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Mass spectrometric determination of ergosterol in a prairie natural wetland

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Abstract

Fungi are the main decomposers of plant material in an aquatic system. Levels of ergosterol, a compound generally specific to the cell membranes of fungi can be used as an indirect measure of their presence and biomass. Described is a procedure utilising reversed-phase liquid chromatography with positive-ion atmospheric pressure chemical ionization tandem mass spectrometry (LC–APCI–MS–MS) for full quantification and confirmation of ergosterol in various wetland matrices. Solid and liquid samples (0.2–1 g dry weight and 10 ml) were subjected to alkaline saponification followed by serial extraction using pentane (3×10 ml). The procedure was applicable to quantitative analysis of wetland samples with little or no clean up. Under low energy (CID) collision induced dissociation conditions the major product-ion formed from m/z 379.4 $[M+H-H_2O]^+$ was m/z 69.4 $[(CH_3)_2CHCH{=}CH]^+$. Selected reaction monitoring (SRM) of this transition along with the retention time were used to confirm that ergosterol was widely distributed at ppm levels (2.4 to 303 μg ash free dry mass (AFDM)) in matrices of decaying willow leaves, *Scirpus* stems (living and dead) and sediment collected at the water–sediment interface. Comparison between LC–APCI–MS–MS (SRM), LC–APCI–MS using selected ion monitoring (SIM) and liquid chromatography with ultraviolet absorption detection (LC–UV) indicated that SRM analysis was the most selective technique.

Keywords: Mass spectrometry; Ergosterol

1. Introduction

Bacteria and fungi are the main decomposers of plant material in aquatic environments. In some systems, fungi can be predominant, accounting for up to 90% of the total microbial biomass production [1–3]. Ergosterol, the chemical structure given in Fig. 1, is a cell membrane component largely restricted to fungi [4,5] thus making it an ideal index molecule for these micro-organisms. Ergosterol is believed to undergo rapid degradation after cell death and thus levels of ergosterol present are generally considered to be directly correlated to living fungal biomass [6,7]. Most research to date has focused on fungal biomass in decaying plant leaves or grasses in salt-marshes, streams or in forest soils where there is a high plant litter content [1,2,8]. However, little work has been reported for the detection of ergosterol in environmental matrices other than those noted and only one on extraction efficiencies [9] in northern prairie wetlands.

Various methods have been reported for the de-

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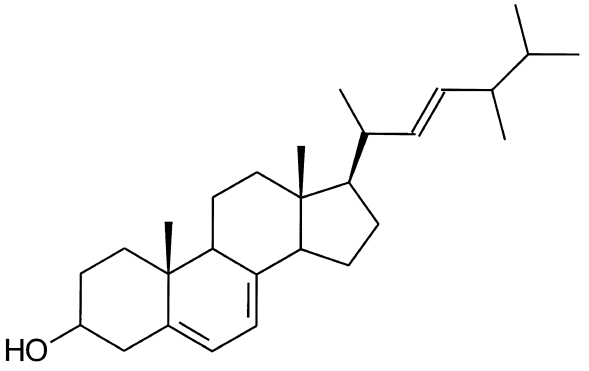

Fig. 1. Chemical structure of ergosterol.

termination of ergosterol [10–14], most of which are based on conventional high-performance liquid chromatography with ultraviolet detection (HPLC–UV) [11] and/or gas chromatography with mass spectrometry detection [12–14]. For the gas chromatographic methods it is common to form a trimethylsilyl derivative or methyl ester to improve the peak shape and detection [5]. There has been one application using atmospheric pressure chemical ionization (APCI) for the determination of plasma membrane ergosterol of the yeast *Saccharomyces cerevisiae* with tandem mass spectrometry [15]. However, confirmations were based on loop injection of samples with no liquid chromatographic separation of ergosterol. In the present work, reversed-phase liquid chromatography with positive-ion atmospheric pressure chemical ionisation tandem mass spectrometry (LC–APCI–MS–MS) is utilized for the confirmation and quantification of ergosterol as a more specific approach to conventional HPLC–UV analysis. For comparison, we also report the results obtained from conventional HPLC–UV determinations and LC–APCI–MS using SIM of two ions ($[M+H]^+$ and $[M+H-H_2O]^+$).

2. Materials and methods

Field studies were conducted at wetlands (St. Denis National Wildlife Area 40 km east of Saskatoon, Saskatchewan, Canada 106°06′ W, 52°02′ N) that typifies the prairie ecozone. The dominant plant flora contributing a significant amount of organic matter production in these wetlands is *Scirpus lacustris* spp. *acutus*, the hardstem bulrush. Six samples in triplicate were collected including green and dead *Scirpus* stem, dead *Scirpus* tips, ambient water, sediment at the sediment–water interface and *Scirpus* roots for a total of 18 samples. Sub-sampling for determination of percent moisture and ash free dry mass (AFDM) was also completed. All samples were transported on ice to the laboratory. Samples for extraction were placed in scintillation vials (Fisher Scientific, Nepean, Ontario) containing 10 ml of methanol and extracted for ergosterol the next day. Water was collected in Teflon lined bottles and 10 ml aliquots used for extraction. All materials were stored at 4 °C overnight. For AFDM determinations the material was placed in zip-lock bags and weighed immediately upon arrival at the lab and placed in drying ovens. Ash free dry mass of plant material was measured by combusting for 4 h at 450 °C [3,18]. Extraction efficiencies [9] on spiked *Scirpus* and sediment were in the range of 93.9±11.6% SD. Ergosterol was not detected in the eight method quality control blanks analysed.

2.1. Extraction of ergosterol

Sediment at the water–sediment interface, standing live and dead *Scirpus* stems, *Scirpus* roots, and pond water were extracted for ergosterol in triplicate as per the procedure previously described [16] with the exception that samples were concentrated to 2 ml (rather than 1 ml) and divided into two 1 ml portions: one for mass spectrometric analysis and the other for HPLC–UV analysis.

Briefly, the procedure is as follows: environmental samples were placed into 35 ml pressure reaction tubes (Alltech, Deerfield, IL) containing 15 ml of methanol, 5 ml of 40 mg ml^{-1} KOH in 95% ethanol followed by vortexing and sonication for 1 min each. The tubes were then placed in an 85 °C water bath for 30 min and hand mixed after 15 min. The tubes were allowed to cool and the contents filtered through Whatman # 41 paper into separatory funnels. The filter was rinsed with 5 ml of methanol. The mixture was extracted with pentane three times (3×10 ml) and the solvent was evaporated using a gentle stream of dry nitrogen gas. The dried extract was re-dissolved in 5 ml of methanol, filtered

through a 0.2 μm pore-size syringe filter and the extract concentrated to 2 ml by evaporation with nitrogen. A volume of 15 μl was utilized for MS–MS and MS analyses whereas a volume of 100 μl of the extract was injected into the HPLC–UV system for the determination of ergosterol concentrations.

2.2. LC–APCI–MS–MS conditions

A method similar to that described by Toh and colleagues [15] was employed. However, unlike Toh's application that used loop injection, chromatographic separation was also added to provide retention time as an additional parameter for confirmation and to reduce possible ionization problems associated with loop injection of complex extracts. Samples were injected in 15 μl aliquots using a Waters 2690 separations module (Milford, MA.). Eluent consisted of 100% methanol at a flow-rate of 200 μl min^{-1}. Separation was achieved by a Waters Xterrra C_{18} analytical column (Milford, MA.), 3.5 μm, 2.1×100 mm. At these conditions ergosterol eluted at 4.24 min (Fig. 2). The column effluent was delivered to the APCI interface of a Micromass Quattro Ultima (Micromass, UK) triple quadrupole mass spectrometer set to positive ion mode. Interface conditions were as follows: Corona voltage of 7.2 kV and a source and desolvation temperature of 130 and 500 °C, respectively. Nebulizer gas of nitrogen was set to maximum flow-rate while the flow-rates for the cone and desolvation nitrogen were set to 151 and 181 l h^{-1}, respectively. Sampling cone voltage was maintained at 11 V. Selected reaction monitoring (SRM) was employed for confirmation and quantitation of ergosterol. Monitoring the transition of the precursor ion at m/z 379.4 to the product ion m/z 69.4 (Fig. 3a), at a dwell time of 0.50 s and an inter-channel delay of 0.10 s. The collision cell housing pressure was increased to 3.38×10^{-4} mbar using argon as the collision gas with a collision energy of 78 V applied (laboratory frame of reference). Quantification was based on a five point ($n=3$) external standard method with a linear calibration ($r^2=0.9998$) over a concentration range 0.10–1.00 μg ml^{-1}.

2.3. LC–APCI–MS conditions

Selected ion monitoring (SIM) data was also acquired in order to compare values with that of the MS–MS values obtained. Mass spectrometer conditions were similar to the MS–MS conditions with the following exceptions; the collision cell energy was reduced to 8 V with no argon gas present, MS1

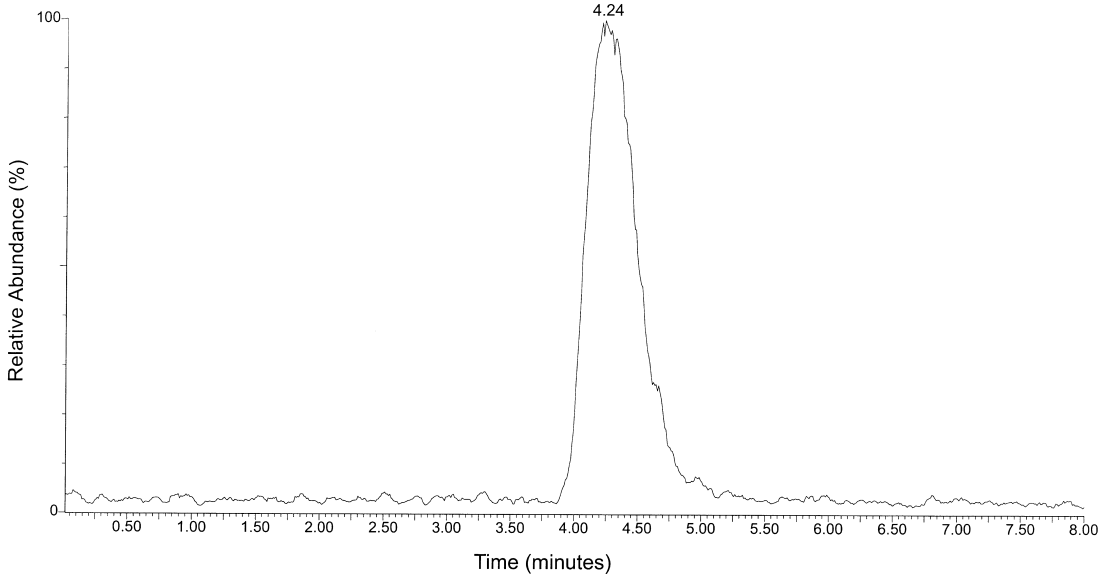

Fig. 2. MS–MS chromatogram of ergosterol.

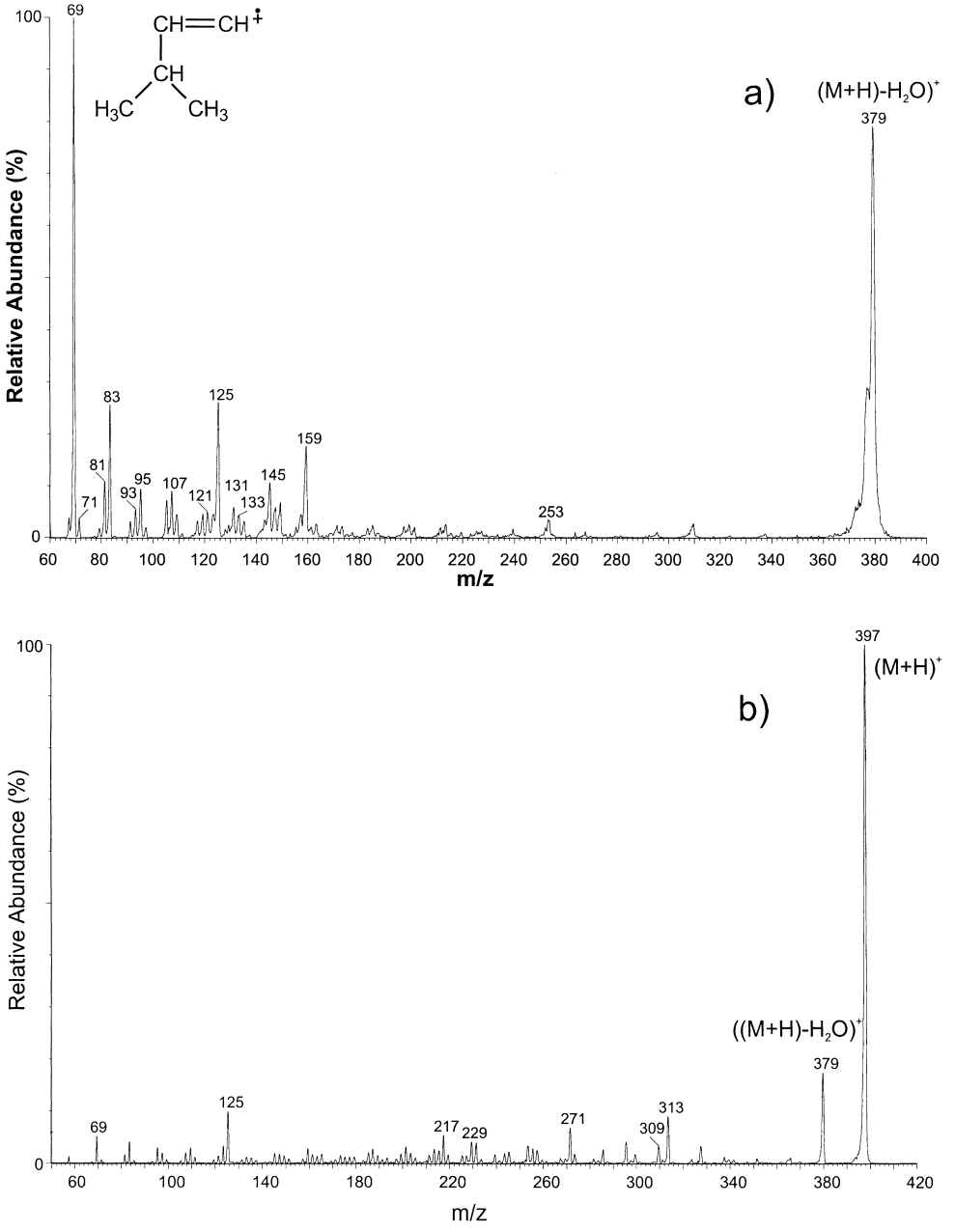

Fig. 3. (a) MS–MS mass spectrum of the products obtained from m/z 379.4. (b) MS–MS mass spectrum of the products obtained from m/z 397.4.

was set to transmit two ion channels, 397.4 and 379.4 with a dwell and inter-channel delay times of 0.50 and 0.10 s respectively. Quantification was also based on a five point ($n=3$) external standard method with a linear calibration ($r^2=0.9986$) over a concentration range of 0.10–1.00 μg ml^{-1}.

2.4. HPLC–UV conditions

In addition to mass spectral determinations, analyses were also conducted using a conventional LC-UV method. The HPLC–UV was comprised of a Bio Rad Model #1350 pump (Hercules, CA.) with a reversed-phase Supelcosil LC_{18} 5 μm 15 cm×4.6 mm HPLC column (Supelco, Bellefonte, PA). The mobile phase was HPLC grade methanol with a flow-rate of 1.8 ml min^{-1}. The ergosterol retention time was 4.50 min and detection utilized a Dionex (Sunnyvale, CA) variable wavelength detector set at 282 nm. Quantification was based on a five point ($n=5$) external standard method with a linear calibration ($r^2=0.9999$) over a concentration range of 1.0–10.0 μg ml^{-1}.

3. Results and discussion

Full scan data of ergosterol (spectrum shown in Fig. 4), shows m/z 397.4 and a base peak at m/z 379.4 corresponding to $[M+H]^+$ and $[M+H-H_2O]^+$, respectively. Prior to selected reaction monitoring (SRM) studies, product ion scans were obtained to assess suitable transitions from both the m/z 379.4 ion $[M+H-H_2O]^+$ and the m/z 397.4 ion $[M+H]^+$ (see Fig. 3a and b). The m/z 379.4 to m/z 69.4 transition was chosen for quantitative analysis over the m/z 397.4 to m/z 379.4 (loss of H_2O) transition due to improved sensitivity and a more selective transition. Detection limits calculated on unsmoothed data were 0.50 ng on column.

In the previous work [15] the protonated molecule was not observed under APCI conditions. This difference is likely due to a combination of factors. In this work a desolvation temperature of 500 °C, cone voltage of 11 V and Z-spray source geometry were utilized as compared to a relatively cool desolvation temperature (200 °C), higher cone voltage setting (23 V) and linear geometry of the APCI source used in the previous method. These differences not only allowed the observation of the dehydrated ergosterol ion but also the protonated molecule, improving the number of diagnostic ions for SIM analysis. For selected ion monitoring the m/z 379.4 was used as the quantitation mass and m/z 397.4 as the confirmation mass. Detection limits

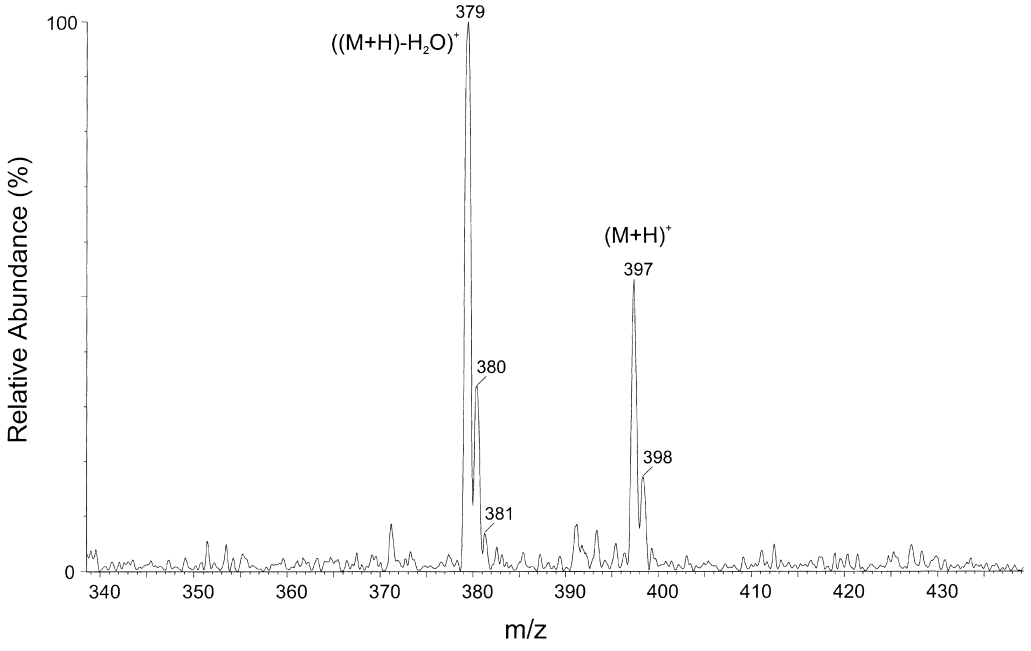

Fig. 4. Full scan mass spectrum of ergosterol.

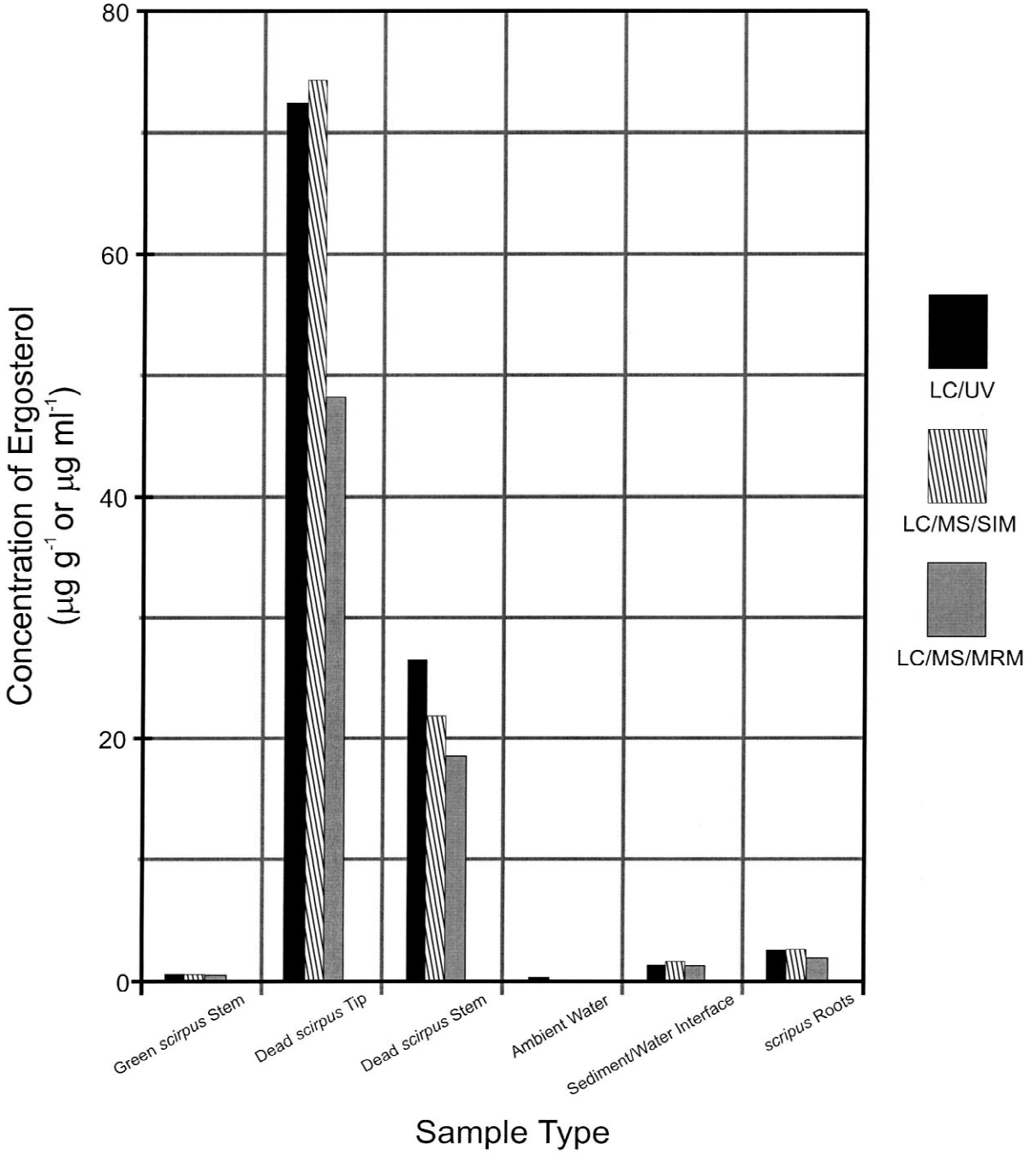

Fig. 5. Comparison of the average concentration of ergosterol (μg g^{-1} wet mass or μg ml^{-1}) obtained using three methods of instrumental analysis.

calculated on unsmoothed data were 0.13 ng on column.

Although the detection limits of the conventional LC-UV method were sufficient, in some matrices detection was subject to interferences from co-eluting components. For example, all replicates of water samples analysed by LC–UV gave a positive detection of ergosterol. It is noted, however, that ergosterol was not detected in the same sample extracts analysed by the more selective LC–APCI–MS–MS technique.

All three of the analytical methods provided detection of ergosterol in the matrices investigated (Fig. 5). Monitoring a specific transition was the most selective method as evidenced by lower average values based on wet weight obtained for SRM analysis of ergosterol for each matrix type as illustrated in Fig. 5. The comparative data suggests that monitoring wavelength 282 nm using the UV detector is more specific for three out of the six sample types analysed as compared to monitoring two ions with SIM. One explanation of this observation is possible matrix interference resulting in an increased abundance of the quantitation mass (m/z 379). For example, the average relative abundance of ions at m/z 379:397 for the ''sediment–water'' interface sample was 92.5% in contrast to a value of 72.3% observed for the authentic standard in methanol. To possibly correct for this interference calibration based on standard addition would be required if SIM analysis were to be used for quantification of ergosterol. Likewise, the SIM and MS–MS quantification could be further refined in future development of the technique by using a suitable internal standard.

Standing dead S*cirpus* stems had the largest levels of ergosterol with the tips and stems accounting for an average of 303 and 90 $\mu g\ g^{-1}$ AFDM, respectively. These numbers are consistent with what other researchers have found (100–2000 $\mu g\ g^{-1}$) and are indicative of the majority of decomposition and carbon sequestration by the fungi occurring while the plant is standing [17–19]. The sediment from the sediment–water interface also had significant amounts of ergosterol with 39 $\mu g\ g^{-1}$ AFDM. This may have been associated with the small quantities of plant material, which were observed in the sediment, that had been colonised by aquatic fungi. It has been noted that ergosterol levels temporarily decrease once a plant dislodges and falls into the water. This is believed to occur because of colonisation by aquatic fungi following the dislodging or death of terrestrial species but has not been experimentally confirmed. Persistence of ergosterol in the environment has not clearly been established and it is possible that the ergosterol observed was from dead fungal husks. The occurrence of ergosterol on green stems may be attributed to remnants of fungal material on the surface of the plant or of either symbiotic or parasitic fungi.

4. Conclusions

Conventional HPLC–UV methods for the determination of ergosterol was subject to interference due to the complexity of the natural matrices. Similarly, quantification by monitoring two ions using SIM was subject to mass interference and would require calibration based on standard addition as a corrective measure. The LC–APCI–MS–MS method provided the most selective analysis of ergosterol in natural matrices with a detection limit of 0.50 ng ergosterol on column. Quantitative analysis by tandem mass spectrometry, specifically SRM, is thus the recommended procedure to minimise erroneous results from matrix interference.

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