Eur Biophys J (2007) 36:517–527
DOI 10.1007/s00249-006-0090-6

ARTICLE

The suitability of different DHB isomers as matrices for the MALDI-TOF MS analysis of phospholipids: which isomer for what purpose?

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Received: 30 April 2006 / Revised: 28 July 2006 / Accepted: 25 August 2006 / Published online: 20 September 2006

Abstract Although the analysis of large biomolecules is the prime application of matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS), there is also increasing interest in lipid analysis. Since lipids possess relatively small molecular weights, matrix signals should be as small as possible to avoid overlap with lipid peaks. Although 2,5-dihydroxybenzoic acid (DHB) is an established MALDI matrix, the question whether just this isomer is ideal for lipid analysis was not yet addressed. UV absorptions of all six DHB isomers were determined and their laser desorption spectra recorded. In addition, all isomers were used as matrices to record positive and negative ion mass spectra of selected phospholipids (phosphatidylcholine and -serine): In the order 2,5-, 2,6-, 2,3- and 2,4-DHB, the quality of the positive ion lipid spectra decreases. This correlates well with the decreasing acidity of the applied DHB isomers. The 3,4- and 3,5- isomers give only very weak positive ion signals especially of acidic lipids. In contrast, the most suitable matrices in the negative ion mode are 2,5-, 2,4- and 3,5-DHB. 2,6-DHB does not provide any signal in the negative ion mode due to its marked acidity. Finally, differences in the crystallization behavior of the pure matrix and the matrix/lipid co-crystals were also monitored by atomic force microscopy (AFM): 2,5-DHB gave the smallest crystals and the skinniest layer. It is concluded that basically all DHB isomers can be used as MALDI matrices but the 2,5-isomer represents the most versatile compound.

Keywords MALDI-TOF MS · Phospholipids · DHB isomers · UV spectra · pK_a values · AFM

Dedicated to Prof. Dr. Klaus Arnold on the occasion of his 65th birthday.

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Introduction

The analysis of biomacromolecules (e.g. proteins or polysaccharides) and/or less volatile compounds as lipids by mass spectrometry (MS) was impossible over many decades (Schiller and Arnold 2000a). Nowadays, however, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization (ESI) MS are techniques that help to overcome these problems. Both techniques are so called ''soft-ionization'' methods, i.e. they induce only a very small extent of analyte fragmentation (Cristoni and Bernardi 2003).

The prime application of MALDI-TOF MS is protein analysis (''proteomics'') (Reinders et al. 2004), but it was also successfully used for carbohydrate (Harvey 1999) and DNA analysis (Gut 2004) as well as the characterization of synthetic polymers (Wu and Odom 1998). The special advantages of MALDI-TOF MS are the fast and convenient performance as well as its robustness to impurities, for instance, salts, detergents, etc. This tolerance depends, however, on the type of the used matrix (Karas et al. 1993).

Finding the most appropriate matrix for a certain analytical problem is still a ''try and error'' process because the mechanism of ion generation is so far only

barely understood (Knochenmuss and Zenobi 2003). There are several important properties by which a MALDI matrix must be characterized: (a) the matrix should have a strong absorbance at the wavelength with which the laser irradiates the sample, (b) matrix and analyte should be soluble in the same solvent to provide an even co-crystallization between the matrix and the analyte in order to enhance the reproducibility of spectra and (c) the matrix must have sufficient acidic/basic properties to enable the generation of quasi molecular ions from the analyte.

Since a few years, MALDI-TOF MS is also established in lipid and phospholipid (PL) analysis (Schiller et al. 1999, 2004), although some problems related to quantitative analysis and potential ''lipidomics'' studies still have to be solved (Schiller et al. 2006). Lipids (molecular weight about 500–1,500 Da) have a much lower molecular weight than proteins. Therefore, signals derived from the applied (low molecular weight) matrix may strongly interfere with the signals of the lipids of interest. Accordingly, it is an additional prerequisite of lipid analysis by MALDI-TOF MS that the yield of matrix signals is as low as possible.

There were so far no systematical reports on the ideal matrix for lipid analysis by MALDI-TOF MS. Although many different matrices (Fig. 1), including 4-nitroaniline (Estrada and Yappert 2004), sinapinic acid or α-cyano-hydroxy-cinnamic acid (Harvey 1995), 5-ethyl-2-mercaptothiazole (Raju et al. 2001), 6,7-dihydroxy-coumarine (Harvey 1995) or the recently developed ionic liquid matrices (Li et al. 2005) can be used, in the majority of studies of lipids, dihydroxybenzoic (DHB) acid was applied. The 2,5-isomer seems most useful (Marto et al. 1995; Schiller et al. 2004) and there is only a single paper describing the application of the 3,5-isomer (Tanaka et al. 2004). All six isomers of DHB together with the corresponding pK_a values are shown in the lower part of Fig. 1.

In a few cases, the free 2,5-dihydroxybenzoic acid was also replaced by the corresponding lithium salt (Cvacka and Svatos 2003). There were also attempts to further improve the matrix properties of 2,5-DHB, e.g. by the addition of 2-hydroxy-5-methoxy-benzoic acid (Tsarbopoulos et al. 1994). It was suggested that the crystal lattice of 2,5-DHB is disordered by such additives leading to a softer ionization process and, accordingly, a lower yield of fragmentation products (Karas et al. 1993).

The influence of the sample preparation (dried droplet and thin-layer preparation) on the quality of mass spectra of proteins recorded with different DHB isomers was elucidated in a comprehensive study by the Hillenkamp group (Horneffer et al. 1999). In this study, the authors have used a variety of different methods including X-ray crystallography to monitor the inclusion of the analyte into the matrix. The most important result was that the direct entrapment of the analyte into the matrix crystal is not an absolute necessity but the interactions along the matrix/analyte borderline are sufficient to obtain reasonable spectra. The second result was that among the five different DHB isomers under investigation, only 2,5-DHB

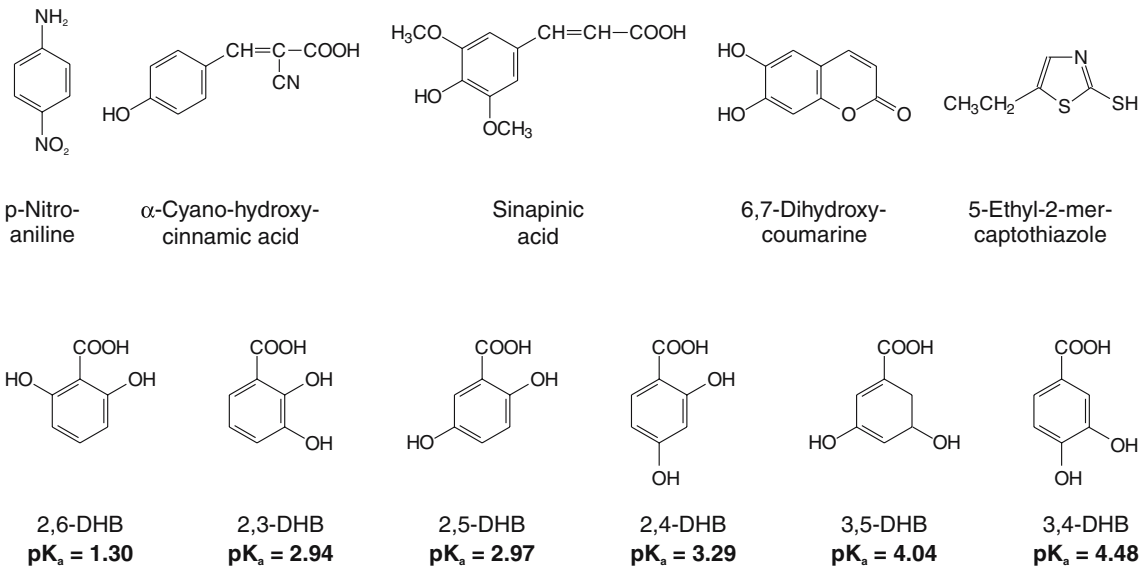

Fig. 1 So far used matrix compounds in lipid and phospholipid analysis by MALDI-TOF MS (*upper row*). The *bottom row* shows all isomers of dihydroxybenzoic acid (DHB) used in this study in combination with the corresponding pK_a-values determined in water (d' Ans et al. 1992). For details see text

represents a suitable matrix for proteins at a laser excitation wavelength of 337 nm and by using the dried droplet preparation technique.

These results are not necessarily valid for lipids because (a) lipids have much smaller molecular weights than proteins as well as different interactions with the matrix and (b) all lipid preparation steps are performed in non-aqueous solvents. Additionally, the different acidities of the individual DHB isomers were not considered.

In the present paper, the suitability of all DHB isomers (2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dihydroxybenzoic acid) (Fig. 1) as matrices for lipid analysis by MALDI-TOF MS was investigated. It will be shown that in contrast to proteins and depending on the application, 2,5-DHB may be replaced by the other isomers: For the positive ion spectra, the 2,5-, 2,6- and 2,3-DHB isomers represent the matrices of choice, whereas 2,5-, 2,4- and 3,5-DHB are the best matrices in the negative ion mode. These differences are particularly caused by differences in the pK_a values of the individual DHB isomers, and also by a different crystallization behavior of the matrix with the analyte. It will be emphasized that the assumption that at least one ortho group must be present in a compound to allow its application as a MALDI matrix (Krause et al. 1996) could not be confirmed in our study.

Materials and methods

Chemicals

All DHB isomers as well as trifluoroacetic acid (TFA) were obtained in highest commercially available purity from Fluka Feinchemikalien GmbH (subcompany of Sigma-Aldrich Chemie GmbH, Taufkirchen). They were used as supplied. The same holds for the applied solvents (chloroform and methanol) and salts (NaCl).

Dipalmitoyl-*sn*-phosphatidylcholine (PC 16:0/16:0 (DPPC)) and 1-palmitoyl-2-oleoyl-*sn*-phosphatidylserine (PS 16:0/18:1 (POPS)) were purchased from AVANTI Polar Lipids Inc. (Alabaster, Alabama, USA) as 10 mg/ml solutions in $CHCl_3$ and further diluted by the addition of appropriate amounts of $CHCl_3$.

Matrix preparation

0.5 mol/l (77.1 mg/ml) solutions of the DHB isomers were prepared in pure methanol under vortexing. In order to increase the ion concentration, all solutions were saturated with solid NaCl. Matrix solutions were prepared freshly each day.

MALDI-TOF mass spectrometry

Aliquots of individual DHB solutions were directly brought onto commercially available gold-coated MALDI targets. For experiments with phospholipids, a 20 µl aliquot of the corresponding PL mixture (1 mg/ml DPPC or 2.5 mg/ml POPS in $CHCl_3$) was mixed with 20 µl of the corresponding matrix solution and subsequently directly deposited onto the MALDI target. In order to improve the crystallization, the solvent was rapidly removed under a warm stream of air (Schiller et al. 1999).

All MALDI-TOF mass spectra were acquired on a BRUKER Autoflex™ workstation (Bruker Daltonics, Bremen, Germany). The system utilizes a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage was 20 kV in all cases. Hundred and twenty-eight single laser shots were averaged for each mass spectrum. The applied laser strength was varied according to the absorption properties of the matrix in order to obtain comparable signal-to-noise ratios (discussed below in more detail). To achieve enhanced resolution, all spectra were acquired in the reflector mode under ''delayed extraction'' conditions. A more detailed methodological description is given in Schiller and Arnold (2000a).

UV spectroscopy

All UV measurements were performed on a UV-VIS Spektrophotometer Cary 50 (Varian, Darmstadt) in quartz cuvettes. All measurements were performed with 20 mg/l (129.9×10^{-6} mol/l) solutions of the individual DHB isomers in methanol. This concentration was used because it provided reasonable UV absorption in all cases.

AFM studies

Atomic force microscopy (AFM) was used to image the crystallization behavior of the individual DHB isomers and to monitor differences in the presence of PL. Solutions of the different DHB isomers in methanol or the corresponding DHB/PL mixtures were deposited onto a mica surface and allowed to dry at room temperature. Mica was used in order to warrant a clean surface for the individual samples. All AFM studies were performed on an Asylum Research MFP-3D atomic force microscope. All DHB and DHB/PL surfaces were characterized in the contact mode. The used tips were Olympus TR400PSA with a spring constant in the range of 0.01–0.08 N/m (Binnig et al. 1986).

Results and discussion

Comparison of UV spectral properties of the individual DHB isomers

As mentioned above, one of the prerequisites for an organic compound to be a useful MALDI matrix is a sufficiently high absorbance at the laser wavelength (Schiller and Arnold 2000a). Since nitrogen lasers emitting at 337 nm are most frequently installed in commercially available MALDI-TOF mass spectrometers, the absorbance of the matrix at 337 nm is most important. In Fig. 2, the absorption spectra of 129.9 μM solutions of the individual DHB isomers in methanol are shown: Obviously, 2,5-DHB provides the strongest absorption at 337 nm, followed by the 2,3-, the 2,6- and the 3,5-isomers. In contrast, neither the 2,4- nor the 3,4-isomer show a significant absorption at 337 nm, but are characterized by an intense absorption at smaller wavelengths. Although it was reported (Horneffer et al. 1999) that in the solid state a slight shift to smaller wavelengths occurs, the absorbance at 337 nm (in solution) is a crude measure of the suitability of a given compound as matrix.

Its strong absorption at 337 nm is most probably the reason why—with a very few exceptions (Tanaka et al. 2004)—exclusively the 2,5-DHB isomer was so far used as MALDI matrix. A high absorbance at 337 nm indicates that moderate laser intensities may be used, while higher laser intensities (i.e. less attenuation) are required if the other DHB isomers are used. An enhanced laser intensity would, however, also lead to pronounced fragmentation of the analyte, resulting in more complex spectra.

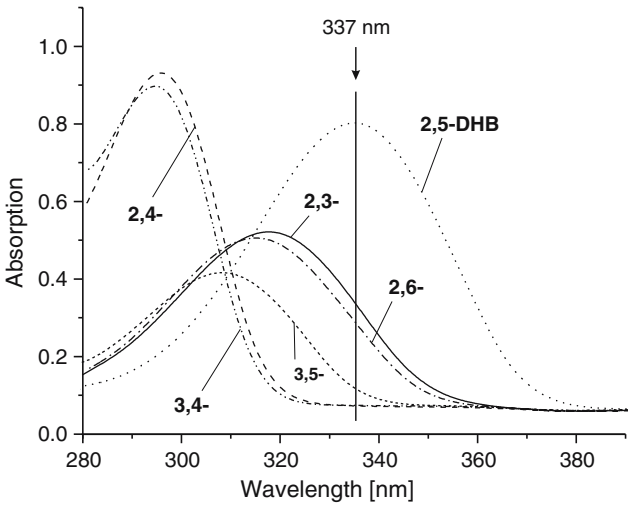

Fig. 2 Absorption spectra of the individual isomers of DHB used in this study. All measurements were performed with 20 mg/l (129.9 μM) solutions of the individual DHB isomers in methanol. The *vertical line* corresponds to $\lambda = 337$ nm. This is the excitation wavelength of lasers commonly used in commercially available MALDI-TOF mass spectrometers

Comparison of the laser desorption spectra of the individual DHB isomers

As stated above, another important prerequisite of a MALDI matrix is the low yield of matrix peaks in the mass range of the analyte. Highly characteristic peaks of all investigated DHB isomers are listed in Table 1 together with possible assignments for the peak origin. Hereby, "M" denotes the molecular weight of the neutral DHB molecule (154 g/mol).

For all six DHB isomers, there were no peaks detected at *m/z* values higher than 775.0. Therefore, the interference with lipid peaks is rather weak. Furthermore, comparing the different DHB matrices, no marked differences for the abundance of the peaks listed in Table 1 are found (data not shown). Accordingly, from this point of view, there is no reason to prefer one selected DHB isomer as MALDI matrix.

However, the intensities of the given peaks are highly influenced by the applied laser intensity and the buffer composition, i.e. the ion concentration (primarily the $Na^+$and K^+ concentration) of the individual DHB preparations and increase at higher ionic strengths (Petković et al. 2001). Therefore, all DHB preparations in this paper were made with NaCl-saturated solutions to warrant defined conditions. A more quantitative data analysis could not be performed because differences in the absorption properties require different laser intensities and this would also influence the corresponding peak intensities. It must be emphasized that the peak intensities decrease considerably with increasing *m/z* values.

For DHB, matrix peaks are primarily generated by oligomerization processes of DHB in the gas phase. Obviously, the tendency of DHB to undergo oligomerization is weak in contrast to compounds with an olefinic side chain (e.g. sinapinic acid). Additionally, the generation of cluster ions might be limited by the number of aggregated DHB molecules and the stability of the corresponding clusters might decrease with their molecular weight. Both effects may lead to decreasing matrix peak intensities at increasing *m/z* values.

Indeed, a comprehensive discussion of the matrix spectra is beyond the scope of this paper, particularly since the molecular origins of signals arising from the pure DHB matrix are still a matter of debate (Hao et al. 1998; Petković et al. 2001).

Table 1 Assignment of the *m/z* values detected in the positive and negative ion laser desorption mass spectra of the applied DHB isomers

Positive ions *m/z* ratio	Assignment	Negative ions *m/z* ratio	Assignment
413.0	$2M - 3H^+ + 3Na^+ + K^+$	465.0	$3M - 3H^+ + 2Na^+ - NaOH$
429.0	$2M - 3H^+ + 2Na^+ + 2K^+$	471.0	$3M - 2H^+ + K^+ - CO$
449.0	$3M + Na^+ - 2H_2O$	483.0	$3M - 2H^+ + Na^+$
465.0	$3M + K^+ - 2H_2O$	499.0	$3M - 2H^+ + K^+$
551.0	$3M - 3H^+ + 4Na^+$	505.0	$3M - 3H^+ + 2Na^+$
567.0	$3M - 3H^+ + 3Na^+ + K^+$	543.0	$3M - 4H^+ + 2Na^+ + K^+$
583.0	$3M - 3H^+ + 2Na^+ + 2K^+$	641.0	$4M - 4H^+ + 3Na^+ - NaOH$
599.0	$3M - 3H^+ + Na^+ + 3K^+$	675.0	$4M - 3H^+ + Na^+ + K^+$
727.0	$4M - 4H^+ + 5Na^+$	681.0	$4M - 4H^+ + 3Na^+$
743.0	$4M - 4H^+ + 4Na^+ + K^+$	691.0	$4M - 3H^+ + 2K^+$
759.0	$4M - 4H^+ + 3Na^+ + 2K^+$	729.0	$4M - 4H^+ + 3K^+$
774.9	$4M - 4H^+ + 2Na^+ + 3K^+$	745.0	$4M - 6H^+ + 2Na^+ + 3K^+ - CO$

"M" indicates the molecular weight of the neutral DHB molecule (154 g/mol). No matrix gives peaks at higher *m/z* values than 775. Peaks at lower *m/z* values are not shown because they are outside the typical mass range of lipids. Please note that peak intensities decrease with the *m/z* ratio

Positive ion spectra of phospholipids

In Fig. 3, positive ion MALDI-TOF mass spectra of a 1:2.5 (w/w) mixture of DPPC and POPS recorded with different DHB isomers are shown. Both these PL were used because they possess a quite similar molecular weight but differ in their charge state with DPPC being a neutral PL and POPS representing a negatively-charged PL at physiological pH (7.4). It is known that charge differences lead to different detectabilities of a given compound as positive or negative ions (Müller et al. 2001): DPPC is exclusively detected as positive ion with high sensitivity, whereas POPS can be detected as positive as well as negative ion. The reduced detectability of POPS as positive ion is the reason why an excess of POPS in comparison to DPPC was used.

Spectra are sorted in the order of increasing pK_a values of the corresponding DHB isomers used as matrix, i.e. 2,6-DHB (**a**), 2,3-DHB (**b**), 2,5-DHB (**c**), 2,4-DHB (**d**), 3,5-DHB (**e**) and 3,4-DHB (**f**). The pk_a values of the individual DHB isomers in water are as follows: 2,6-DHB: 1.30; 2,3-DHB: 2.94; 2,5-DHB: 2.97; 2,4-DHB: 3.29; 3,5-DHB: 4.04 and 3,4-DHB: 4.48 (ĺAns et al. 1992). The reader should note that these data were determined in solution and may differ from the recently determined gas phase basicities of the DHB isomers (Yassin and Marynick 2005).

The acidity may be assessed by using the pK_a value. Strictly, this value is defined and usually determined in water. However, the given pK_a values may also serve as measures of the acidity of a certain compound in methanol solution, although only relative differences may be considered under these conditions.

It must also be emphasized that the provided pK_a values were determined in water, whereas in our experiments the solvent was methanol. Therefore, only relative differences in acidities should be considered.

The spectra shown in Fig. 3 differ significantly: The DPPC signals (*m/z* = 734.6 and 756.6 according to the $H^+$and the Na^+adduct, respectively) are detectable in all spectra, although the intensities obtained with the two upper DHB isomers (3,5-DHB and 3,4-DHB shown in traces (3e) and (3f), respectively) are comparably low even at enhanced laser intensities. In contrast, there are considerable differences with respect to the detection of the POPS: It is evident that the intensities of the POPS peaks (*m/z* = 784.5, 806.5 and 828.5) (Schiller et al. 2006) decrease when the pK_a value of the used DHB isomer increases. These differences are quite small when traces (3a–c) are compared, but are more marked if less acidic DHB isomers (3d–f) are used.

The sodium salt of POPS has a molecular weight of 783.5 and, accordingly, the peaks at *m/z* = 784.5 and 806.5 correspond to the H^+ and the Na^+ adduct of POPS, respectively. The peak at *m/z* = 828.5 represents the exchange of a H^+ by a Na^+ ion. This peak pattern is characteristic of acidic PL (Schiller et al. 2004). One should note that the most acidic isomer (2,6-DHB) provides the best detectability of the POPS in comparison to the DPPC. This could actually be expected because charge compensation of POPS is required in order to be able to detect POPS as a singly charged positive ion. The lower the pK_a value of the DHB matrix, the higher is its tendency to release protons and the more H^+ are available for charge compensation. Therefore, the intensity of the POPS

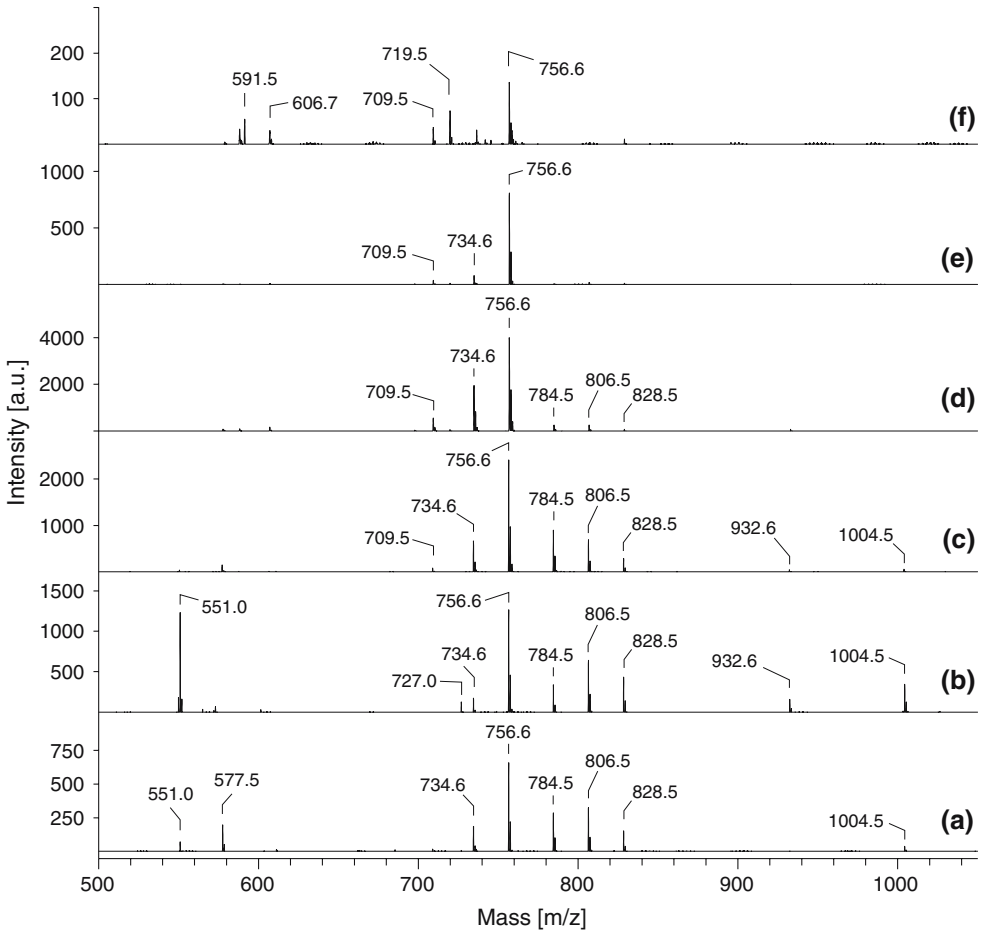

Fig. 3 Positive ion MALDI-TOF mass spectra of a 1:2.5 (w/w) mixture of DPPC and POPS recorded with the different isomers of DHB as matrix: 2,6-DHB (**a**), 2,3-DHB (**b**), 2,5-DHB (**c**), 2,4-DHB (**d**), 3,5-DHB (**e**) and 3,4-DHB (**f**). The pK_a values of the DHB isomers increase from the bottom to the top. Peaks are marked by the corresponding *m/z* values

peaks increases with decreasing pK_a values of the matrix. This is also the reason why TFA (as a strong organic acid) is often added to the MALDI matrix as an additional cationizing agent (Schiller et al. 2006). Although the use of the 2,3-DHB isomer as matrix provides also a high spectral quality, it is evident that this matrix leads to relatively high matrix peaks in the investigated mass range. The peak at *m/z* = 551 is very characteristic of the positive ion spectra of DHB (cf. Table 1).

It is also of interest to note that the intensities of the characteristic DHB adducts of DPPC at *m/z* = 932.6 and POPS at *m/z* = 1004.5 (corresponding to the addition of the positively charged sodium adduct of sodium dihydroxybenzoate to the neutral DPPC or POPS molecule) depends significantly on the type of the DHB isomer. These adducts possess higher intensities when more acidic matrix compounds are used. This indicates that the generation of these complex matrix cluster ions is favored under acidic conditions (as well as a high ionic strength).

However, when discussing absolute signal intensities, one has to keep in mind that the obtained intensities (Fig. 3) are also influenced by the applied laser intensity. In general, this parameter cannot be kept constant but must be changed using the quality of the spectral resolution and the baseline as measures of an appropriate laser intensity. Additionally, it is also a good idea to check the adequate setting of the laser intensity by the intensities of degradation products of the applied lipids. For instance, it is known that an excess of laser intensity leads to the generation of lysophospholipids. Therefore, the laser intensity was not constant for all traces in Fig. 3: spectra with 3,4-DHB as matrix were recorded with a much higher laser intensity in comparison to 2,5-DHB because the individual DHB isomers possess different UV absorption properties (cf. Fig 2).

On the other hand, it would be interesting to compare the required laser intensities when different matrices with characteristic UV absorption properties are used. However, it is very difficult to assess the different laser energies that are required if different DHB isomers are used. This problem is also coming from the fact that on the majority of MALDI devices the control of the laser energy is possible only on a

relative scale. Only very little absolute data are known. For instance, the minimum (threshold) laser irradiance necessary to produce protein ions from the sample is about 10 mJ/cm^2 for a 10 ns laser pulse width (Hillenkamp et al. 1993). Since no data on lipids were published so far, we do not want to speculate about further details when different DHB isomers are used.

Equally which matrix is used, the generation of fragmentation products is rather weak. One useful fragmentation marker is the signal at m/z = 577.5 that corresponds to the cleavage of the polar headgroup from the POPS (Al-Saad et al. 2003). In contrast to POPS, PC species do not provide major amounts of fragmentation products. Although not yet carefully investigated, similar tendencies are expected when other lipid classes with comparable charge characteristics would be used.

The presented results do not support the established opinion about the structural requirements of matrix compounds: it is commonly accepted that a suitable MALDI matrix must possess at least one ortho-group, since only under these conditions an intramolecular proton shift along the resulting intramolecular hydrogen bond may occur leading to a transition state crucial for ionization (Krause et al. 1996). Since we were able to record a mass spectrum with 3,5-DHB (trace 3e) that does not possess an ortho group, the presence of ortho groups is obviously not absolutely required. Although this is so far very speculative, the suitability of this isomer might also be caused by intermolecular proton shifts along intermolecular H-bonds, as those may be expected to be formed in the crystalline state.

Anyway, we conclude that acidic compounds are the matrices of choice for positive ion spectra.

Negative ion phospholipid spectra

Although recording positive ion spectra is much more common than negative ion spectra, many compounds are also detectable in the negative ion mode. The comparison between positive and negative ion mass spectra provides often important information about the chemical structure of an unknown compound and can be used to confirm a tentative peak assignment (Müller et al. 2001).

The negative ion MALDI-TOF mass spectra of the same DPPC/POPS mixture as in Fig. 3 are shown in Fig. 4. As in Fig. 3, one of the six investigated isomers of DHB served as matrix for each trace and the representation is in the order of increasing pK_a values of the respective DHB isomer. However, since in the presence of 2,6-DHB no negative ion spectrum could be obtained, this isomer was completely omitted. Its considerable acidity (pK_a = 1.30) is one convincing explanation that the 2,6-DHB isomer is not capable of producing intense negative ion signals.

Under negative ion conditions, it is evident that exclusively the POPS is detectable whereas the DPPC is not detectable at all. The POPS is identified by the signals at m/z = 760.5 and 782.5 that correspond to the abstraction of one H^+ or one Na^+ ion, respectively, from the neutral POPS molecule (M = 783.5 for the corresponding sodium salt). The spectral quality differs between the individual spectra, whereby it is somewhat surprising that only very small intensities are obtained when the least acidic 3,4-DHB isomer (4e) is used. One would have expected that the yield of negative ions would be favored under these conditions.

Spectra in traces (4b–d) are of comparable quality although the measured intensities differ considerably. In contrast, the spectral quality by using 2,3-DHB (trace 4a) and 3,4-DHB (trace 4e) is obviously lower because there is in both cases a more marked contribution of matrix peaks (for instance, at m/z = 681) and further peaks that could not be assigned so far. Additionally, the intensities in the presence of the 3,4-isomer are very low, most probably because of its small UV absorbance.

In analogy with the positive ion spectra, the negative ion spectra exhibit also cluster ions between the matrix and the POPS at m/z = 936.5 and 958.5. These peaks are generated by the addition of one negatively charged matrix molecule to the neutral form of POPS.

It is known that phosphatidylcholines may be basically also detected as negative ions subsequent to the addition of a negatively charged DHB molecule, i.e. as a cluster with the DHB matrix (Schiller et al. 2002). However, in the presence of higher amounts of PS that yields by far stronger negative ion signals, we failed to detect such negatively charged cluster ions between the PC and the matrix. It is concluded that 2,5-, 2,4- and 3,5-DHB are useful compounds to record negative ion spectra of lipids. Among these compounds, the 2,5-isomer provides the highest intensities, i.e. the lipids are detected with the highest sensitivity.

AFM studies

As mentioned above, beside the before addressed features, one further prerequisite for a suitable matrix substance is an even co-crystallization with the analyte molecules. Consequently, the final question addressed in this study was the different crystallization behavior of the individual DHB isomers in the absence and presence of lipid. In Fig. 5, different atomic force microscopic (AFM) images of three selected DHB isomers

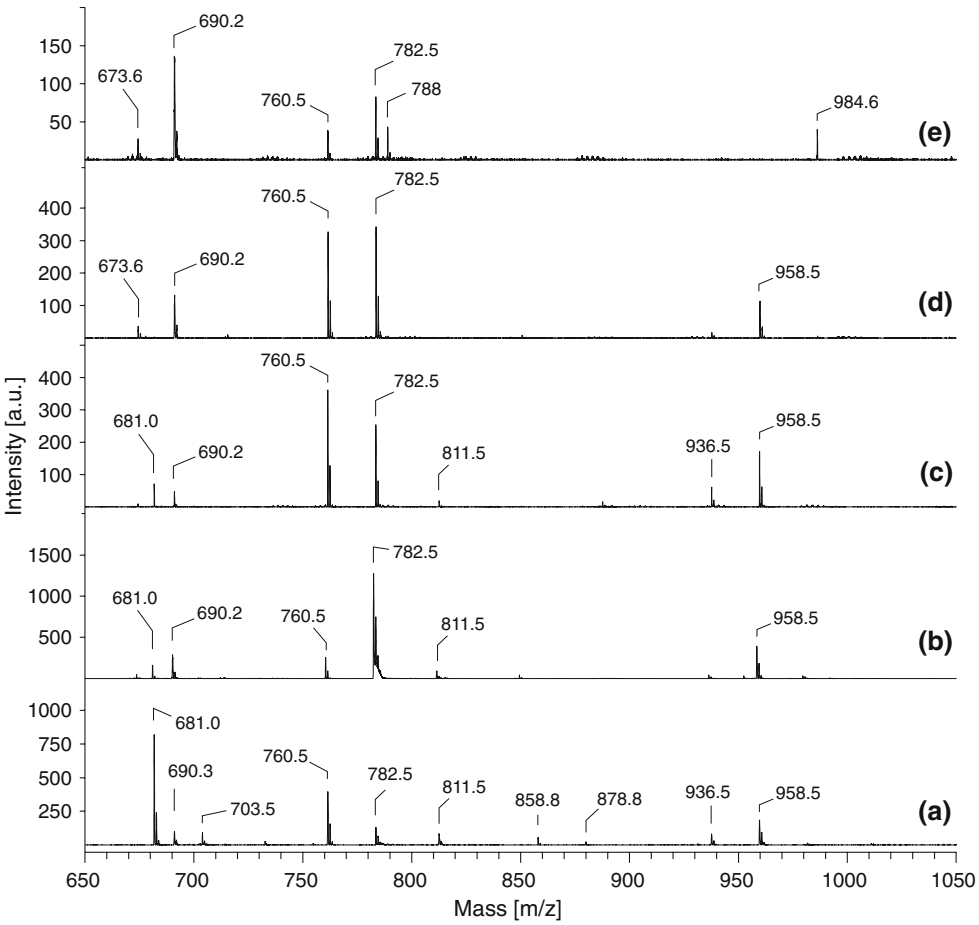

Fig. 4 Negative ion MALDI-TOF mass spectra of a 1:2.5 (w/w) mixture of DPPC and POPS recorded with the different isomers of DHB: 2,3-DHB (**a**), 2,5-DHB (**b**), 2,4-DHB (**c**), 3,5-DHB (**d**) and 3,4-DHB (**e**). The pK_a values increase from the bottom to the top. Peaks are marked by the corresponding *m/z* values. Please note that it was impossible to record negative ion spectra in the presence of the 2,6-isomer. Therefore, this isomer is not shown in this figure

(3,4-, 2,5-, and 2,4-DHB) are shown. These three DHB isomers were selected because the 2,4- and the 2,5-isomer give mass spectra of a high quality, whereas the spectral quality obtained with 3,4-DHB is very poor. Images on the left were obtained in the absence of lipid, whereas images on the right were recorded in the presence of lipid. It is obvious from the comparison of the AFM images that the 2,5-DHB isomer provides the most suitable crystal shape: The crystals of the 2,5-DHB isomer possess a smooth, regular surface, whereas the 2,4-DHB isomer shows a more inhomogeneous structure with a rough surface. In the presence of the lipid, smaller, homogeneous crystals are obtained in the case of 2,5-DHB. In contrast, the 2,4-isomer is characterized by an ordered crystalline structure that is extended in the presence of the lipid, whereby the height of the layer is considerably increasing. Clearly, this reduces the homogeneity of the sample and enhances the variations from shot to shot. The 3,4-DHB isomer shows a completely different behavior and this—in combination with the very low UV absorption at the laser wavelength—is most likely the reason why this isomer is not very suitable as MALDI matrix.

Similar investigations on the crystallization behavior of DHB isomers were already carried out by confocal laser scanning microscopy (Horneffer et al. 2001). In this study, the entrapment of labeled avidin into crystals of 2,6- and 2,5-DHB was investigated. A much more homogeneous distribution was found in the case of the 2,5-isomer, whereas a rather uneven crystallization resulted from the 2,6-isomer. However, this study (Horneffer et al. 2001) is difficult to compare with our study because these authors used water as solvent, whereas organic solvents were used in our case. Additionally, this study was performed with proteins instead of lipids. Nevertheless, the 2,5-isomer of DHB seems most suitable from the AFM results.

Summary

Three parameters seem to be most relevant for the assessment of the suitability of an organic component as MALDI matrix, namely (a) the absorbance at the laser wavelength, (b) the acidity and (c) the homogeneous co-crystallization with the analyte of interest. Summarizing, all obtained data indicate that 2,5-DHB

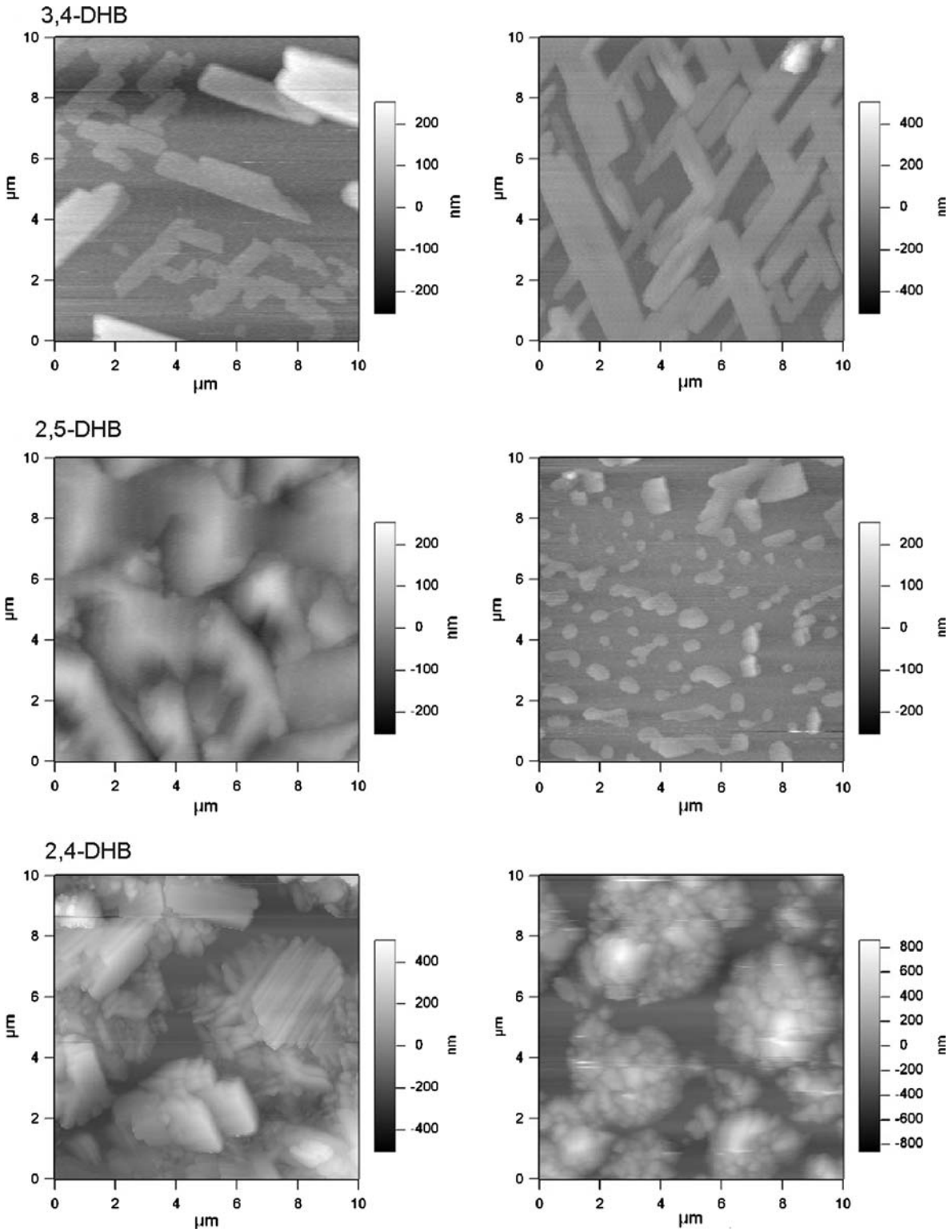

Fig. 5 AFM images of crystals of different DHB isomers (bottom, 2,4-DHB; middle, 2,5-DHB; top, 3,4-DHB) crystallized on mica surfaces. Images on the left correspond to the pure DHB surfaces in the absence of phospholipids, whereas images on the right represent co-crystals of matrix-lipid mixtures prepared as described in Materials and methods. Dimensions in the plane are given by numbers on the *horizontal* and the *vertical axes*, whereas the *vertical grey scale bars* indicate the relative heights of the visualized structures

is still the MALDI matrix of choice, even if all further isomers may also be used. However, especially the 2,4- and the 3,5-isomers should not be used for positive ions spectra, whereas the 2,6- and the 2,4-isomers are not suitable for negative ion detection. The 2,6-isomer is, however, a real alternative to 2,5-DHB in the positive ion mode due to its considerable acidity.

Acknowledgments This work was supported by the Deutsche Forschungsgemeinschaft (Schi 476/5-1) and the Federal Ministry of Education and Research (BMBF, Grant 982000-041).

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