

# Characterization of intact *Penicillium* spores by matrix-assisted laser desorption/ionization mass spectrometry

Hun-Yun Chen and Yu-Chie Chen\*

Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan

Received 23 March 2005; Revised 22 September 2005; Accepted 4 October 2005

The fungal spores of *Penicillium expansum*, *P. chrysogenum*, *P. citrinum*, *P. digitatum*, *P. italicum*, and *P. pinophilum* were characterized by using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOFMS). These fungal spores are frequently found in grain and fruit. The mass spectra of these six species were directly obtained from the intact spores without any pretreatment. The results obtained indicate that 2,5-dihydroxybenzoic acid and sinapinic acid are suitable matrices for the analysis of *Penicillium* spores. Characteristic ions representing the different species were obtained with sufficiently high reproducibility that these ions can be employed to identify the different fungal species. On the basis of these characteristic ions obtained from these authentic *Penicillium* spores, the approach was applied to characterize the fungal species contaminating the surfaces of fruit. It was demonstrated that the fungal spores directly scratched from the surfaces of fruit contaminated by unknown fungi can be rapidly identified using MALDI-TOFMS analysis without any tedious pretreatment. Copyright © 2005 John Wiley & Sons, Ltd.

Matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOFMS) has been widely used to characterize microorganism species.<sup>1–21</sup> Most studies are focused on the analysis of bacteria that are pathogenic to human beings.<sup>1–18</sup> Only a few reports have discussed the effectiveness of employing the technique to characterize fungal spores.<sup>19–21</sup> Unlike the MALDI mass spectra obtained from bacteria, the mass spectral profiles of intact fungal spores obtained by direct analysis are less complicated. Nevertheless, they are sufficient to differentiate different fungal species on the base of the limited ions. Welham and co-workers presented the first paper describing use of MALDI-TOFMS to characterize different fungal spores;<sup>19</sup> the mass spectral profiles obtained from *Penicillium* spp., *Scytalidium dimidiatum*, and *Trichophyton rubrum* indicated the potential to identify the fungal species by simply using MALDI mass spectra. In a previous study<sup>20</sup> we demonstrated that the MALDI-TOFMS results obtained for aflatoxigenic and non-aflatoxigenic *Aspergillus* spores have different mass spectral profiles.

*Penicillium* species such as *P. citrinum*, *P. italicum*, and *P. digitatum* frequently contaminate citrus fruits;<sup>22–24</sup> these

fungal spores are frequently found on the surfaces of these fruits. Additionally, *P. expansum*<sup>25,26</sup> and *P. pinophilum*<sup>27</sup> are commonly found on apples. This work is focused on the analysis of fungal spores from six *Penicillium* spp., i.e. *P. expansum*, *P. chrysogenum*, *P. citrinum*, *P. italicum*, *P. digitatum*, and *P. pinophilum*, by using MALDI-TOFMS. The fungal spores from different strains within each of these six species were also compared.

## EXPERIMENTAL

Sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB), bradykinin, and ubiquitin were purchased from Sigma (St. Louis, MO, USA), while  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) was purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany). Melittin was purchased from Fluka (Buchs, Switzerland). Potato dextrose agar (PDA) was purchased from Difco (MD, USA).

Fungal spores were purchased from the Culture Collection and Research Center (CCRC) in Taiwan, and cultured in this laboratory. The medium for growing fungal spores was composed of PDA (24 g/L) mixed with granulated agar (15 g/L, Becton Dickinson, MD, USA). Cultivation on agar plates was performed with this medium at ca. 30°C for 5–10 days. A pipette tip was employed to scratch the spores cultured on a medium plate. A volume of 3  $\mu$ L of MALDI matrix solution was used to wash off the spores from the tip.

\*Correspondence to: Y.-C. Chen, Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan.  
E-mail: yuchie@mail.nctu.edu.tw  
Contract/grant sponsor: Department of Health, Executive Yuan of Taiwan; contract/grant number: DOH93-TD-F-113-005.

The matrix was dissolved (30 mg/mL) in different solvents such as ACN/(0.1% TFA/H<sub>2</sub>O) (2:1, v/v), ACN/(1% TFA/H<sub>2</sub>O), and methanol/(1% TFA/H<sub>2</sub>O) (2:1, v/v). A volume of 0.5  $\mu$ L of the mixture containing spores and matrix solution was deposited on a sample target. After the volatile solvent had evaporated, the sample was ready for MALDI-TOFMS analysis.

Bradykinin, melittin, and ubiquitin were used for internal mass calibration. Since internal standards can affect the intensities of the ion peaks from the samples, two identical samples were analyzed in parallel by MALDI-TOFMS but only one of these samples had the internal standards added.

All the mass spectra were obtained using a Biflex III time-of-flight mass spectrometer (Bruker Daltonics, Germany). The mass spectrometer was equipped with a 337-nm nitrogen laser, a 1.25-m flight tube, and a sample target with the capacity to load 384 samples simultaneously. The accelerating voltage was set to 19 kV.

## RESULTS AND DISCUSSION

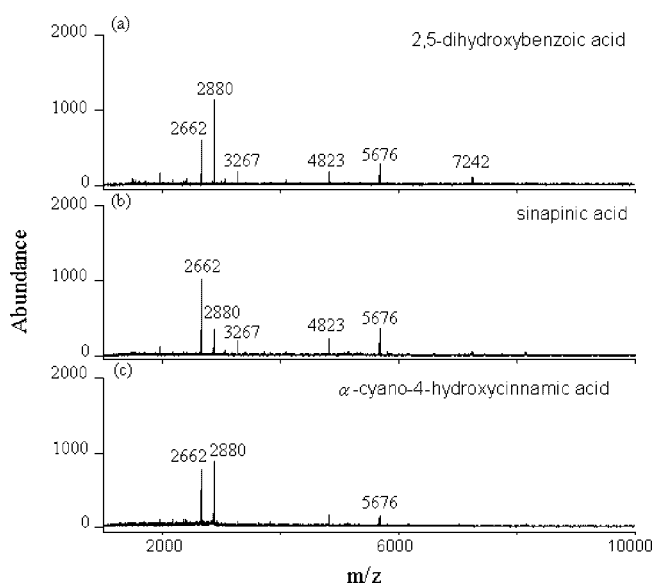
The selection of matrix and the solvent for dissolving the matrix can affect the MALDI mass spectra of intact bacterial cells, as shown previously by Musser and co-workers.<sup>28</sup> Thus, as a first step, several matrices were tested for the analysis of fungal spores by MALDI-TOFMS. Figures 1(a)–1(c) present the MALDI mass spectra of fungal spores from *P. expansum* (CCRC 32045), which had been directly scratched from agar plates and then mixed with solutions of 2,5-DHB, SA, and CHCA, respectively. Figure 1(a) displays more ions in the mass spectrum than those in Figs. 1(b) and 1(c). The peaks at  $m/z$  2662, 2880, 4823, and 5676 reproducibly appear in Figs. 1(a)–1(c). These results indicate that both 2,5-DHB and SA are suitable matrices for such MALDI-TOFMS analyses; all the mass spectra displayed in the following were obtained using 2,5-DHB as MALDI matrix. Additionally, several solvents such as ACN/(1% TFA/H<sub>2</sub>O) (2:1, v/v), ACN/

(0.1% TFA/H<sub>2</sub>O), and methanol/(1% TFA/H<sub>2</sub>O) were used to dissolve the matrix. When ACN/(1% TFA/H<sub>2</sub>O) (2:1, v/v) was used, more ions appeared in the MALDI mass spectra of intact spores than those when using matrix dissolved in the other two solvents. Thus, 2,5-DHB dissolved in ACN/(1% TFA/H<sub>2</sub>O) (2:1, v/v) was used for MALDI-TOFMS analysis of intact spores in this study.

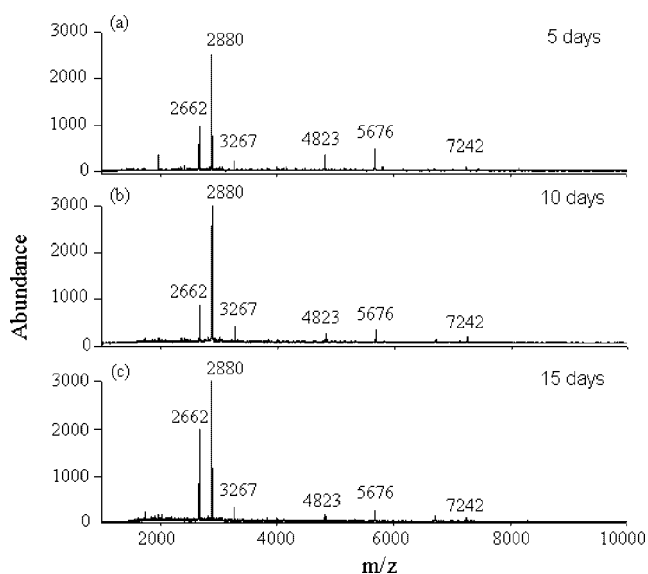
In order to investigate whether the age of the spores would affect the MALDI mass spectral patterns, the spores of *P. expansum* obtained on different days following initiation of the culture were analyzed by MALDI-TOFMS. Figures 2(a)–2(c) present the MALDI mass spectra of fungal spores from *P. expansum* (CCRC 32045) cultured for 5, 10, and 15 days, respectively. The ions at  $m/z$  2662, 2880, 3267, 4823, 5676, and 7242 repeatedly appear in all the MALDI mass spectra. Since these three mass spectral profiles are similar to one another, the fungal spores obtained in the remainder of the work were cultured for 5 days before performing MALDI-TOFMS analyses.

In order to compare the spectra of different strains within the same fungal species, several strains of *P. chrysogenum* were analyzed using MALDI-TOFMS. Figures 3(a)–3(d) present the MALDI mass spectra of fungal spores from four strains of *P. chrysogenum*, i.e. CCRC 30298, ATCC 10002, ATCC 7813, and ATCC 11625. The ion peaks at  $m/z$  3140 and 5173 appear in each mass spectrum with very good reproducibility, which indicates that these two ions can be used as biomarker ions for *P. chrysogenum*.

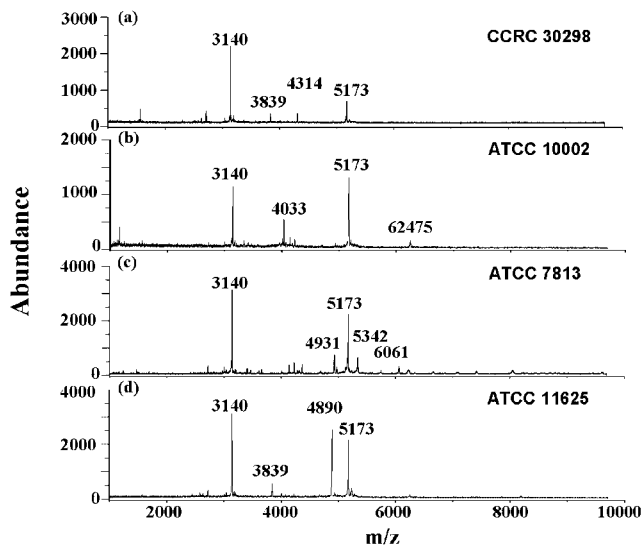
In addition to *P. expansum* and *P. chrysogenum*, fruit is frequently contaminated by *P. italicum*, *P. digitatum*, *P. citrinum*, and *P. pinophilum*. Figures 4(a)–4(d) present the MALDI mass spectra of fungal spores from four strains of *P. italicum*, i.e. ATCC 48814, CCRC 32575, CCRC 32630, and CCRC 35176, respectively. The peak at  $m/z$  2994 dominates the mass spectra. Furthermore, this peak is the only one that appears in each mass spectrum, indicating that this ion can be used as the biomarker ion to represent *P. italicum*.



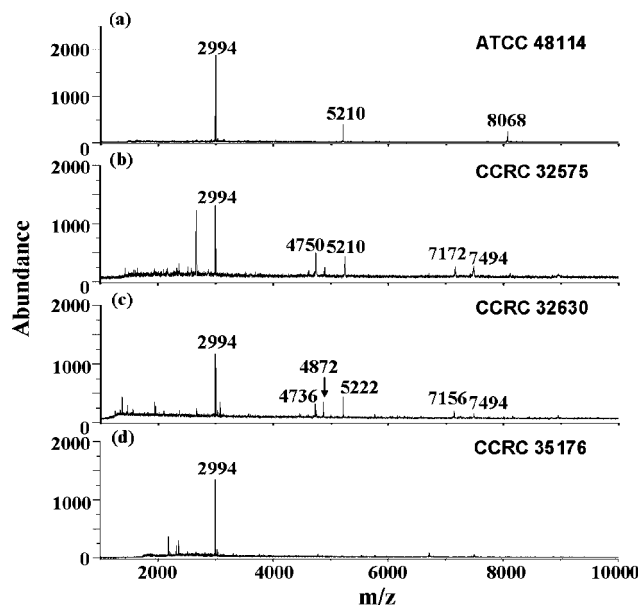
**Figure 1.** MALDI mass spectra of fungal spores from *P. expansum* (CCRC 32045) directly mixed with matrices (a) 2,5-DHB, (b) SA, and (c) CHCA.



**Figure 2.** MALDI mass spectra of fungal spores from *P. expansum* (CCRC32045) cultured for (a) 5, (b) 10, and (c) 15 days.

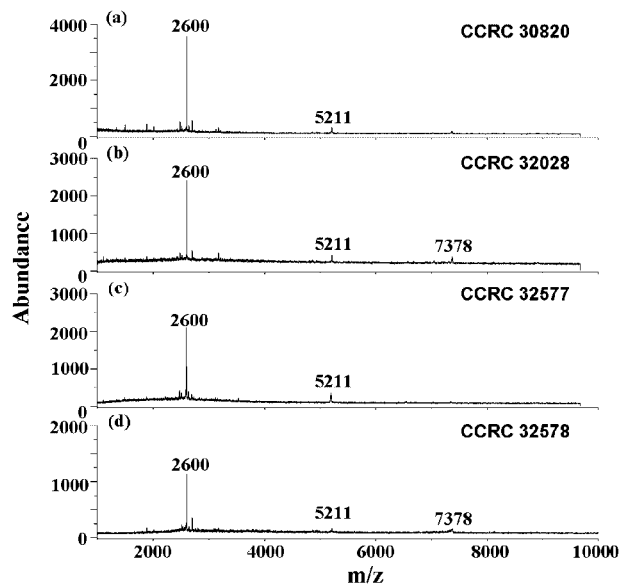


**Figure 3.** MALDI mass spectra of fungal spores from four different strains of *P. chrysogenum*: (a) CCRC 30298, (b) ATCC 10002, (c) ATCC 7813, and (d) ATCC 11625.

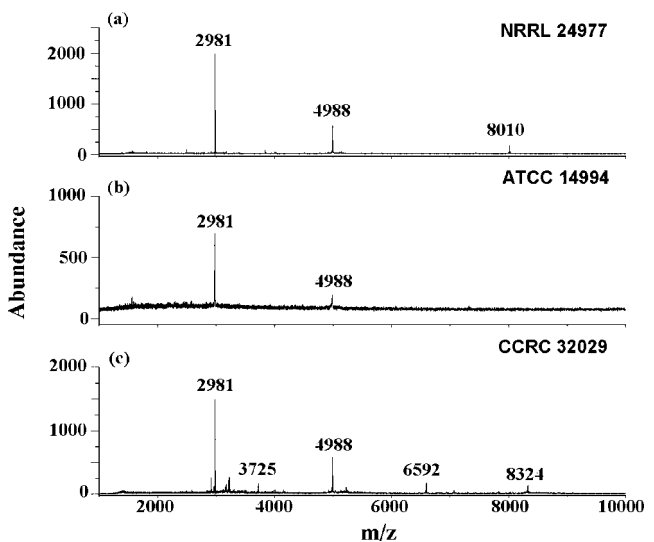


**Figure 4.** MALDI mass spectra of fungal spores from four different strains of *P. italicum*: (a) ATCC 48814, (b) CCRC 32575, (c) CCRC 32630, and (d) CCRC 35176.

Figures 5(a)–5(d) display the MALDI mass spectra of fungal spores from four strains of *P. digitatum*, i.e. CCRC 30820, CCRC 32028, CCRC 32577, and CCRC 32578, respectively. All the mass spectra resemble one another, and the peaks at  $m/z$  2600, 5211, and 7378 appear in all the mass spectra; thus these ions can represent *P. digitatum*. Figures 6(a)–6(c) display the MALDI mass spectra of fungal spores from different strains of *P. citrinum*, i.e. NRRL 24977, ATCC 14994, and CCRC 32029. The peak at  $m/z$  2981 dominates all the mass spectra, while the peak at  $m/z$  4988 also appears in each mass spectrum; this indicates that these ions are the characteristic ions for *P. citrinum*. Additionally, *P. pinophilum* is frequently found on the surfaces of apples. Figures 7(a)–7(c) display the



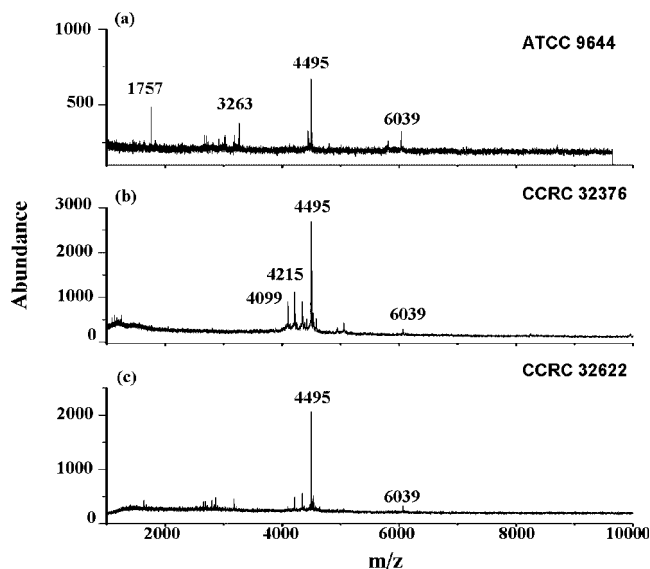
**Figure 5.** MALDI mass spectra of fungal spores from four different strains of *P. digitatum*: (a) CCRC 30820, (b) CCRC 32028, (c) CCRC 32577, and (d) CCRC 32578.



**Figure 6.** MALDI mass spectra of fungal spores from three different strains of *P. citrinum*: (a) NRRL 24977, (b) ATCC 14994, and (c) CCRC 32029.

MALDI mass spectra of different strains of *P. pinophilum* including ATCC 9644, CCRC 32376, and CCRC 32622. The peak appearing at  $m/z$  4495 dominates the mass spectra, while the less intense peak at  $m/z$  6069 appears in each mass spectrum, so these ions can be used as biomarker ions for *P. pinophilum*.

The biomarker ions recommended as biomarkers for these six *Penicillium* species are listed in Table 1; the mass spectral profiles are similar for different strains within the same species, while the biomarker ions for different *Penicillium* species are different from one another. Note that the majority of the biomarker ions are below 5 kDa. This mass region is similar to that found in the previous studies of biomarkers for fungal spores,<sup>19,20</sup> and is significantly lower than that generally reported in most bacteria studies that generally find the biomarker ions in the mass region above 5 kDa. The



**Figure 7.** MALDI mass spectra of fungal spores from three different strains of *P. pinophilum*: (a) ATCC 9644, (b) CCRC 32376, and (c) CCRC 32622.

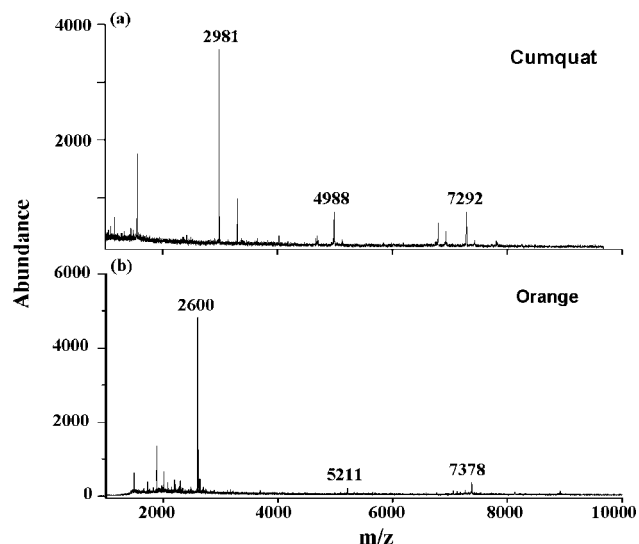
differences may arise from the different compositions of the cell walls.

Based on these results, this approach was applied to characterize the fungal spores contaminating fruit. We used the fruit samples purchased from a supermarket, that were obviously contaminated by fungi, as the realistic test samples. Figure 8(a) presents the MALDI mass spectrum of the sample obtained by scratching the fungal spores from the surface of a rotted cumquat. The peaks appearing at  $m/z$  2981 and 4988 imply that the fruit was contaminated by *P. citrinum* (cf. Fig. 6). Figure 8(b) displays the MALDI mass spectrum of the sample obtained by scratching the fungal spores from the surface of a rotted orange contaminated by fungi. The peak at  $m/z$  2600 dominates the MALDI mass spectrum, and the smaller peak at  $m/z$  5211 belonging to one of the biomarker ions of *P. digitatum* also appears in the MALDI mass spectrum, indicating that the fungal species contaminating this orange was *P. digitatum*.

These results suggest that, on the basis of the characteristic ions of *Penicillium* spp., the spores directly scratched from the surface of rotted fruit can be rapidly identified. The interference ions (other than those assigned to fungal spores) are very sparse. It has been reported previously that both *P. citrinum* and *P. digitatum* are the fungi commonly found in citrus fruit.<sup>22–24</sup> The present approach provides a rapid method to characterize the fungal species.

**Table 1.** List of biomarker ions of *Penicillium* spores appearing in MALDI mass spectra

Species	Biomarker ions ( $m/z$ )
<i>P. expansum</i>	2662, 2880, 3267, 5676, 7242
<i>P. chrysogenum</i>	3140, 5173
<i>P. italicum</i>	2994
<i>P. digitatum</i>	2600, 5211, 7378
<i>P. citrinum</i>	2981, 4988
<i>P. pinophilum</i>	4495, 6069



**Figure 8.** MALDI mass spectra of the samples of fungal spores scratched directly from the surfaces of rotted fruit: (a) cumquat and (b) orange.

## CONCLUSIONS

This work reports the biomarker ions, observed using MALDI-TOFMS analysis, that represent six *Penicillium* species. Based on these biomarker ions, rapid screening of fruit for contamination by fungal spores, without any tedious sample pretreatment, was demonstrated. Simply scratching of the sample from the rotted surface and mixing this sample with MALDI matrix solution are the only two steps required before performing the MALDI-TOFMS analysis. Note that the fungal spores tested in this work are the most common species found on citrus fruit and apples. The biomarker ions obtained in this study provide information for a reliable and rapid identification of the species of fungal spores that contaminate the surfaces of these fruit when using MALDI-MS. This approach provides a much more rapid screening than the traditional methods such as immunoassay.

## Acknowledgements

We thank the Department of Health, Executive Yuan of Taiwan (under contract no. DOH93-TD-F-113-005) for supporting this research financially. We also thank Yi-Ting Wu for assistance in preparing the figures.

## REFERENCES

- Fenselau C, Demirev PA. *Mass Spectrom. Rev.* 2001; **20**: 157.
- Elhanany E, Barak R, Fisher M, *et al.* *Rapid Commun. Mass Spectrom.* 2001; **15**: 2110.
- Bacher G, Korner R, Atrih A, *et al.* *J. Mass Spectrom.* 2001; **36**: 124.
- Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees KJ, Lay JO Jr. *Rapid Commun. Mass Spectrom.* 1996; **10**: 1227.
- Liang X, Zheng K, Qian MG, Lubman DM. *Rapid Commun. Mass Spectrom.* 1996; **10**: 1219.
- Krishnamurthy T, Ross PL. *Rapid Commun. Mass Spectrom.* 1996; **10**: 1992.
- Welham KJ, Domin MA, Scannell DE, Cohen E, Ashton DS. *Rapid Commun. Mass Spectrom.* 1998; **2**: 176.

8. Wang Z, Russon L, Li L, Roser DC, Long SR. *Rapid Commun. Mass Spectrom.* 1998; **12**: 456.
9. Easterling ML, Colangelo CM, Scott RA, Amster IJ. *Anal. Chem.* 1998; **70**: 2704.
10. Arnold RJ, Reilly JP. *Rapid Commun. Mass Spectrom.* 1998; **12**: 630.
11. Lynn E-C, Chung M-C, Tsai W-C, Han C-C. *Rapid Commun. Mass Spectrom.* 1999; **13**: 2022.
12. Nilsson CL. *Rapid Commun. Mass Spectrom.* 1999; **13**: 1067.
13. Dai Y, Li L, Roser DC, Long SR. *Rapid Commun. Mass Spectrom.* 1999; **13**: 73.
14. Winkler MA, Uher J, Ceca S. *Anal. Chem.* 1999; **71**: 3416.
15. Demirev PA, Ho Y-P, Ryzhov V, Fenselau C. *Anal. Chem.* 1999; **71**: 2732.
16. Lazar LM, Ramsey RS, Ramsey JM. *Anal. Chem.* 2001; **73**: 1733.
17. Wahl KL, Wunschel SC, Jarman KH, Valentine NB, Petersen CE, Kingsley MT, Zartolas KA, Saenz AJ. *Anal. Chem.* 2002; **74**: 6191.
18. Madonna AJ, Cuyk SV, Voorhees KJ. *Rapid Commun. Mass Spectrom.* 2003; **17**: 257.
19. Welham KJ, Domin MA, Johnson K, Jones K, Ashton DS. *Rapid Commun. Mass Spectrom.* 2000; **14**: 307.
20. Li T-Y, Liu B-H, Chen Y-C. *Rapid Commun. Mass Spectrom.* 2000; **14**: 2393.
21. Amiri-Eiasi B, Fenselau C. *Anal. Chem.* 2001; **73**: 5228.
22. Wyatt MK, Parish ME. *Food Microbiol.* 1995; **12**: 237.
23. Wyatt MK, Parish ME, Widmer WW, Kimbrough J. *Food Microbiol.* 1995; **12**: 347.
24. Stange RR, Midland SL, Sims JJ, McCollum TG. *Physiol. Mol. Plant.* 2002; **61**: 303.
25. Sanderson PG, Spotts RA. *Phytopathology* 1995; **85**: 103.
26. Chen L, Ingham BH, Ingham SC. *J. Food. Sci.* 2004; **69**: C669.
27. Vinas I, Vallverdu N, Monllao S, Usall J, Sanchis V. *Mycopathologia* 1993; **123**: 27.
28. Williams TL, Andrzejewski D, Lay JO Jr, Musser SM. *J. Am. Soc. Mass Spectrom.* 2003; **14**: 342.