sample preparation are the main criteria for success in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Despite intense research and the testing of several hundred potentially promising compounds, no general scheme has evolved so far that would allow an *a priori* prediction of the performance of a matrix for a given analytical problem. The only obvious but rather trivial requirement is a minimum absorptivity at the laser wavelength. Of further matrix functions, analyte ionization has been looked at most intensely. Several models such as photoionization of the matrix [1] as the primary step as well as proton

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Dedicated to Professor Michael T. Bowers on the occasion of his 60th birthday.

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transfer from electronically excited matrix molecules have been suggested [2–6], but no coherent model has evolved so far that could explain the bulk of experimental evidence and would allow a prediction of matrix functionality. At least for solid matrices, the localization of the analyte in the matrix crystals has been suspected to be one of the key factors in the MALDI process, decisive for success or failure. In an earlier publication it was shown by Strupat et al. [7] that large single crystals of 2,5-dihydroxybenzoic acid (2,5-DHB) matrix, grown from protein containing solutions, incorporate the protein at a concentration equal to that of the mother liquor up to a saturation concentration of $\sim$(5–10) $\times$ 10^{-5} mol/L. A similar but not quantified incorporation was also demonstrated by Beavis and co-workers [8–10] for sinapinic acid, a member of the cinnamic acid UV matrices, and by Strupat et al. [11] a quantitative incorporation for the IR-matrix succinic acid. X-ray diffractograms did not indicate any measurable change in the matrix crystal structures because of the analyte incorporation for all used concentration [7,8,11]. Dai et al. have investigated analyte incorporation into different standard preparations of 2,5-DHB and sinapinic acid by confocal laser scanning microscopy (CLSM) [12]. As expected from the earlier studies of these two matrices they found a preparation dependent more or less inhomogenous analyte incorporation. Their results must be interpreted with some care because the pH dependence of the FITC fluorescence labels was not taken into account, nor was index matching used for the microscopy. A more detailed discussion of CLSM for the analysis of MALDI samples will be published later. Based on these findings it is generally postulated in the literature that analyte incorporation into the matrix crystal lattice is a prerequisite for a successful MALDI analysis. In response to the results described in this publication, Beavis and co-workers in a very recent publication [13], have described an observation made nine years ago, which they now interpret as suggesting a desorption of very thin protein surface layers, adsorbed at crystal faces of (*trans*)cinnamic acid.

In earlier experiments we had found the 2,5-DHB isomer to perform best among different positional isomers of DHB tested (unpublished result). Krause et al. [14] found that 2,5- and 2,6-DHB, ortho-hydroxy isomers, were good matrices at 337 nm laser wavelength, whereas 3,5-DHB does not work as a matrix. In this article results on the wavelength dependent absorption, the crystal structure, the protein incorporation, and the MALDI performance under different sample preparation procedures are reported for five different positional isomers of DHB using four UV-laser wavelengths in the range of 266–355 nm and one at 2.94 μm in the IR.

2. Experimental

2.1. Materials

2,4-DHB and 2,5-DHB were obtained from Fluka, Buchs, Switzerland, 2,6-DHB from Janssen Chimica, Geel, Belgium, 3,4-DHB was purchased from Aldrich, Gillingham, Great Britain, and 3,5-DHB from Merck, Frankfurt, Germany. All matrix compounds were purified twice by activated carbon followed by recrystallization from an aqueous solution. A mixture of water and ethanol (9:1, v/v) was used as a solvent for all experiments except for the thin layer preparations. Suitable aliquots of horse heart cytochrome *c* (Fluka, Buchs, Switzerland, 12360 Da), dissolved in water to a concentration of 10^{-5} mol/L, were added to the preparations as described further down.

2.2. Crystal growth

Crystals of the five positional isomers were grown from solution by controlled cooling of the mother liquor from 36 to 4 °C over a seven day period. Details of the process are described elsewhere [15]. In short, 2,4-, 2,6-, and 3,4-DHB crystals were grown from saturated solutions of 15, 12, and 22 g/L concentrations, respectively. The concentrations of 50 g/L for 2,5-DHB and 100 g/L for 3,5-DHB were somewhat below saturation at the start temperature. For the incorporation studies cytochrome *c* (cc) was added to the matrix solution at a molar analyte-to-matrix ratio ranging from 1.2 $\times$ 10^{-5} to 1.5 $\times$ 10^{-4}.

2.3. Crystallography

The crystal data of 2,4- and 3,4-DHB were determined by x-ray crystallography with a Siemens diffractometer P3 with a 0.71073 Å resolution in collaboration with the Institute of Inorganic Chemistry (Professor Bernt Krebs) at the University of Münster. The crystallographic data of the 2,5-DHB [16] and 2,6-DHB [17,18] were taken from the literature (Chemical Abstract Services and Cambridge Structural Database [19]). X-ray crystallography was also used to determine which of the three structures for 2,6-DHB, described in the literature, are formed under the growth conditions used in these studies. Crystal structures were visualized with the program DIAMOND [20].

2.4. Spectrophotometry

As described previously, a Cary 15 (Varian Subsidiary, CA, USA) spectrophotometer was used to determine the amount of protein incorporated into matrix single crystals [7]. In short, a calibration curve was recorded for the heme absorption of the cc at the Soret band (400 nm) for known protein concentrations in the (acidic) matrix solution. Crystals, grown from a cytochrome *c* containing mother liquor, were carefully washed, dried, weighed, and redissolved in a known volume of solvent. The average cc content in the crystals was determined from the absorption of these solutions. A solution of the respective matrix of equal concentration but without protein was placed into the reference beam of the spectrophotometer for all measurements. This method allows to quantitate cytochrome *c* incorporation into single crystals down to a molar analyte-to-matrix ratio of 10^{-5}. A Beckman UV 5270 spectrophotometer (Beckman Instruments Inc., USA) equipped with a two port integrating spheres was used for the diffuse reflection measurements. For these measurements a suitable amount of sample powder was mixed with an aqueous slurry of $BaSO_4$ and dried as a thick layer onto a microscopic slide.

2.5. Analysis of single crystals by UV- and IR-MALDI-MS

Before the MALDI analysis all crystals of suitable size were first checked for homogeneity of crystal appearance by polarization light microscopy. The single crystals were then mounted onto a stainless steel sample holder with electrically conducting, double-sided adhesive tape. Only crystals oriented with a large enough face parallel to the target surface were investigated for any given matrix. MALDI spectra of single crystals were recorded at a laser repetition rate, slow enough to avoid sample charging.

2.6. Standard preparation (dried droplet)

For the dried droplet (dd) preparations, the respective matrices were dissolved to a concentration of 10 g/L (6.5×10^{-2} mol/L). 2.0–2.5 μL of the matrix solution and 0.5 μL of an aqueous 10^{-5} mol/L protein solution were mixed on the MALDI target and dried in a stream of cold air to result in a final analyte-to-matrix ratio of $\sim 4 \times 10^{-5}$.

2.7. Standard preparation (thin layer)

For thin layer (thl) preparations, matrices were dissolved in ultrapure acetone (UVASOL, Merck, Darmstadt, Germany) at a concentration of 100 g/L (6.5×10^{-1} mol/L). $\sim$10 μL of the solution were spotted onto the stainless steel target. After drying, 0.5 μL of the 10^{-5} mol/L aqueous protein solution were dispensed onto the dried matrix layer [21]. Depending on the water solubility of the chosen matrix, the thin matrix layer redissolved to some extent. In these cases the resulting morphology resembles a dd preparation near the rim and has a more homogeneous center. Water solubility increases in the sequence 2,6-, 2,4-, 3,4-, 2,5-, and 3,5-DHB.

2.8. Time-of-flight MALDI mass spectrometer

Two in-house built time-of-flight MALDI systems, equipped with different lasers, were used for the experiments. Both instruments are relatively similar

in construction and performance [22,23]. Only linear mode was used in this investigation. Ions are accelerated to 5 or 20 keV (depending on the instrument used) in the ion source and postaccelerated to a total ion energy of 20–35 keV onto a conversion dynode in front of the detector. Electrons and secondary ions created at this dynode are detected by a secondary electron multiplier (EMI 9643/4A, Electron Tube Ltd., Great Britain). The detector signals are preamplified and digitized by a transient recorder (LeCroy 9450, LeCroy, Chestnut Ridge, NY, USA). At the laser wavelengths 337 nm (N_2 laser, $\tau = 3$ ns, LSI Inc., Franklin, USA) and 308 nm (XeCl excimer laser, $\tau = 15$ ns, EMG 102MSC, Lambda Physik, Göttingen, Germany) a flat-top laser beam profile on the target with a diameter of 200 μm was used [22]. The laser beams at 266 and 355 nm ($\tau = 15$ ns, frequency quadrupled/tripled Nd:YAG laser, System 2000, JK-Lasers Ltd., Rugby, Warwickshire, Great Britain) and at 2.94 μm ($\tau = 80$ ns, Er:YAG-laser, Spektrum GmbH, Berlin, Germany) had near-Gaussian beam profiles.

3. Results

3.1. Optical absorption

Fig. 1 shows the UV-absorption spectra of the five isomers 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-DHB obtained in solution and in diffuse reflection for solid sample material. No significant differences were observed between spectra obtained from an aqueous versus a water/ethanol solution (9:1, v/v). All spectra of the solid samples exhibit a substantial band broadening and a more or less pronounced redshift, as expected. Plotted are the values of $\log(1/R)$ where R is the intensity of the reflected light from a matrix doped sample normalized to that of the pure $BaSO_4$ preparation. No quantitative data can be extracted from the diffuse reflection spectra. In Fig. 1 they are individually scaled to have a value equal to the decadic molar absorption coefficient of the solution spectra at the long wavelength peak. Of the five isomers, 2,5-DHB is the only one with an absorption at and beyond 355 nm. 2,6-DHB has a substantial absorption at 337 nm; all other isomers have significant absorption at shorter wavelengths only. At 266 nm all positional isomers exhibit a measurable absorption. At the 308 nm their absorption is expected to be comparable, based on the quantitative solution values. The absolute values of the molar absorption coefficients of the DHB isomers in the infrared are not available from the literature and difficult to measure, particularly for morphologically heterogeneous, solid samples. At 2.94 μm the absorption is dominated by that of the hydroxyl groups and should be fairly independent of their position in the isomers. As a rule of thumb the absorption of DHB matrices at 2.94 μm should be smaller than that in the UV at the long wavelength maximum by two to three orders of magnitude [24].

3.2. Crystal structure

The crystallographic structural data of four of the five isomers are summarized in Table 1. Those of 2,5- and 2,6-DHB were obtained from the literature. For 2,6-DHB three different crystal structures depending mostly on the solvent were found. The x-ray crystallography revealed that the *Pnma* structure is formed for the used water/ethanol mother liquor. 2,6-DHB, therefore, is the only isomer of which the crystals form extended hydrophobic planes, similar to sinapinic acid [8]. The data for 2,4- and 3,4-DHB were also recorded as part of this project. (The detailed crystal data have been deposited into the Cambridge Structural Database and are available there. The deposition numbers are 103056 for 2,4-DHB and 103057 for 3,4-DHB. For 3,4-DHB the space group without atomic parameters was published before by Agmon and Herbstein [25].) For 3,5-DHB no crystallographic data are available in the literature, nor could any be obtained experimentally. It is assumed that this compound incorporates large quantities of water upon crystallization in the liquor and loses its structure upon drying when removed from the liquor. No measurable structural changes were observed after addition of protein (molar analyte-to-matrix ratio $\leq 1.5 \times 10^{-4}$) to the mother liquor for any of the isomers. In cases of measurable protein incorporation,

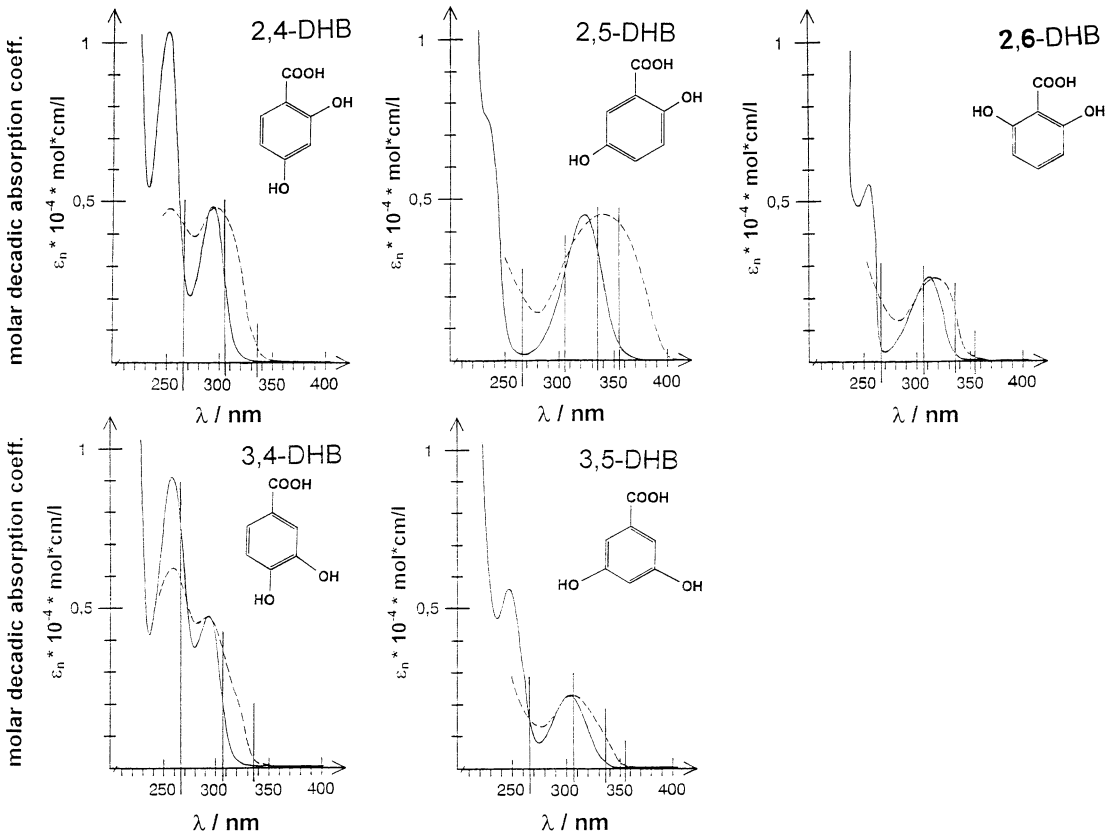

Fig. 1. UV-absorption spectra of five positional isomers of dihydroxybenzoic acid; solid lines: spectra obtained from a water/ethanol solution (9:1, v/v); dashed lines: diffuse reflection spectra of solid samples dispersed in $BaSO_4$. For the solution spectra the values for the molar decadic absorption coefficients are absolute. For the diffuse reflection spectra log $(1/R)$ is plotted (see the text for details), normalized to the value of the absorption coefficient in solution at the long wavelength maximum.

the single crystals had a pink appearance resulting from the heme absorption of the cc. In 2,4-, 2,5-, and 2,6-DHB the ortho-hydroxyl groups form a hydrogen bond with the neighboring carboxyl function as suggested in the literature [14].

Two different habits with equal crystal structure were observed for the 2,4-DHB crystals. Habit 1 formed bunches of long translucent needles of several 10 μm across and up to 1 mm long, originating from a common center. Habit 2 formed flat thin plates of up to 4×5 mm in size. Single needles or plates appeared dark between crossed polarizers of suitable orientation, indicating that they were single crystals. 2,5-DHB crystallized in a single habit forming slabs of typically $0.3 \times 0.5 \times 1.5$ mm in size. Even though the habit was usually not perfect with steps on the faces, they were single crystals with full extinction between suitably oriented crossed polarizers. 2,6-DHB formed long thin needles of 10 μm thickness and several millimeters in length. Polarization microscopy showed that they were single crystals. 3,4-DHB formed orthorhombic crystals of up to a few millimeters in size. At least the smaller ones were typically single crystals. Polycrystals of 3,5-DHB grew in very thin single crystal plates of 0.5×0.5 mm in size which formed stacks of up to 10 mm in height.

3.3. Protein incorporation

Besides 2,5-DHB which had been shown before to incorporate cc quantitatively up to a saturation con-

Table 1
Selected crystal data for single crystals of different DHB isomers grown from a water/ethanol-solution (9:1, v/v)

DHB isomer	Space group	|a| (Å)	|b| (Å)	|c| (Å)	α (deg)	β (deg)	γ (deg)	Number of observed habits	Hydrophobic domains	H_2O incorporation	Ref.
2,4	$P\bar{1}$	7.02 (6)	9.54 (7)	11.19 (9)	96.7 (8)	104.3 (4)	98.8 (5)	2	No	1 per DHB dimer	
2,5	$P2_1/c$	23.945 (2)	4.908 (1)	5.621 (1)	90.0	100.981 (8)	90.0	1	No	No	[16]
2,6[a]	$Pnma$	6.778 (1)	9.411 (1)	11.890 (2)	90.0	90.0	90.0	1	Yes	1 per DHB molecule	[17]
2,6[b]	$Pna2_1$	14.174 (3)	12.132 (2)	3.8280 (10)	90.0	90.0	90.0		No	No	[17]
2,6[b]	$P2_1/c$	5.4084 (5)	5.2240 (7)	22.986 (4)	90.0	94.69 (3)	90.0		No	No	[18]
3,4	$P\bar{1}$	8.04 (5)	8.13 (4)	12.69 (2)	71.5 (8)	76.7 (9)	72.1 (7)	1	No	1 per DHB molecule	

[a] Crystal structure as formed in MALDI preparations.
[b] Crystal structures found in the literature for 2,6-DHB grown from different solvents.

centration of (5–10) $\times$ 10^{-5} molar analyte-to-matrix ratio [7,11], 2,4-DHB was the only one among the other four isomers which incorporated the protein at concentrations above 10^{-5} molar ratio (Fig. 2). However, the incorporation was less quantitative than for 2,5-DHB with large statistical fluctuations from crystal to crystal. Within these fluctuations the incorporation appeared to be equal for both habits of the 2,4-DHB crystals. All other DHB isomers incorporated less than the spectrophotometric detection limit of 10^{-5} molar ratio of protein to matrix (see Table 2).

3.4. MALDI-MS from large single crystals

Crystals of all five isomers were analyzed by UV, as well as IR MALDI. Except for 3,5-DHB, where conglomerates of crystals were investigated, all crystals were checked by polarization microscopy for homogeneity and mounted on stainless steel substrates with the largest side facing the laser beam. No attempts were made to investigate the orientation dependence of the single crystals, because for most of the crystals only one face was large enough to accommodate the full laser beam (diameter 200 μm) and because of mounting problems. UV-MALDI spectra were recorded with two wavelengths, 337 and 308 nm. The 2.94 μm wavelength of the Er:YAG laser was used in the infrared.

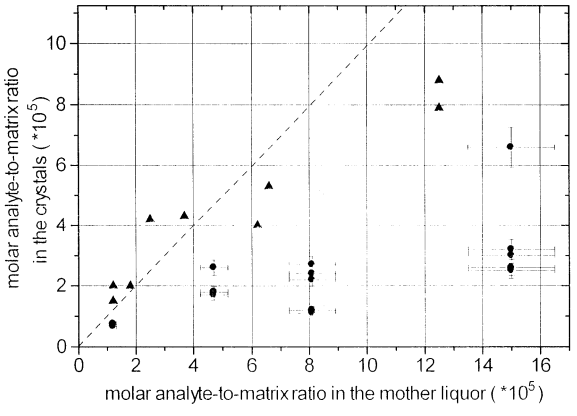

Fig. 2. Incorporation of cytochrome *c* into single crystals of 2,4- (closed circle) and 2,5-DHB (closed triangle) [7]. The dashed line represents an equal analyte-to-matrix ratio in the mother liquor and the crystals, i.e. quantitative incorporation. For details see the text.

The results of the MALDI-MS of single crystals are summarized in Table 2. The classification of the performance is based mainly on the signal intensity of the cc signals and, to a lesser extent, on the signal-to-noise ratio and the intensity of matrix signals in the low mass range, which could saturate the detector and/or signal processing electronics and, thereby, interfere with the MALDI analysis. Peak resolution was difficult to compare, because the spectra were recorded with two different instruments, of which one had only a 5 kV acceleration potential. Intense matrix signals were obtained from crystals of all five isomers. Very good spectra of the cc holoenzyme were recorded with UV-MALDI for 2,5-DHB as reported before [7,11]. A large number of spectra of equal quality could be obtained from one and the same crystal location. At 337 nm very weak signals of the cc apoenzyme were registered occasionally from crystals of 2,4-DHB. Besides strong matrix signals these spectra always showed intense signals of the heme at

Table 2
Protein incorporation into single crystals and MALDI performance of single crystals of dihydroxybenzoic acid positional isomers, grown from protein containing mother solution (+: good protein signals; ○: poor protein signals; −: no protein signals)

	UV-MALDI 308 and 337 nm		IR-MALDI 2.94 μm		
Matrix	1st shot[a]	15th shot[a]	1st shot[a]	15th shot[a]	Incorporation, measured by spectrophotometry[b]
2,4-DHB	+[c]	+[c]	+	+	some $>10^{-5}$
2,5-DHB	+	+	+	+	$>10^{-5}$, quantitative up to $\sim 10^{-4}$
2,6-DHB	−	−	−	−	$<10^{-5}$
3,4-DHB	○	○	○	−	$<10^{-5}$
3,5-DHB[d]	○	○	○	○	$<10^{-5}$

[a] Number of shots onto a given spot.
[b] Molar ratio of analyte-to-matrix molecules in the crystals to that in the mother liquor.
[c] Strong protein signals at 308 nm only, weak signals for 337 nm; for details see the text.
[d] Orientation dependent, for details see the text.

mass 618.5 ± 1 Da, typical for a high laser fluence (results not shown). No cc signals were obtained from the other three isomers at this wavelength. At 308 nm good signals of the cc holoenzyme were detected from single crystals of 2,4-DHB, even for successive exposures of the same location on the crystal. For 3,4-DHB some signal of the cc holoenzyme was recorded from nonwashed crystals. After careful washing of the crystals only a very weak signal remained. 3,5-DHB is a special case. Samples, for which only the major surface of the top crystal plate was exposed gave results similar to 3,4-DHB. cc holoenzyme signals were observed for the first one or two shots only and disappeared after washing. For a perpendicularly mounted crystal stack, for which the laser beam irradiated the edges of many crystal plates (and the space between them), strong cc signals were obtained even for many successive exposures. Signal intensity as well as signal-to-noise ratio was markedly decreased after washing the samples. No cc signal was obtained for 2,6-DHB under any of the tested experimental conditions. The differences between 308 and 337 nm for the 2,4-, 3,4-, and 3,5-DHB isomers can possibly be attributed to the decreased absorption at the longer wavelength and the fact that the N_2 laser at 337 nm had only a limited output energy. It is puzzling, however, that the matrix ion signals in 308 nm spectra with a strong cc signal were substantially lower than those of 337 nm at maximum laser energy and no cc signal. This seems to indicate that at least partially different physical processes are induced by the shorter wavelength and the total energy, deposited into the sample per unit volume, is not the only parameter of importance. It must be kept in mind, however, that the absorption of these crystals is expected to be nonisotropic and only one orientation was looked at.

By IR-MALDI strong signals of the cc holoenzyme were obtained from 2,4- and 2,5-DHB. Depending on the thickness of the crystals, up to 30 spectra of comparable quality could be obtained from one and the same spot for both matrices. This limited number of successful desorption events reflects the larger amount of material desorbed by the IR-laser beam, exceeding that by the UV wavelengths by 2–3 orders of magnitude. Only very weak cc signals were recorded and only for the first one or two exposures of a given location on a 3,4-DHB single crystal. These signals disappeared if the crystals were thoroughly washed before mounting. The results with 2.94 μm for 3,5- and 2,6-DHB essentially resemble those obtained at 308 nm.

3.5. MALDI-MS from standard preparations

For practical applications of MALDI-MS the growth of single crystals is rarely a viable option. The performance of different standard preparations of the five DHB positional isomers has, therefore, been investigated for the influence of the sample morphology of dd and thl preparations and of the laser

Table 3
Protein signal detection from MALDI standard preparations of DHB positional isomers at different wavelengths (+: good protein signals; ○: weak protein signals; −: no protein signals)

Isomer	Preparation	266 nm	308 nm	337 nm	355 nm	2.94 μm
2,4	dd[a]	+	+	−	−	+
	thl[b]	○	○	−	−	ni[c]
2,5	dd	+	+	+	+	+
	thl	○	○	○	○	ni
2,6	dd	ni	−	−	ni	−
	thl	ni	+	+	ni	○
3,4	dd	○	○	−	−	○
	thl	○	○	−	−	ni
3,5	dd	○	○	−	−	○
	thl	○	○	−	−	ni

[a] dd: dried droplet preparation.
[b] thl: thin layer preparation.
[c] ni: not investigated.

wavelength. The main results are summarized in Table 3. All spectra were recorded at or near the respective threshold fluences, which vary by more than a factor of 10 depending on the isomer, the wavelength and the preparation. The least differences in MALDI performance were observed at 308 nm where all isomers exhibit a comparable absorption [Figs. 3(a)–(e)]. A more or less pronounced peak broadening and corresponding loss in mass resolution of the cc signals is observed for all isomers at the wavelengths of 308 and 266 nm (results not shown). Spectra obtained with delayed ion extraction at 308 nm revealed that this is caused by single or multiple adduct formation of matrix fragments (M–OH) of mass 137 Da. Adduct formation is most pronounced for 3,4-DHB and least pronounced for 2,5-DHB as shown in the insets to Fig. 3(d) and (b), respectively. This adduct formation is indicative of matrix photochemistry, induced by the shorter wavelengths, but not at 337, 355 nm, or 2.94 μm and is in agreement with the observation of similar adduct formation for nicotinic acid at 266 nm. Only 2,5-DHB exhibits a strong absorption beyond ~330 nm and accordingly it is the only DHB isomer performing optimally at 337 and 355 nm. At 2.94 μm the absorption of the isomers is dominated by the hydroxyl groups and is expected to be relatively independent of their position of these groups on the aromatic ring.

2,4-DHB performs best at 266 and 308 nm as expected, with a quality of the spectra almost equaling that of the 2,5-DHB isomer at these wavelengths; at 337 and 355 nm spectra could be obtained albeit of inferior quality. Comparable spectra were obtained for dd and thl preparations. The morphology of dd preparations depends on the solvent as well as on the protein addition. For no or low ($<1.5 \times 10^{-5}$ molar ratio) cc addition the sample crystallizes into a meshwork of long, thin needles of <20 μm lateral extension covering the whole sample area. At higher cc concentrations a rim of larger crystals of typically 100 μm in size is formed with a needle meshwork in the center. The transition concentration from a pure needle to a rim/needle preparation appears to depend on the type and particularly the mass of the analyte, increasing with decreasing mass. cc spectra of comparable intensity could be obtained from the needles as well as from the larger crystals, despite of a approximately ten times smaller cc concentration used for the needle preparation. Spectra of equal quality could also be recorded from the more homogeneous microcrystalline thl preparations.

2,5-DHB is the only isomer performing nearly equally well at all four wavelengths with a slight preference for the two longer ones. For dd preparations best spectra are obtained from the larger crystals at the rim of the preparation as described in the literature. thl preparations give comparable results including spectra obtained from the microcrystalline center of the partially recrystallized sample.

2,6-DHB forms just a few isolated single crystals of ~200 μm across and several millimeters in length. Often these crystals are removed from the target mechanically by a single laser exposure. cc signals are very rarely obtained from such exposures, independent of the UV wavelength. When prepared as a thl, 2,6-DHB gives excellent and reproducible spectra at 337 nm, comparable in quality to spectra obtained from 2,5-DHB dd samples (Fig. 4). At 308 nm strong signals are obtained, but mass resolution is rather poor because of the adduct formation discussed above [Fig.

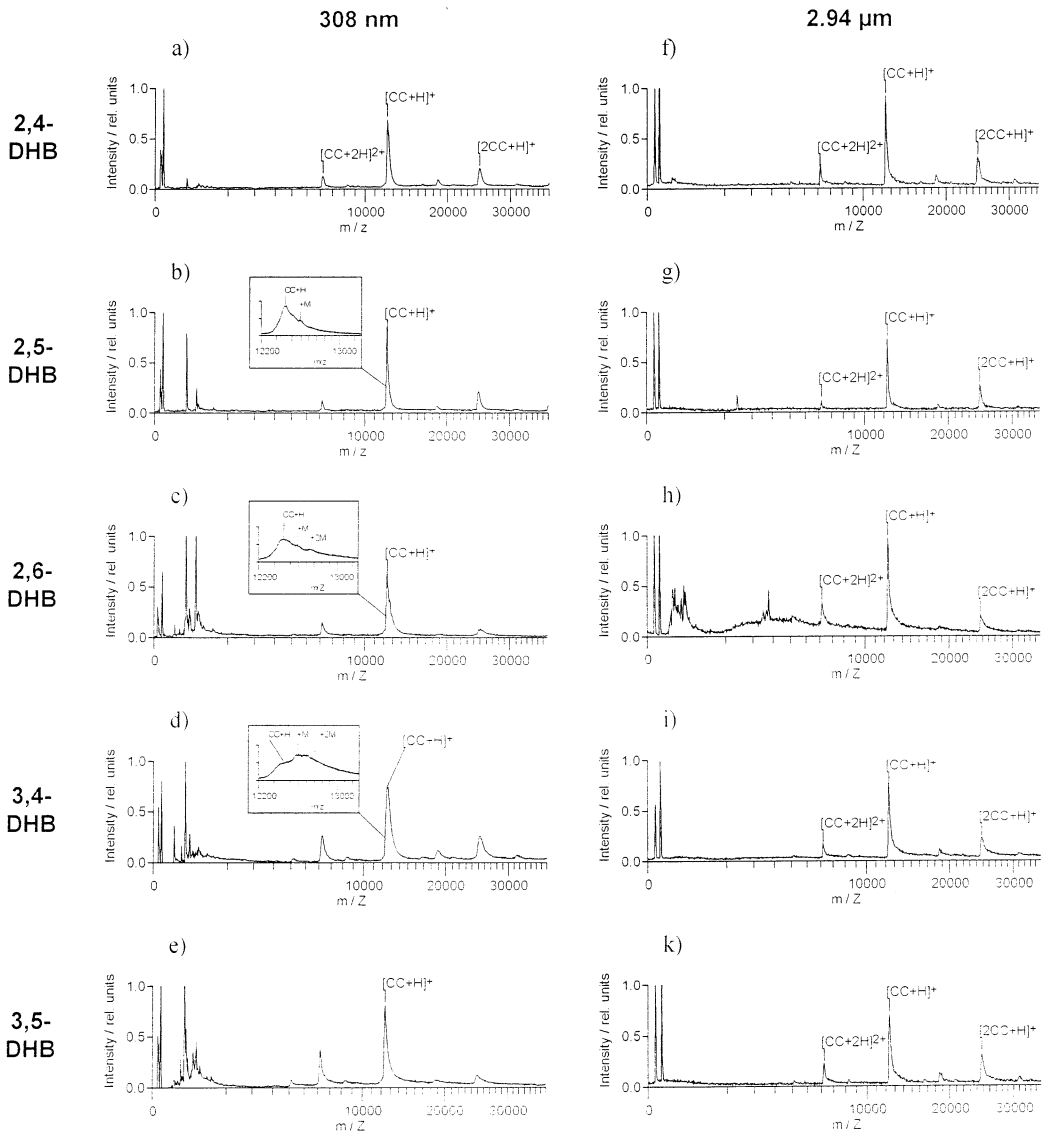

Fig. 3. Positive ion MALDI mass spectra of five DHB positional isomers recorded at wavelengths of 308 nm in the UV [(a)–(e)] and 2.94 μm in the IR [(f)–(k)]. 2.5 μL of 6.5 $\times$ 10^{-2} M matrix solutions were mixed with 0.5 μL of a 10^{-5} M solution of cytochrome *c* for the dried droplet preparation. 0.5 μL of a 10^{-5} M protein solution was spotted onto a thin matrix layer prepared from 10 μL of a 6.5 $\times$ 10^{-1} M matrix solution in acetone for the thin layer; Delayed ion extraction with 18–20 kV acceleration voltage. Sum of 15 single spectra each; "M" denotes the 137 Da fragment of the matrices. (a), (f) 2,4-DHB, dried droplet preparation; (b), (g) 2,5-DHB, dried droplet preparation; (c), (h) 2,6-DHB, thin layer preparation; (d), (i) 3,4-DHB, dried droplet preparation; (e), (k) 3,5-DHB, dried droplet preparation.

3(c)]. 2,6-DHB is the least water soluble among the five isomers and is, therefore, best suited for the thl preparation. The wavelengths of 266 and 355 nm were not tested for this matrix.

As expected from its absorption spectrum, 3,4-DHB performs well only at the shorter wavelengths of 266 and 308 nm. thl preparations are slightly superior to dd ones, which look very similar to the 2,5-DHB dd preparations. For dd preparations best results are obtained from positions at the outermost rim of the

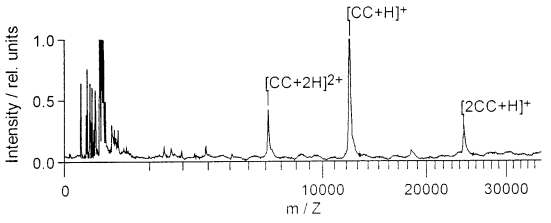

Fig. 4. Positive ion UV-MALDI mass spectrum of cytochrome *c* from 2,6-DHB matrix at $\lambda = 337$ nm; thin layer preparation as described in Fig. 3; 5 kV acceleration voltage; sum of ten single spectra.

sample with spectral quality decreasing the closer the desorption site was to the sample center. For thl preparations best results are obtained from the transition zone at the inner edge of the crystalline rim to the polycrystalline center region. The spectra quality is quite inferior to that of 2,5-DHB.

No protein signals were obtained from 3,5-DHB preparations at 337 and 355 nm wavelength. Only spectra of poor quality could be obtained at 266 and 308 nm, independent of the preparation and the location of the desorption site on a given sample. Similar to the observations with single crystals the IR-MALDI spectra of the different isomers at 2.94 μm wavelength resemble each other closely, as shown in Fig. 3(f)–(k). The dried droplet preparation worked well for all but the 2,6-isomer which had to be prepared as a thin layer. For dd preparations, the signal intensity depends substantially on the desorption site with best signals obtained from the rim of the preparations. For the 2,6-DHB thl samples, spectra could reproducibly be obtained from all sites and with best spectra quality typically for the third to tenth exposure of a given site, after which all material was removed from this site.

4. Discussion

The project was started with the assumption that incorporation of analytes into the matrix, especially into the crystals of solid state matrices, is a necessary prerequisite for successful UV-MALDI-MS and with the expectation that there would be some sort of correlation between the crystal structure of a compound and its ability to incorporate analytes such as proteins up to a molar ratio of 10^{-5}–10^{-4}. The collected experimental evidence does not seem to support either of the two. On the contrary, the results strongly suggest that analyte incorporation may be helpful, but is not a general prerequisite for the generation of large analyte ions. For 2,6-, 3,4-, and 3,5-DHB incorporation was found to be below 10^{-5} molar analyte-to-matrix ratio if any incorporation occurs at all. In UV-MALDI with these matrix isomers analyte ions appear to derive from molecules in close proximity, most probably contact to matrix molecules, e.g. by adsorption at matrix surfaces. In IR-MALDI not even this direct contact might be necessary. The larger the surface-to-volume ratio of the matrix preparation, the higher the chance for an analyte molecule to be in contact with such a surface. 2,6-DHB is the best example for this principle. When grown slowly from solution, large prismatic single crystals are formed with a low surface-to-volume ratio. No spectra could be obtained from such crystals, grown out of a protein containing solution, under any of the conditions tested. Even for dried droplet preparations with their long needles, protein signals were recorded only very rarely. If, however, a thin layer preparation was made by fast evaporation of the solvent, consisting of a large number of submicrometer size crystals with a very large surface-to-volume ratio, excellent spectra could be obtained. Protein spectra out of 3,4- and 3,5-DHB crystals have a much better quality when recorded by IR-MALDI, for which immediate contact does not seem to be so critical, presumably because much more material is desorbed and a better mixing takes place in the expanding plume. For 3,4-DHB the location of the protein in a dd sample preparation was checked by energy dispersive x-ray microanalysis (EDX) in a scanning electron microscope, looking at the S K_α line of the two cysteines and two methionines in the cc. Signals were recorded exclusively from the outer rim of the preparation and from interfaces and clefts of the single crystals. The fact, that protein spectra could be obtained for many successive shots onto the same location from stacks of 3,5-DHB crystal plates at 308 nm and 2.94 μm when the plate interfaces were

oriented perpendicular to the substrate surface, but not for a parallel orientation, also supports the assumption that the proteins are adsorbed to the crystal surfaces rather than incorporated. Additional support comes from the observation, that very small, if any, signal was observed for 3,4- and 3,5-DHB after careful washing. Results of the UV-MALDI-MS of standard preparations also support this model. Except for 2,4- and 2,5-DHB, for which at least some incorporation has been shown, all other isomers perform somewhat better if prepared in a microcrystalline thin layer than in dried droplet preparations.

The results of this project do not seem to suggest any model for the mechanism of protein incorporation in the matrix crystals where it is measurable (sinapinic acid [8–10], 2,4-DHB, this work, 2,5-DHB [7], and succinic acid [11]). All five isomers present distinctly different habits. One would have expected the incorporating isomers 2,4- and 2,5-DHB to be closest in crystal structure, but it is 2,4- and 3,4-DHB which have the most similar structure, both belonging to the point group $P\bar{1}$. Yet 2,4-DHB incorporates protein at least partially and 3,4-DHB does not. 2,6-DHB is the only DHB isomer which exhibits large hydrophobic planes in its crystals. In analogy to the protein incorporation into sinapinic acid, reported to be related to such hydrophobic planes [8], protein incorporation could have been expected, but was not observed.

5. Conclusions

In conclusion, the experiments have revealed that all of the five isomers are functional matrices for MALDI-MS with UV and IR wavelengths. Under the conditions tested, 2,4-, 2,5-, and 2,6-DHB, all of which have an ortho-hydroxyl group, give somewhat better quality spectra if suitable wavelength and preparation methods are applied as compared to the 3,4- and 3,5-DHB matrices which is in good agreement with the literature [14]. Matrix adduct formation appears to be a general problem for the shorter UV wavelengths of 266 and 308 nm. The use of UV matrices with strong absorption and optimal performance at 337 or 355 nm, should therefore be given preference for practical applications. The results presented in this article strongly support the notion that a successful UV-MALDI analysis requires an intimate contact between analyte and matrix molecules in the sample. For the 2,5-DHB and partially for the 2,4-DHB positional isomers, this is achieved by protein incorporation into the matrix crystals. For the other three isomers, adsorption at matrix surfaces appears to be at least the dominating mechanism. The larger the surface-to-volume ratio of the matrix crystals in the preparation, the better the result for a given non- or poor-incorporating matrix. The recently reported MALDI analysis of noncovalently bound complexes [26] supports this concept. The observed “first shot” phenomenon in these experiments was also interpreted as a precipitation of the complexes onto the crystal faces of the microcrystalline preparations during the last phase of solvent evaporation. The relevance of the results on the protein incorporation into large single crystals must be interpreted with some care. In standard sample preparations the solvent is removed in seconds to minutes. Incorporation may be quite different under such conditions as compared to the very slow growth of crystals from a saturated mother liquor. In a further set of experiments, confocal laser scanning microscopy will be used to investigate the protein incorporation into matrix crystals under standard preparation conditions to resolve this uncertainty. In IR-MALDI the larger amount of desorbed material and so a better mixing in the plume seems to relax the requirement for the immediate contact between analytes and matrix molecules. This may be one reason why IR-MALDI of proteins, electroblotted onto polymer membranes, works so much better than UV-MALDI.

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