MALDI Post-Source Decay and LIFT-TOF/TOF Investigation of α-Cyano-4-Hydroxycinnamic Acid Cluster Interferences

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Large signals from α-cyano-4-hydroxycinnamic acid (CHCA) matrix complexes with sodium and potassium ions were found to interfere with sensitive matrix-assisted laser desorption/ionization (MALDI) analysis of a hydrochloric acid digest of gelatine preparations. The nature of some selected matrix clusters was investigated by conventional post-source decay and LIFT-TOF/TOF experiments. The matrix clusters fragmented readily by neutral evaporation to give smaller sized matrix cluster species without matrix disintegration. Their characterization distinguished them from peptide signals, in particular from those that had the same nominal mass and differed only in the fractional part of the mass as encountered for gelatine-derived peptides. Knowledge of the molecular composition of these cluster species allowed using them for internal calibration of the MALDI mass spectra. The hydrolytic peptides could be analyzed with increased sensitivity when using 2,5-dihydroxy benzoic acid (DHB) as the MALDI matrix. (J Am Soc Mass Spectrom 2004, 15, 336–343) © 2004 American Society for Mass Spectrometry

During the last decade, MALDI mass spectrometry has advanced into a highly sensitive technique for peptide analysis [1]. The amount of peptide required for a successful MALDI experiment usually ranges from atto- to picomoles deposited on-target using a large variety of sample preparation techniques. In a recent study, the analysis of low zeptomoles of total peptide loading using a nanolitre microspot deposition technique was reported and raised the fundamental question of the detection limit of MALDI mass spectrometry [2]. Sensitivity of MALDI depends mainly on the sample preparation procedure as well as the instrumental parameters and can be described as the amount of material deposited on-target that can still be distinguished from background noise, also referred to as "chemical noise". This noise consists mainly of unresolved ion species spread across the entire *m/z* scale [3]. Insights into the nature of the background noise have been gained by MALDI ion trap MS/MS experiments of randomly selected background noise [4]. Characteristic losses of matrix and (matrix-H_2O) were observed indicating that the chemical background noise largely consists of variable matrix cluster species. In addition, it could also be demonstrated that these clusters are relatively unstable and fragment readily at low activation energies. Furthermore, the formation of non-covalent matrix adducts of peptides and proteins is also a widely observed phenomenon [5]. By using a high pressure MALDI Fourier transform mass spectrometer it was demonstrated, however, that matrix molecules can be easily detached from a polypeptide by mild collisional activation illustrating that these analyte–matrix complexes are also relatively unstable [6]. The origin of matrix–matrix and analyte–matrix complexes is qualitatively described in a widely accepted mechanism for ion formation in MALDI, which is based on the emission and ionization of molecular clusters [7, 8]. Whereas most clusters generated by MALDI are not observed in the spectrum, certain matrix cluster species seem to be rather more stable and appear as discrete signals, particularly when these clusters are comprised of matrix, sodium, and potassium ions [9]. The formation of matrix clusters from 2,5-dihydroxy benzoic acid (DHB) and α-cyano-4-

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hydroxycinnamic acid (CHCA) follows the general formula $M_{Cluster} = nM - xH + yK + zNa$ with $x = y + z - 1$ and $y + z \leq n + 1$ possibly also including water losses [9]. Such a calculation may be useful in distinguishing these clusters from peptide signals. Nonetheless, these signals may complicate data analysis by increased spectral complexity but their value as internal mass calibrants in MALDI TOF mass spectrometry has been recognized [10]. Moreover, knowledge about matrix cluster masses may also be important when analyzing protein digests in order to exclude these signals from database searches for enhanced confidence scores [11].

Herein, we report the MALDI TOF analysis of peptides derived from a hydrochloric acid digest of gelatine preparations [12]. Poor sensitivity was achieved with CHCA and in many cases signals arising from CHCA matrix clusters dominated the spectra, particularly when lower peptide concentrations were analyzed. The resulting spectra were found to be misleading as some of the matrix cluster interferences and peptide signals coincidentally were of identical nominal mass. Conventional post-source decay and LIFT-TOF/TOF experiments of some major CHCA cluster signals supported their identification and distinguished them from peptide signals. Furthermore, fragmentation studies shed some light into the nature of these clusters that mainly yielded smaller clusters as a result of neutral evaporation. The use of DHB matrix as an alternative to CHCA enabled the sensitive detection of the marker peptides without interference using MALDI mass spectrometry.

Experimental

Materials

The digestion of porcine gelatine with hydrochloric acid and the subsequent sample processing is described elsewhere [12]. MALDI mass spectrometry grade α-cyano-4-hydroxycinnamic acid (CHCA, molecular weight 189.2) and 2,5-dihydroxybenzoic acid (DHB, molecular weight 154.1) were obtained from Fluka (Poole, UK) and were used without further purification. Acetonitrile, acetone, ethanol and trifluoroacetic acid (TFA) were of analytical grade and from BDH (Poole, UK). Water was purified to a specific conductivity of 18.2 MΩ/cm on an Elga Maxima system (High Wycombe, UK).

Sample Preparation

A MALDI sample preparation protocol using target plates with a 600 μm hydrophilic anchor (AnchorChip 600, Bruker, Coventry, UK) was employed in order to maximize sensitivity and to ensure reproducible spot characteristics. A 10 μl aliquot of a porcine gelatine digest at 100,000, 10,000, 1000, 100, 10, and 1 μg/ml was desalted using C18 ZipTips (Millipore, Watford, UK). The digests were acidified with 1 μl of 1% aqueous TFA to enhance peptide binding on the C18 resin. Conditioning, loading, and washing steps were according to the manufacturer's recommendation. Peptides were eluted from the C18 bed with 1 μl of acetonitrile:0.1% aqueous TFA, 50:50 (vol:vol) directly onto either a thin layer of CHCA crystals formed from 0.3 μl of a 5 mg/ml solution of CHCA (acetone:0.1% aqueous TFA, 97:3) or a thin layer of DHB crystals formed from 0.5 μl of a 5 mg/ml solution of DHB (acetonitrile:0.1% aqueous TFA, 1:2). After drying under vacuum, the samples were re-crystallized with 0.2 μl of either ethanol:acetone:0.1% aqueous TFA (6:3:1) for CHCA layers or ACN:0.1% aqueous TFA (1:9) for DHB layers to promote analyte–matrix mixing within the area of the hydrophilic anchor.

Conventional MALDI TOF Mass Spectrometry

Mass Spectra were acquired with a Bruker MALDI-TOF Autoflex (Coventry, UK) operated in the positive reflectron mode. Ions were generated by a nitrogen laser emitting at 337 nm and were accelerated at 20 kV. In order to avoid detector saturation, low mass material was deflected; the detector voltage was 1.75 kV. All other parameters were set for an optimized mass resolution at about *m/z* 1000. Usually, 200 laser shots were averaged to record a mass spectrum. Signals from sodium adducts of polypropylene glycol were used for external multipoint calibration, compensating for the non-linearity of the time-of-flight analyzer [13]. In addition, an internal two-point correction of CHCA MALDI spectra using matrix cluster masses was performed after their further characterization by PSD and TOF/TOF experiments as described below.

MALDI Post-Source Decay TOF Mass Spectrometry

For MALDI post-source decay analysis, the pulsed ion deflector on the Autoflex was set to allow the selection of the precursor ion in a ±5 Da mass window. Pulsed ion extraction of 80 ns was employed for enhanced spectral resolution. The reflector voltage was stepped from 20 kV down to 1.28 kV in 15 segments in order to focus metastable fragments on the detector. The spectra obtained at each reflector voltage setting were pasted together in XMASS employing a calibration file from the PSD fragments of the ACTH clip(18-39). The mass assignment of fragments was performed in XMASS using an annotation macro for PSD spectra.

MALDI LIFT-TOF/TOF Mass Spectrometry

LIFT mass spectra [14, 15] were acquired on a Bruker Ultraflex TOF/TOF mass spectrometer (Coventry, UK) operated in the positive ion mode. Metastable fragmentation was induced by a nitrogen laser (337 nm) without the further use of collision gas. Precursor ions were

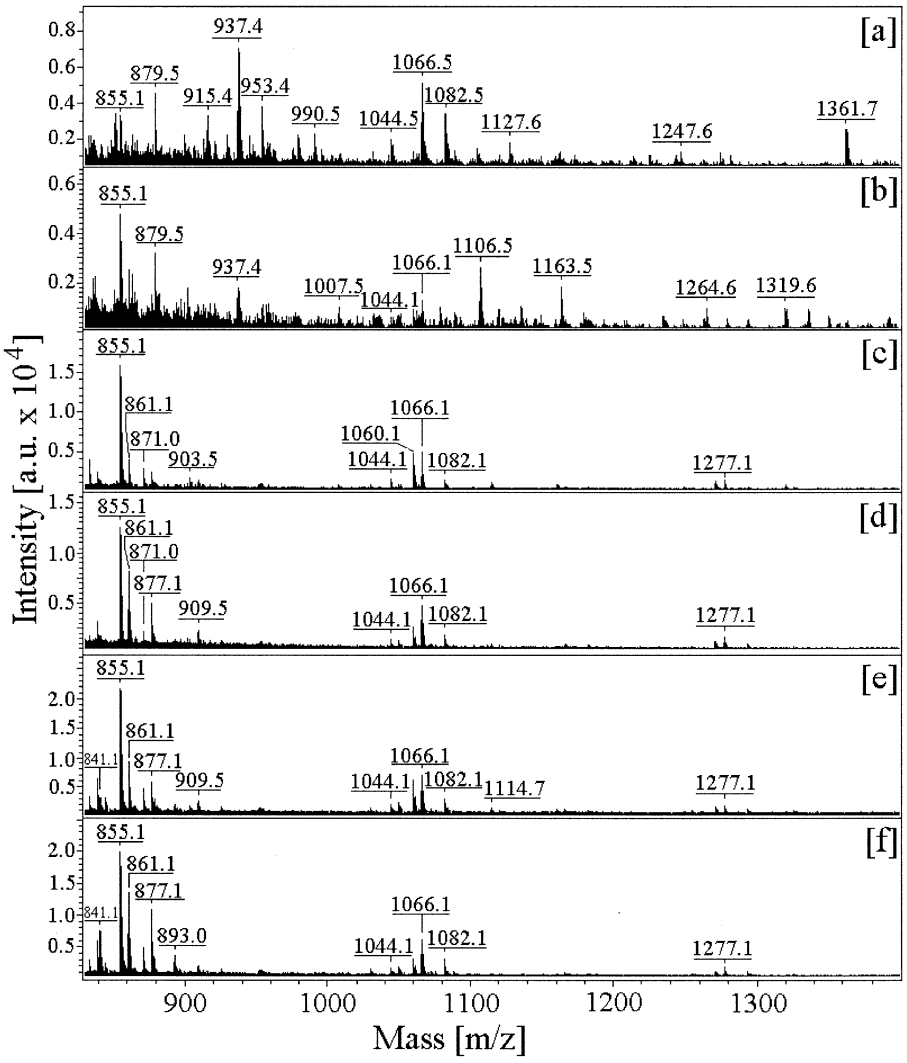

Figure 1. Conventional MALDI TOF MS of gelatine digests at a concentration of (**a**) 100,000 μg/ml, (**b**) 10,000 μg/ml, (**c**) 1000 μg/ml, (**d**) 100 μg/ml, (**e**) 10 μg/ml, and (**f**) 1 μg/ml using CHCA matrix and thin layer AnchorChip preparation. The mass assignments were internally corrected using matrix cluster masses after their further characterization by PSD and TOF/TOF experiments as described in the text.

accelerated to 8 kV and selected in a timed ion gate. In the LIFT-cell the fragments were further accelerated to 19 kV. The reflector potential was 29 kV.

Results and Discussion

MALDI TOF Mass Spectrometry Using CHCA

MALDI sample preparation of porcine gelatine digests employed AnchorChip target technology [16] using a modified thin layer sample preparation of CHCA [17], a commonly used MALDI matrix for peptide analysis particularly suitable for PSD analysis [18]. On-target sample enrichment using the AnchorChip technology was found to be essential to obtain interpretable signals using CHCA matrix. A typical MALDI spectrum recorded from a HCl digest of a 100 mg/ml porcine gelatine standard using such a sample preparation is shown in Figure 1a. The signal pattern was relatively complex representing the enormous variety of products resulting from the non-specific hydrochloric acid hydrolysis. Many low abundant peptides were detected, but most significantly, ions such as *m/z* 1044 or the respective sodium adduct at *m/z* 1066 were detected as major marker components [12]. Despite desalting with C18 ZipTips and using analytical grade reagents and containers to avoid contamination, ubiquitous sodium ions could not be entirely removed as was evident from the relatively large intensity of *m/z* 1066. Various commercially available qualities of CHCA were also investigated as a potential source for sodium contamination, one of which was doubly recrystallized, however, all CHCA batches showed a comparable tendency towards

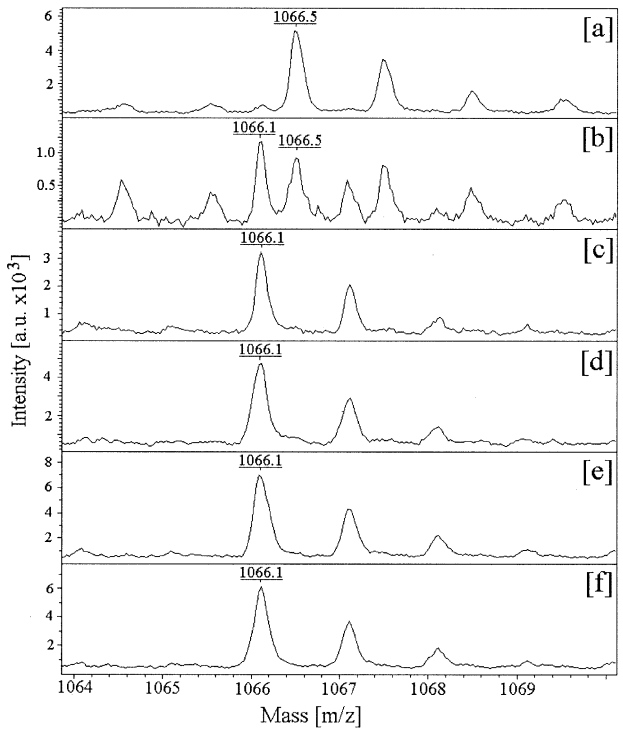

Figure 2. Isotopic envelopes of the signals at *m/z* 1066 in a 6 Da mass window at a gelatine digest concentration of (**a**) 100,000 μg/ml, (**b**) 10,000 μg/ml, (**c**) 1000 μg/ml, (d) 100 μg/ml, (e) 10 μg/ml, and (f) 1 μg/ml using CHCA matrix and thin layer AnchorChip preparation. The presence of two distinct ion species at *m/z* 1066.5 and 1066.1 is clearly demonstrated in (**b**). The mass assignments were internally corrected using matrix cluster masses after their further characterization by PSD and TOF/TOF experiments as described in the text.

sodium adduct formation. When analyzing the hydrolytic products from less concentrated gelatine standard preparations such as 10,000, 1000, 100, 10, and 1 μg/ml, by MALDI, the spectra gradually appeared to be less complex and better signal-to-noise ratios were obtained for many of the signals (Figure 1b–f). MALDI signals at *m/z* 1044 and 1066 were observed at all gelatine digest concentrations, however, their intensity did not decrease with lower gelatine concentrations. Remarkable also was the observation of a few new signals at these lower gelatine concentrations, such as those at *m/z* 861 and 1277.

A closer investigation of the isotopic envelopes of the signal at *m/z* 1066 at all gelatine concentrations is presented in Figure 2a–f. Evidently, the fractional part of the ion signal at *m/z* 1066 changed from 0.5 to 0.1 Da from higher to lower gelatine concentrations. At the 10 mg/ml gelatine concentration two ion species at *m/z* 1066 were detected in coexistence (Figure 2b). However, at lower analyte concentrations only a signal at *m/z* 1066.1 could be observed (Figure 2c–f). The fractional part of peptide masses can be predicted [19] and usually ranges from 0.4 to 0.7 Da for peptides between 1000 and 1100 Da [11]. This is a result of the contribution of near-integral masses of the elements typically found in

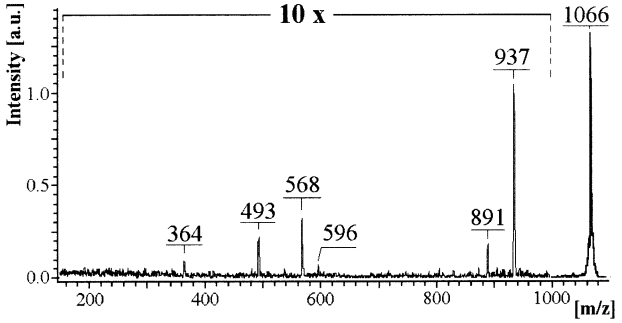

Figure 3. PSD MALDI spectrum of the sodiated peptide marker signal at *m/z* 1066 from a CHCA preparation at 100 mg/ml porcine gelatine digest. The intensity of the mass region from *m/z* 150 to 1000 was expanded ten times.

peptides, such as C, H, N, O, and S [10, 19]. This knowledge is useful for distinguishing peptides from molecules with a largely different elemental composition, such as those with large amounts of low-fractional mass elements such as the alkali metals, sodium, and potassium. It was therefore suspected that the ion signal at *m/z* 1066.1 was not in fact a peptide but a cluster of CHCA matrix molecules with alkali metal ions. A comparable phenomenon was observed with the peptide ion signal at *m/z* 1044.5 where the fractional part of the ion signal also changed from 0.5 to 0.1 Da from higher to lower gelatine concentrations. Again, at the 10 mg/ml gelatine concentration two ion species at *m/z* 1044 were detected in coexistence and at lower analyte concentrations only a signal at *m/z* 1044.1 could be observed. It appeared that the peptides of interest were entirely suppressed and replaced by a set of new signals in the MALDI spectra at and below a concentration of 1 mg/ml of the gelatine digest. This was found to be misleading when the analytical goal was to detect gelatine-derived peptides at *m/z* 1044/1066. This phenomenon was further investigated in order to fully characterize these interferences.

MALDI Post-Source Decay TOF Mass Spectrometry

A fragmentation fingerprint by post-source decay (PSD) would further characterize the proposed marker ions and support their discrimination from interferences as described above. Unfortunately, PSD of *m/z* 1044.5 from concentrated gelatine digests using CHCA matrix showed no reasonable fragmentation yields, probably because of comparatively low abundance. In contrast, the sodium adduct $[M + Na]^+$ at *m/z* 1066.5 showed moderate fragmentation under PSD conditions using CHCA matrix (Figure 3). PSD signals were observed at *m/z* 364, 493, 568, 596, 891, and a very abundant signal at *m/z* 937. It was not attempted to elucidate the complete sequence by means of PSD of the sodiated marker peptide ion at *m/z* 1066. However, the fragmentation seemed characteristic and a particularly useful

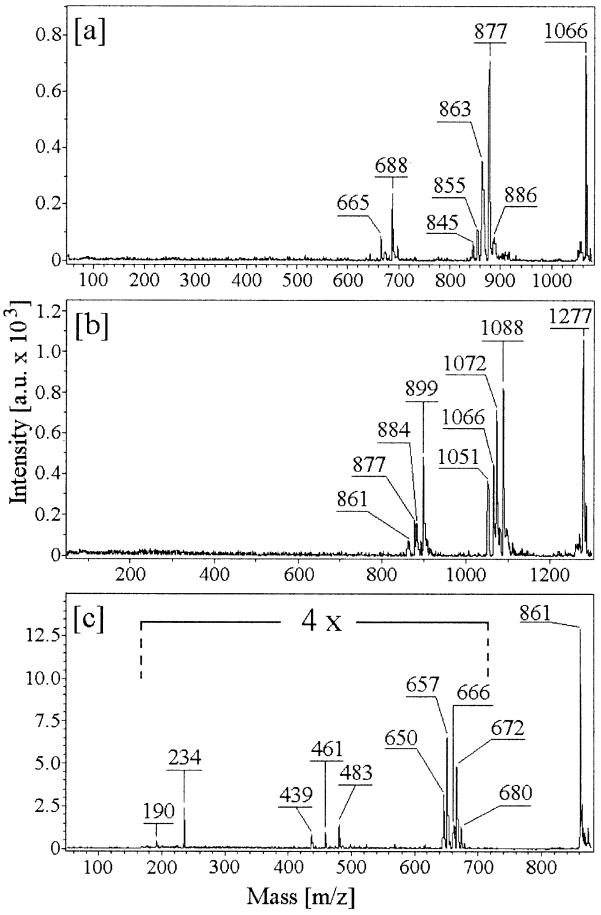

Figure 4. PSD MALDI spectra of CHCA clusters recorded from a 1 mg/ml porcine gelatine digest preparation using CHCA matrix. CHCA cluster at (**a**) *m/z* 1066, (**b**) *m/z* 1277, and (**c**) *m/z* 861 (intensity scale of low mass region expanded four times).

feature was the possibility to elucidate the C-terminal residue [20–22]. The main product ion from *m/z* 1066.5 was a sodiated b-type ion annotated as $[b_{n-1} + OH + Na]^+$ at *m/z* 937 resulting from a sodium mediated C-terminal rearrangement and loss of a glutamic acid residue. This indicated that the protonated/sodiated ion pair at *m/z* 1044.5/1066.5 was indeed a peptide or was at least peptide-like.

A PSD spectrum of the interference signal at *m/z* 1066.1 detected at a gelatine digest concentration of 1 mg/ml was also acquired (Figure 4a). Clearly, the fragmentation fingerprint was different from that of *m/z* 1066.5 (Figure 3), with major signals at *m/z* 665, 688, 845, 855, 863, 877, and 886. The interference was identified to be a cluster of CHCA matrix molecules with alkali metal ions and has the composition of $[5M - 3H + 2K + 2Na]^+$. The occurrence of CHCA matrix clusters has been reported previously and follows the general formula $M_{Cluster} = nM - xH + yK + zNa$ with $x = y + z - 1$ and $y + z \leq n + 1$ [9]. The CHCA clusters and also their PSD fragments reported in this study agree well with this formula. Conventional MALDI TOF spectra that contained the matrix cluster signal at *m/z* 1066.1 also exhibited signals at *m/z* 861 and 1277 that could be classified as matrix cluster signals (see Figure 1b–f). According to the above formula, the matrix cluster signals correspond to $[4M - 3H + 1K + 3Na]^+$ for *m/z* 861 and $[6M - 4H + 2K + 3Na]^+$ for *m/z* 1277, respectively. The PSD spectra of *m/z* 861 and 1277 confirm their identity as CHCA clusters (Figure 4b and c). The PSD fragment ion signals of the analyzed CHCA clusters to which fragment structures could be proposed are summarized in Table 1. Most of these complexes contained more sodium ions than potassium ions, in contrast to many of the cluster signals reported in the literature [10]. Sodium and potassium salts are ubiquitous impurities and their complete removal can be very difficult. Loss of water or cyano groups from a CHCA molecule within a cluster were not observed; even the unassigned masses did not calculate to matrix fragments containing such losses. All assigned fragmentations could be attributed to neutral evaporation, more specifically to loss of intact neutral CHCA molecules, deprotonated sodium, or potassium adducts of CHCA or combinations and multiples thereof. This observation is in good agreement with the experimentally determined neutral evaporation from emitted cluster species [23]. For instance, the major PSD signal from *m/z* 1066 is *m/z* 877 which calculates to a loss of a neutral CHCA unit (189 mass units). In contrast, *m/z* 855 resulting from post-source decay of *m/z* 1066 computes to a loss of a neutral deprotonated sodium adduct of

Table 1. The MALDI post-source decay fragment ions of observed CHCA cluster signals at *m/z* 861, 1066, and 1277 compared with the calculated masses

m/z 861 $[4M - 3H + 1K + 3Na]^+$			*m/z* 1066 $[5M - 3H + 2K + 2Na]^+$			*m/z* 1277 $[6M - 4H + 2K + 3Na]^+$		
Obs'd[a]	Calc'd[b]	Formula	Obs'd[a]	Calc'd[b]	Formula	Obs'd[a]	Calc'd[b]	Formula
672	672	$[3M - 3H + 1K + 3Na]^+$	877	877	$[4M - 3H + 2K + 2Na]^+$	1088	1088	$[5M - 4H + 2K + 3Na]^+$
666	666	$[3M - 2H + 2K + 1Na]^+$	855	855	$[4M - 2H + 2K + 1Na]^+$	1066	1066	$[5M - 3H + 2K + 2Na]^+$
650	650	$[3M - 2H + 1K + 2Na]^+$	688	688	$[3M - 3H + 2K + 2Na]^+$	899	899	$[4M - 4H + 2K + 3Na]^+$
461	461	$[2M - 2H + 1K + 2Na]^+$				877	877	$[4M - 3H + 2K + 2Na]^+$
439	439	$[2M - 1H + 1K + 1Na]^+$				861	861	$[4M - 3H + 1K + 3Na]^+$
234	234	$[1M - 1H + 2Na]^+$						
190	190	$[1M + 1H]^+$						

[a]Observed fragment masses are monoisotopic and were rounded to the nearest integer.
[b]Calculated fragment masses are monoisotopic and were also rounded to the nearest integer for comparison with the observed values.

CHCA, [M − H + Na]. The smallest observed fragment was an isolated proton adduct of CHCA at *m/z* 190 produced from the precursor cluster at *m/z* 861. Noteworthy is also that the PSD fragmentation patterns of all analyzed CHCA complexes show signals clustering around median masses which are usually separated approximately by the mass of one CHCA including a cation, as previously reported for intact matrix clusters without further fragmentation [9].

MALDI LIFT-TOF/TOF Mass Spectrometry

Characterization and discrimination of potential matrix interferences can be an important aid in avoiding spectral misinterpretation as demonstrated above. However, the acquisition of a PSD spectrum may take up considerable time (approximately twenty min) when stepping down the reflector voltage. Alternatively, the product ions from laser-induced metastable decay can be recorded in a more sophisticated MALDI TOF/TOF instrument employing the LIFT technology [14, 15]. A relatively low voltage of 8 kV is applied initially for ion acceleration. Fragments generated from laser-induced dissociation are subsequently raised to a higher potential (19 kV) in the LIFT cell. This enables the rapid (seconds) detection of all fragments without changing the reflector voltage, which compared to conventional PSD is particularly advantageous for the detection of low mass ions of low abundance. To demonstrate the utility of this approach the LIFT spectra of the CHCA cluster signals at *m/z* 1066 and 861 are presented in Figure 5. Generally, major fragment signals obtained by LIFT agree well with PSD fragments (Figure 4) in terms of relative abundance, e.g., signals at *m/z* 877.0, 854.9, and 687.9 from the CHCA cluster at *m/z* 1066.1 (Figure 5a). Similarly, major LIFT fragments from the CHCA cluster at *m/z* 861.1 (Figure 5b) such as *m/z* 671.9, 649.9, and 482.7 are also in good agreement with the respective PSD data. The MALDI LIFT signals are summarized in Table 2. A cluster composition could be assigned for many of the signals lying within 0.3 mass units of the calculated values. Again, no water or cyano group losses were observed. As mentioned above, a major advantage of the LIFT spectra compared with PSD is the observation of low molecular weight fragments of low abundance. This is particularly noticeable for the cluster at *m/z* 1066.1 where further information about the precursor cluster was obtained by the observation of fragments below *m/z* 665, the lowest signal observed by PSD.

MALDI TOF Mass Spectrometry Using DHB

The MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was investigated as an alternative to CHCA as the latter was shown not only to hamper sensitivity but also to produce misleading mass spectra. DHB preparations demonstrated enhanced sensitivity compared with CHCA as illustrated for the lowest analyzed concentration of porcine gelatine digest at 1 μg/ml (Figure 6). The MALDI spectrum shows the protonated and sodiated marker peptide at *m/z* 1044/1066 with an inset expanding the mass region around *m/z* 1066. Clearly, the monoisotopic signal is found at *m/z* 1066.5 and, expectedly, no signal at *m/z* 1066.1 was detected (dashed line). For comparison, the corresponding MALDI spectrum of the same gelatine digest concentration using CHCA showed only matrix cluster signals (Figure 1f). DHB is also known to form clusters with alkali metal ions but to a lesser degree [9]. Using the conditions as described above, a signal at *m/z* 1199.1 was occasionally observed and probably originates from a DHB cluster calculating to $[7M - 3H + 2K + 2Na]^+$. However, the automatic acquisition and processing of MALDI data from gelatine digests using DHB is not impeded by signal interferences in the relevant mass range.

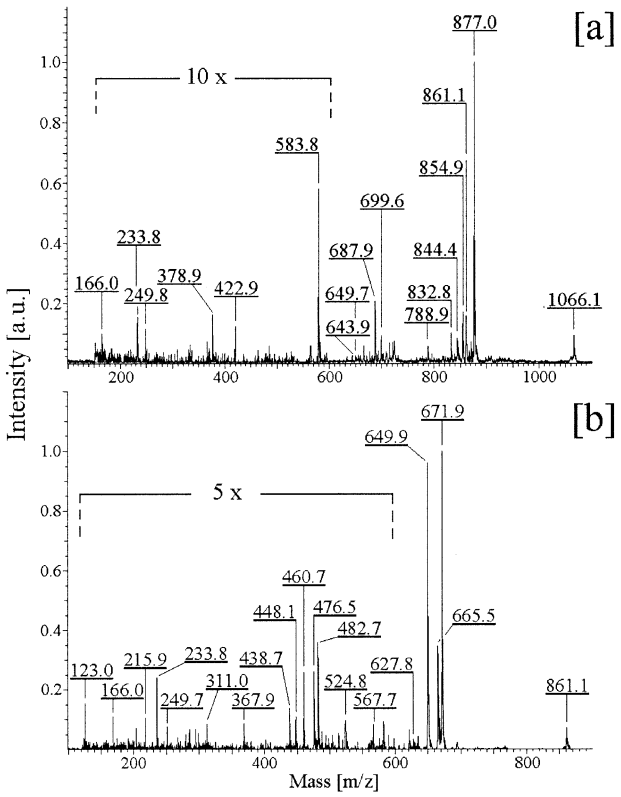

Figure 5. MALDI LIFT-TOF/TOF mass spectra of the CHCA cluster signals at (**a**) *m/z* 1066.1 and (**b**) *m/z* 861 recorded from a HCl digest of 1 mg/ml porcine gelatine. The low mass regions are expanded ten and five times, respectively.

Conclusions

The occurrence of matrix clusters arising from complexes of CHCA with alkali metal ions may severely hamper sensitive MALDI analysis of complex peptide mixtures and produce spectra that may be misinterpreted. CHCA clusters were shown to suppress the peptide signals resulting from hydrochloric acid diges-

Table 2. The MALDI LIFT-TOF/TOF fragment ions of observed CHCA cluster signals at *m/z* 1066 and 861 compared with the calculated masses. Only signals within 0.3 mass units were included

m/z 1066 $[5M - 3H + 2K + 2Na]^+$			*m/z* 861 $[4M - 3H + 1K + 3Na]^+$		
Obs'd[a]	Calc'd[b]	Formula	Obs'd[a]	Calc'd[b]	Formula
877.0	877.0	$[4M - 3H + 2K + 2Na]^+$	671.9	672.0	$[3M - 3H + 1K + 3Na]^+$
854.9	855.1	$[4M - 2H + 2K + 1Na]^+$	649.9	650.1	$[3M - 2H + 1K + 2Na]^+$
832.8	833.1	$[4M - 1H + 2K]^+$	627.8	628.1	$[3M - 1H + 1K + 1Na]^+$
687.9	688.0	$[3M - 3H + 2K + 2Na]^+$	460.7	461.0	$[2M - 2H + 1K + 2Na]^+$
643.9	644.0	$[3M - 1H + 2K]^+$	438.7	439.0	$[2M - 2H + 1K + 2Na]^+$
422.9	423.1	$[2M - 1H + 2Na]^+$	249.7	250.0	$[1M - 1H + 1K + 1Na]^+$
378.9	379.1	$[2M + 1H]^+$	233.8	234.0	$[1M - 1H + 2Na]^+$
249.8	250.0	$[1M - 1H + 1K + 1Na]^+$			
233.8	234.0	$[1M - 1H + 2Na]^+$			

[a]Observed fragment masses are monoisotopic and were rounded to the first decimal place. Masses are presented to one decimal place but this should not be interpreted as the mass accuracy.
[b]Calculated fragment masses are monoisotopic and were also rounded to the first decimal place.

tion of porcine gelatine. The problem was exacerbated when a peptide of interest coincidentally had the same nominal mass as a matrix cluster. In this case, fragmentation analysis of MALDI-generated ions by using conventional post-source decay (PSD) TOF or LIFT-TOF/TOF technology provided powerful means to distinguish peptides from matrix cluster interferences as described for a sodiated peptide (*m/z* 1066.5) and a CHCA cluster (*m/z* 1066.1). Both techniques induced neutral evaporation of matrix clusters to produce smaller sized clusters without noticeable matrix disintegration. Knowledge of the cluster molecular composition allowed these discrete peaks to be used for internal calibration of the MALDI spectra. The computed values for the major analyzed CHCA clusters are 861.0801, 1066.0966, and 1277.1212 Da. For the purpose of internal spectral calibration, the masses of further clusters can be calculated according to the formula provided by Keller and Li [9].

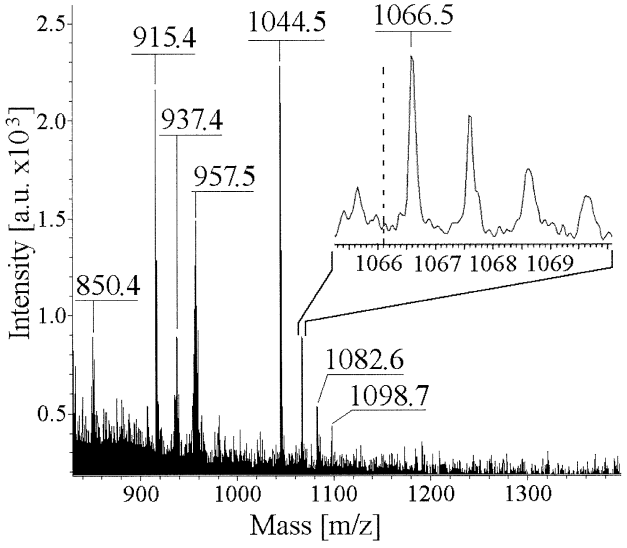

Figure 6. MALDI TOF mass spectrum of a porcine gelatine digest at a concentration of 1 μg/ml using DHB matrix. The inset shows the isotopic envelope of the signal at *m/z* 1066.5. The dashed line illustrates the absence of a signal at *m/z* 1066.1.

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