RAPID COMMUNICATIONS IN MASS SPECTROMETRY, VOL. 10, 1278–1282 (1996)

$K_4[Fe(CN)_6]$/Glycerol—A New Liquid Matrix System for Matrix-assisted Laser Desorption/ Ionization Mass Spectrometry of Hydrophobic Compounds

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Since the introduction of MALDI mass spectrometry, only a few liquid matrix systems have been proposed. This paper reports a new liquid matrix system consisting of glycerol and $K_4[Fe(CN)_6]$. With this binary system, best results are achieved for hydrophobic compounds like triacylglycerols, galactosyl diacylglycerols, phosphatidylcholines, phosphatidylethanolamines, ceramides and several chloroform soluble, synthetic polymers. Only amounts of analyte in the femtomol range have been required to obtain good results with satisfactory mass resolution, mass accuracy and excellent shot-to-shot reproducibility. In all cases, only potassium adducts are formed. No protonated molecular ions could be detected. Potassium adducts of small peptides (up to approx. 3000 Da) and carbohydrates can also be generated with this matrix system but with lower sensitivity than in the case of hydrophobic compounds (picomol to nanomol range). Additionally, sample preparation in the case of hydrophilic compounds is critical and hard to reproduce. This is presumably caused by the destruction of the thin UV absorbing matrix layer by aqueous sample solutions.

Since its introduction by Karas and Hillenkamp[1] and by Tanaka,[2] matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has become one of the most important techniques in the mass spectrometry of involatile biopolymers and synthetic polymers. Much of the success in the rapid development of MALDI can be attributed to the identification of a number of effective matrix systems.[3–10]

Although most of these matrix compounds are solids, liquid matrix systems offer a number of advantages, which makes it worthwhile to search for suitable liquid compounds. The use of a liquid matrix generates more physiological conditions, especially for peptides and proteins (elemental composition and physical characteristics are similar to aqueous solutions), and might, therefore, avoid noncovalent complex dissociation, which is usually observed in the case of solid matrices during the relatively harsh drying and crystallization process. Further, crystallization of a solid matrix leads often to the phenomenon of sweet spots, although newer sample preparation techniques diminish this problem.[11, 12] This phenomenon can be avoided by the use of a liquid matrix system which enables a more homogeneous mixing of analyte and matrix. Finally, the use of liquid matrices offers the attractive possibility of interfacing MALDI mass spectrometry with liquid-phase separation techniques.[13–15]

Despite these advantages, only few approaches have been made towards the use of liquids, presumably caused by the fact that most suitable liquids are too volatile for the vacuum conditions in the mass spectrometer. One possibility is the use of 3-nitrobenzyl alcohol, which gave the best results when used with a 266 nm laser[16–18] although good results can also be obtained with a N_2-laser at 337 nm.[19] A second approach was already published by Tanaka *et al.*[2] in 1988, using a suspension of 30 nm diameter cobalt particles in glycerol. The mass resolution with this system was, however, poor and relatively large amounts of sample were needed for a successful analysis. A similar approach was proposed by Sunner *et al.*[20, 21] using graphite particles suspended in glycerol or diethanolamine, which they call surface-assisted laser desorption/ionization. The authors assume a thermal desorption/ionization process that takes place on the graphite surface. Picomole amounts of peptides and proteins have been analysed successfully.

Another approach is the use of UV-absorbing compounds, such as the dye Rhodamine 3G, 3-nitrobenzyl alcohol or 2-cyano-5-phenyl-2,4-pentadienoic acid, in a non UV-absorbing solvent like glycerol or 1,2,4-butanetriol.[22–24] The use of Rhodamine 3G allows the application of visible laser light at 532 nm. This method suffers, however, from a lack of mass resolution. Additionally, glycerol can itself act as matrix when used in conjunction with infrared MALDI. Thus, 2.94 or 10.6 μm laser light is absorbed by the O–H stretch of the matrix and mass spectra of lysozyme at 14 300 Da has been presented by Hillenkamp *et al.*[25, 26]

We now report a new liquid matrix system that contains potassium hexacyanoferrate and glycerol, where potassium hexacyanoferrate acts as a good UV absorber, when a nitrogen laser (337 nm) is used. This binary system is especially well suited for lipophilic compounds which are soluble in chloroform or other organic solvents.

MATERIALS AND METHODS

Materials

Galactosyl diacylglycerols, dihexadecanoyl α-phosphatidylcholin, dihexadecanoyl α-phosphatidyl-*N*-monomethylethanolamine, lactocerebrosides, maltoheptaose, maltopentaose, melittin, substance P, oxytocin, angiotensin III α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid were supplied by Sigma (Deisenhofen, Germany). Triton X305® was purchased from Serva (Hei-

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CCC 0951–4198/96/101278–05

Received 24 June 1996
Accepted 28 June 1996

delberg, Germany). Tri(*Z*-13-docosenoyl)glycerol was obtained from Croda Surfactants (Goole, U.K.). Glycerol and potassium hexacyanoferrate (II)/(III) were supplied by Merck (Darmstadt, Germany). Castor oil (Austrian pharmacopoeia quality) was purchased from Hestag (Vienna, Austria). Tridodecanoyl glycerol was synthesized according to the literature[27] by the reaction of dodecanoyl acid chloride (Merck, Darmstadt, Germany) and glycerol in anhydrous pyridine.

Apparatus

Positive-ion MALDI mass spectrometric analyses were performed with a Kratos MALDI III reflectron time-of-flight mass spectrometer (Kratos Analytical, Manchester, UK). A nitrogen laser with a pulse width of 3 ns was used to generate photons of 337 nm wavelength which impinged on a stainless steel target. In all experiments the ions generated were extracted by an electric field of 20 kV. The ions were allowed to drift a distance of 140 cm (reflectron mode). Spectra were obtained by adding together the transient records of 40 to 100 individual laser shots.

Sample preparation

5% (v/v) of glycerol was added to a saturated solution of $K_4[Fe(CN)_6]$ in methanol. Then, 0.5 μL of this solution was deposited on the target, where the methanol evaporated after few seconds at room temperature. Afterwards, 0.5 μL of analyte solution, either in water or chloroform, was put on top of the matrix layer. After the solvent had evaporated, the target was inserted into the ion source.

RESULTS AND DISCUSSION

For the $K_4[Fe(CN)_6]$/glycerol matrix, only a thin-layer technique as sample preparation method results in satisfactory mass spectra. No analyte ions could be observed, when matrix and analyte solution were premixed prior to depositing on the target. The matrix system is most suitable for hydrophobic compounds which are soluble in non-aqueous solvents. With peptides and carbohydrates, however, the sensitivity of this matrix system significantly decreases and several attempts were necessary to obtain successful sample preparations. This might be caused by the aqueous solutions which disturb the thin UV-absorbing layer.

The best mass spectrometric results were obtained, when analyses were carried out within one hour after the sample had been introduced into the ion source of the mass spectrometer. After this time, generation of molecular ions was still possible (even after 19 h), but with a decreased signal-to-noise ratio. This observation may be explained by the fact that potassium hexacyanoferrate, as the UV absorbing substance, remains as a solid on the target surface while glycerol is evaporated completely in the meantime.

Mass resolution ($R \approx 1000$ [FWHM] near threshold laser fluence for the production of positive-ions) and mass accuracy (±0.05%) obtained with this matrix system are comparable to the performance obtained with solid matrix systems (e.g. 2,5-dihydroxybenzoic acid or α-cyano-4-hydroxycinnamic acid) under similar experimental conditions. In all cases, for hydrophobic compounds, shot-to-shot reproducibility is good over larger areas of the target. This is in contrast to 'sweet spots' encountered with solid matrices, e.g. 2,5-dihydroxybenzoic acid, that were used for a comparative investigation of hydrophobic analytes. Comparative studies with Fe(III) potassium hexacyanoferrate showed the generation of similar results without any of the reducing effects of Fe(II) potassium hexacyanoferrate on the measured analytes.

The low-mass region of all mass spectra is clearly dominated by the potassium ion at *m/z* 39. Additionally, other peaks related to glycerol, potassium hexacyanoferrate (molecular weight 422.41 Da) and unknown structures occur in the range between *m/z* 100 and *m/z* 430 (e.g. *m/z* 115, $[glycerol+Na]^+$; *m/z* 131, $[glycerol+K]^+$; *m/z* 157; *m/z* 169; *m/z* 205; *m/z* 320; *m/z* 334; *m/z* 429, $[M(K_4[Fe(CN)_6]) - K + 2Na]^+$).

In Fig. 1 a positive-ion mass spectrum of tridodecanoyl glycerol is shown. A total amount as low as 50 fmol was

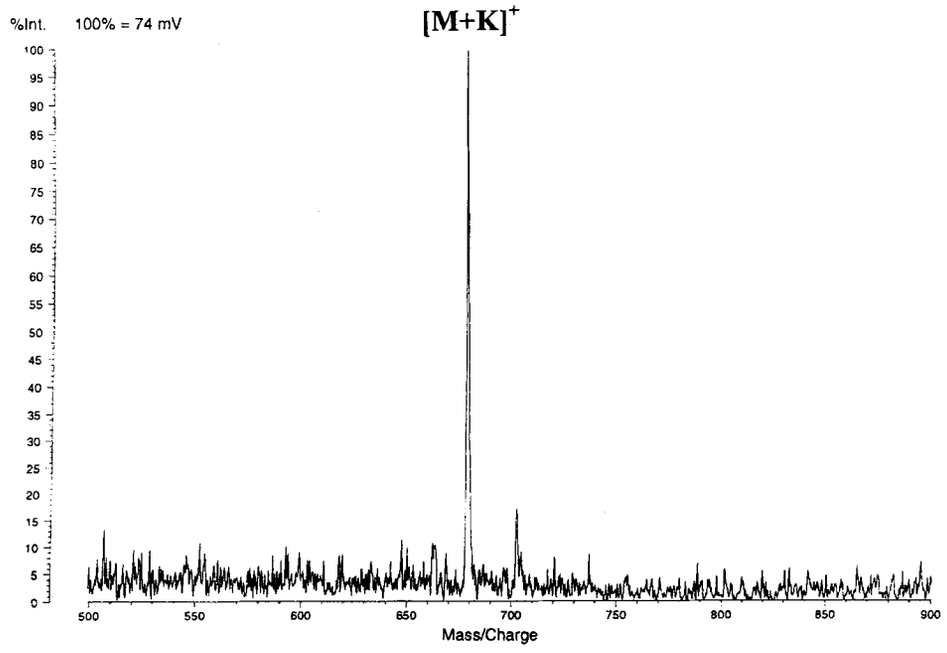

Figure 1. Positive-ion MALDI mass spectrum of tridodecanoyl glycerol (M=638.6 Da); 50 fmol; average of 100 laser shots.

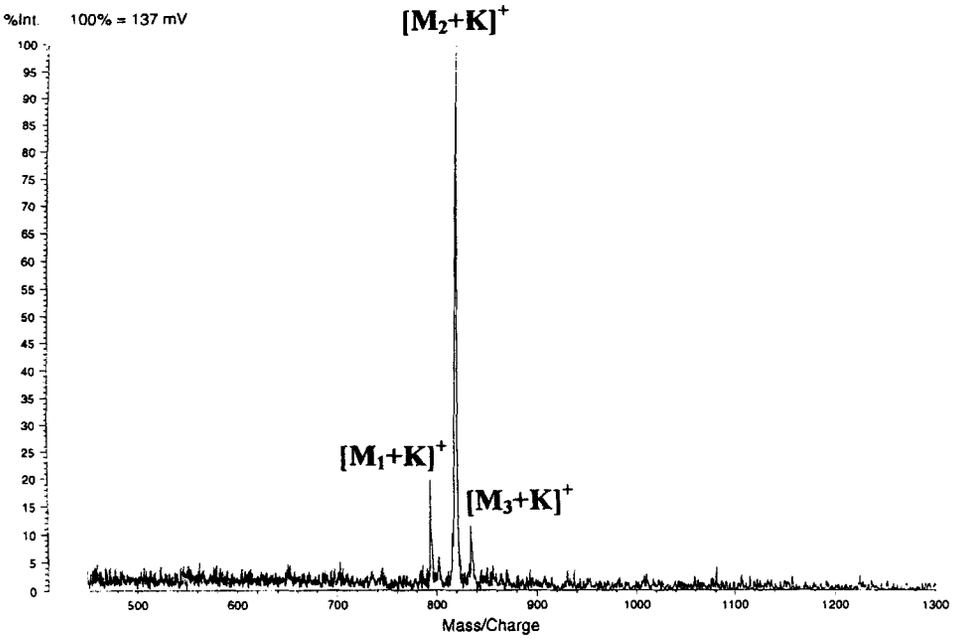

Figure 2. Positive-ion MALDI mass spectrum of a galactosyl diacylglycerol mixture isolated from whole-wheat flour (M_1=754.8 Da, M_2=778.8 Da and M_3=794.8 Da); ≈0.4 ng of mixture; average of 100 laser shots.

sufficient to obtain this spectrum. The molecular mass could easily be determined from the potassium adduct. No protonated molecular ion could be observed. Similar mass spectra can also be obtained from other triacylglycerols with higher molecular masses, e.g. tri(Z-13-docosenoyl)-glycerol or castor oil (mass spectra not shown).

As expected, galactosyl diacylglycerols isolated from whole-wheat flour (Fig. 2) and bovine lactocerebrosides (Fig. 3), form exclusively potassium adducts of the molecular ion, from which molecular weight determination is easily possible, again in the low femtomol range ($[M_1+K]^+$ and $[M_3+K]^+$ in Fig. 2). As shown in Figs 2 and 3, even the investigation of mixtures is easily possible, because there is only a low tendency towards fragmentation of the potassium adducts. With analyte amounts in the picomol range, up to 1000 single-laser-shot mass spectra can be obtained from one target spot. A similar mass spectrometric behaviour can be observed with phospholipids such as diacyl phosphatidylcholines and diacyl phosphatidylethanolamines.

The potassium hexacyanoferrate/glycerol matrix is also suitable for the mass spectrometric characterization of synthetic polymers which are soluble in organic solvents. A mass spectrum of the polymer Triton X305® which consists of a polyethylene chain with an octylphenyl residue on one end and a hydroxy group on the other end is shown in Fig. 4. In this case also, all mass peaks are those of potassium adducts. In this sample preparation, 50 ng of polymer

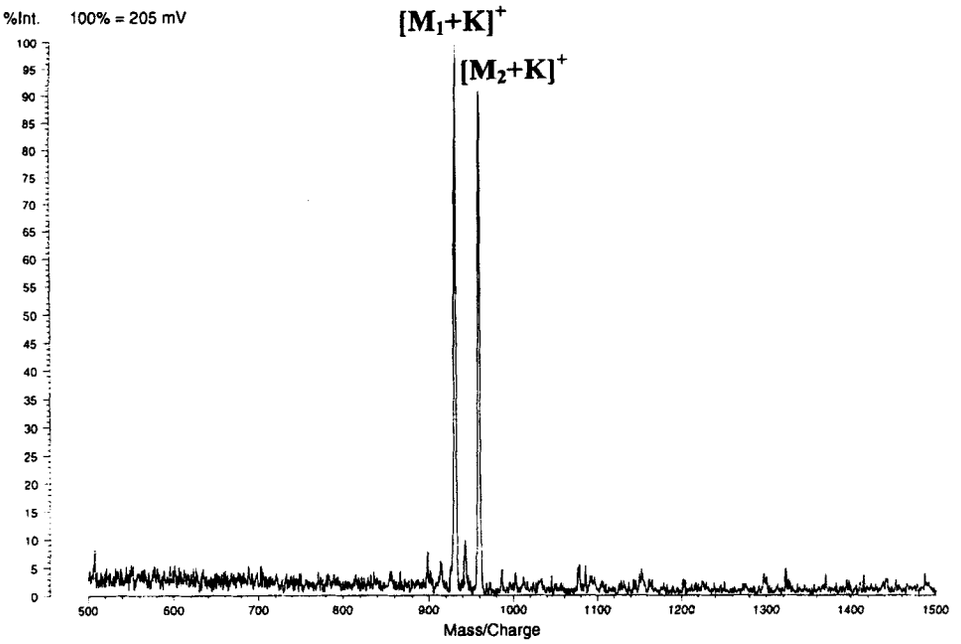

Figure 3. Positive-ion MALDI mass spectrum of two bovine lactocerebrosides (M_1=889.7 and M_2=917.7 Da); 250 fmol of each component; average of 100 laser shots.

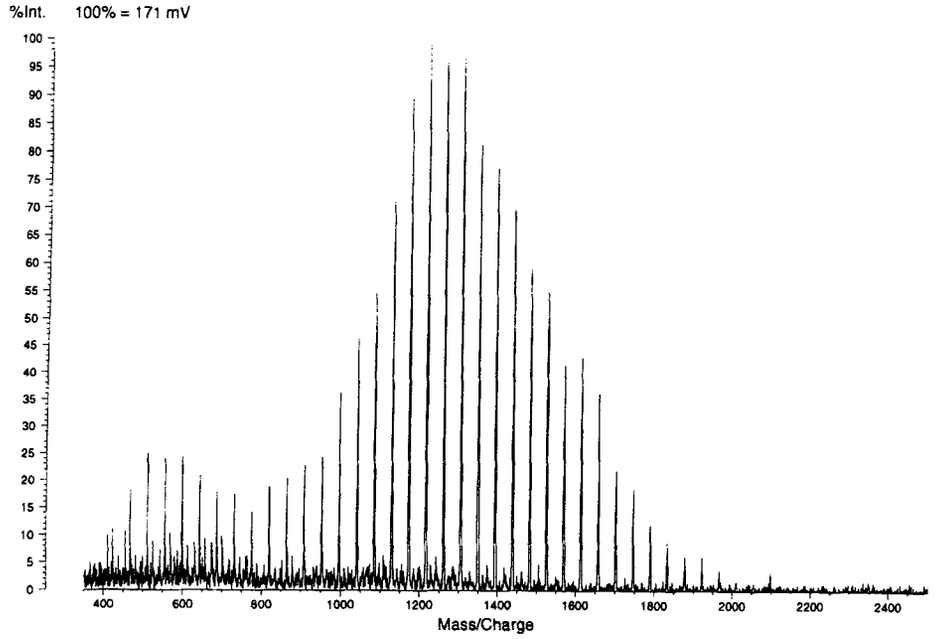

Figure 4. Positive-ion MALDI mass spectrum of Triton X305®; 50 ng of polymer mixture; average of 100 laser shots.

mixture was deposited as a chloroform solution onto the target to obtain the mass spectrum. Thus, the smallest mass peaks correspond to analyte amounts of a few femtomols. In addition, mass spectra from 5 ng sample loadings have been acquired. However, in this case polymer molecules with lower concentrations in the mixture could no longer be detected.

Several peptides have also been successfully analysed. With this compound class, sensitivity significantly decreases, compared to hydrophobic compounds (low picomol range). Figure 5 shows the positive-ion mass spectrum of melittin. Molecular mass determination is easily possible based on the $[M+K]^+$ ion. A small peak at m/z 2870 indicates the sodium adduct of the peptide. Even a peak of low abundance at m/z 2848, corresponding to the protonated molecular ion could be detected. Similar spectra were also recorded for substance P, oxytocin and angiotensin III (mass spectra not shown). Further, the molecular weight determination of neutral carbohydrates, like maltoheptaose and maltopentaose is feasible but more material (low nanomole range) is necessary to obtain a satisfactory signal-to-noise ratio. These oligosaccharides were detected as their potassium adducts.

CONCLUSION

The new liquid $K_4[Fe(CN)_6]$/glycerol matrix system has been shown to be especially suitable for hydrophobic compounds with detection limits in the low femtomol range. No satisfactory sample preparation technique has been

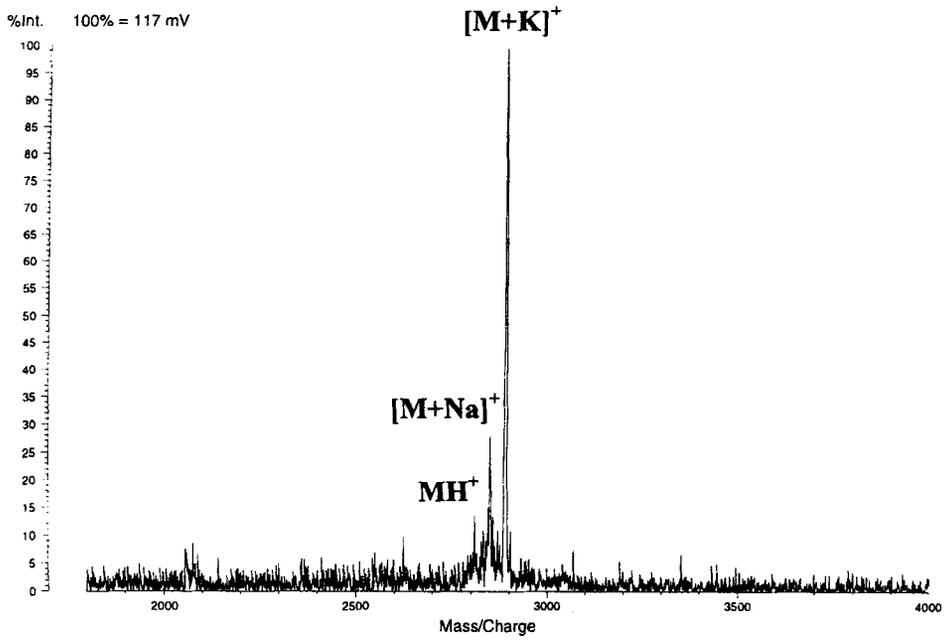

Figure 5. Positive-ion MALDI mass spectrum of melittin (M=2847.5 Da); 50 pmol; average of 50 laser shots.

found for hydrophilic compounds (e.g. peptides and neutral carbohydrates) so far. Additionally, the sensitivity for these compound classes is significantly decreased (pico- to nanomole range). In our opinion this problem is due to the fact that the thin UV absorbing layer is destroyed by the aqueous sample solutions and no suitable UV absorbing matrix/analyte system is formed for the desorption/ionisation process. Replacing glycerol with an appropriate liquid, which is less water soluble, e.g. a long-chain alkyl ether, could be a possible solution to this problem and investigations in this direction are in progress.

Acknowledgements

This work was supported by the Austrian Science Foundation (grant P11183 to G.A.) and the Commission of the European Union (HCM grant ERBCHRX CT94025). The authors thank B. Mayer for providing a sample of Triton X305®.

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