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Extraction and analysis of fungal spore biomarkers in atmospheric bioaerosol by HPLC–MS–MS and GC–MS

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ABSTRACT

Airborne microorganisms, as bacteria and fungi, are ubiquitous components of the atmospheric aerosol particles.

In this paper, we report a method for the simultaneous extraction, purification, separation, identification and quantification of ergosterol, mannitol and arabitol as biomarkers of fungal spores in bioaerosol particles. After sampling by a low volume sampler, filters were spiked with mannitol-^{13}C and dehydrocholesterol as internal standards. Samples were then extracted by accelerated solvent extraction using pure ethanol. The extract was then passed through an amino cartridge and divided in two parts: the apolar fraction, released from the cartridge, was subjected to liquid liquid extraction (by n-hexane), while polar compounds, retained by the cartridge, were eluted by a mixture of methanol–water. The two fractions were joined and analyzed by HPLC equipped with two different columns in series, and coupled to a triple-quadrupole mass spectrometer with Atmospheric Pressure Chemical Ionization source. In addition, the same fractions were analyzed, after derivatization, by GC–MS. The results obtained by the two techniques were finally compared, showing good agreement between them.

Last, the contents of the three biomarkers have been estimated in three atmospheric samples collected in a suburban/rural site and, using literature conversion factors, correlated to fungal biomass.

1. Introduction

Primary biological aerosol particles (also called bioaerosols) are airborne particles that are living (bacteria, biofilms, viruses and fungi) or originate from living organisms (pollens, cellular debris, acres and insect fragments) [1,2]. In particular fungi have been found in the troposphere [3], stratosphere [4], fog [5], cloud water and precipitation [6] and they can affect human health in different ways. Possible effects are: allergic reactions, infections, and toxic responses [7].

Fungal spores have been determined in atmospheric aerosol samples collected in different sites around the world [8,9]. Recent investigations [10–15] demonstrate that fungal spores contribute up to 9.9% of organic carbon (OC) in the coarse fraction (aerodynamic diameter of particles between 1 and 10 μm) of particulate matter. The fungal spore size ranges from 1 to 50 μm [16], but sometimes large amounts of fungal fragments (particles of size smaller than the original spores) are released together with spores from contaminated surfaces.

The risk associated to fungal fragments is even higher since smaller particles can penetrate deeper into the human respiratory system [17].

One of the approaches to assess the occurrence of fungal biomass in atmospheric particulate matter is the use of biomarkers [11]. The basis of this approach is that microorganisms contain compounds that can be used as chemical markers of larger and bioactive microbial structures [18]. This approach includes, among the possible fungal biomarkers, ergosterol [19] and other compounds like the polyols mannitol and arabitol [20]. Ergosterol in fungi and fungal spores is bound to the cell membrane in both the free form and esterified [21].

Polyols or sugar alcohols, as mannitol and arabitol are produced in large amount by many fungi: they constitute energy reserve material and act as cell protective against stressful conditions [1].

The most common methods to detect sugar alcohols in different environmental matrices are based on: a clean up followed by GC–MS, after previous derivatization [22]; the

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HPLC–MS/MS method, after post-column derivatization [23]; the ion chromatography method with amperometric detection [1,24–26]. HPLC–UV [19], HPLC–DAD, or GC–MS [21] are some of the methods proposed for ergosterol. Usually, fungal biomarkers are individually analyzed in HPLC [27,28].

Therefore it is a great challenge to simultaneously analyze all of them in the same matrix, as, at the moment, no one of the biomarkers can be considered fully reliable for the determination of fungal content. Hence the aim of this study has been the development and optimization of a novel analytical strategy for the simultaneous extraction and analysis of the fungal biomarkers, ergosterol, arabitol and mannitol, from the primary biological aerosol by High Performance Liquid Chromatography–Tandem Mass Spectrometry (HPLC–MS/MS) to compare to Gas-chromatography–Mass Spectrometry (GC/MS). This technique has been successfully used in literature [27,29–32] for the single biomarker determination, but has the great disadvantage of a longer analysis time and less reproducible results. Conversely, with our method, sample preparation and analysis time are drastically reduced.

Appropriate factors to convert marker values into biomass are then necessary. Such conversion factors, found in literature and calculated have been established by isolating and cultivating the fungal species known of the system under study and by measuring the load of biomarkers in this system [11,33–37].

We applied the optimized method for the biomarker determination to the analysis of atmospheric particulate matter samples and, through conversion factors, we estimated the mass contribution of fungal components.

2. Materials and method

2.1. Chemicals and reagents

All the chemical and chromatographic reagents were HPLC or analytical grade.

HPLC RS-Plus Methanol and anhydrous Ethanol were purchased by Carlo Erba Reagents, (Milan, Italy), ultra gradient Acetonitrile (ACN) and n-hexane were obtained by ROMIL.

Table 1
Optimized electrical parameters for MS and MS/MS analysis.

Instrumental parameters Polarity	Polyols Negative	Ergosterol Positive
CAD	2	1
NC (kV)	−3	5
TEM	500	500
OR (V)	−40	10
RNG (V)	−220	100
RO_2 (V)	30	−30

Ultra-pure water was produced with a Pure LabTM system (USF Elga, Ransbach-Baumbach, Germany). Formic acid and ammonium formate were obtained from Fluka (Sigma-Aldrich, Milan, Italy). Ammonium formate (6 mM) was prepared by dissolving 378.4 mg of ammonium formate in 1000 mL of water.

N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and pyridine were obtained from Sigma (St. Louis, MO, USA).

Mannitol, arabitol, ergosterol, internal standards mannitol-^{13}C (IS) e dehydrocholesterol (IS) were purchased from Sigma Aldrich (Milan-Italy).

Strata NH_2 cartridges (200 mg/3 mL) were obtained from Phenomenex (Castel Maggiore—BO-Italy).

Stock standard solutions of analytes were prepared by dissolving each compound in methanol (1 mg/mL) and storing them at −20 °C in amber vials. Working solutions were prepared by successive dilution of the stock standard solutions.

2.2. Materials

Teflon filters were weighed before and after sampling using an analytical electronic balance (Sartorius MC-5, $\Delta m = \pm 0.001$ mg) after conditioning under hood (Activa Climatic Cabinet, Aquaria MI, according to UNI EN 12341/2001; UNICHIM 285/2003 and D.M. 60/2002) for 24 h, at $T = 20 \pm 1$ °C and at $50 \pm 5\%$ relative humidity.

Particulate matter extraction was carried out with ethanol by Accelerated Solvent Extraction (ASE 200–Dionex) operating at high pressure (1500 psi) and temperature (100 °C) using two static cycles.

The extracts were evaporated by a Glas-Col SE 500 evaporator (Bioanalitica Strumenti srl, Italy) under a nitrogen stream. A vacuum manifold (Alltech 12-Port model SPE Vacuum manifold; Deerfield, IL) was used to quickly elute the analytes from the SPE cartridge at a constant flow.

2.3. Sampling

Atmospheric particulate matter was collected on PTFE (politetrafluoroetilene) filters (diameter: 47 mm, porosity: 2 μm, PALL Gelman Laboratory) with a FAI-Hydra low volume sampler (FAI Instrument, SRL) equipped with PM_{10} (particulate matter with aerodynamic diameter less than 10 μm) inlet head at 2.3 m^3/h flow. Particles were collected on pre-weighed filters for 24 h. The whole sampling duration was three days so as to have three sampled filters.

2.4. HPLC equipment and condition

LC analysis was carried out by Shimadzu two pump system LC-10 AD (Shimadzu, Kyoto, Japan) at a flow rate between 1 mL/min and 150 μL/min according to the internal diameter of the columns.

Table 2
Precursor, fragment ions and retention times of analytes and internal standards by HPLC–MS–MS in MRM mode.

	Precursor ion m\z (abundance %)	**Qualifier fragment (abundance %)**	**Quantifier fragment (abundance %)**	***tR*** $\bar{x} \pm \sigma$	**Polarity**	
Arabitol	**151 (14)**	**58-89-100 (76)-(72)-(33)**	**71 (100)**	**3.8 ± 0.03**	–	**Exp A**
Mannitol	**181 (22)**	**58-71-100 (38)-(44)-(74)**	**89 (100)**	**6.1 ± 0.01**	–	
Mannitol-C-13 ISTD	*187*		*92*	*6.1 ± 0.02*	–	**Exp B**
Ergosterol	**379 (8)**	**83-125-145 (41)-(25)-(27)**	**69 (100)**	**9.9 ± 0.01**	+	
Deidrocholesterol ISTD	*367*	–	*158*	*10.2 ± 0.03*	+	

Table 3
Fragments (m/z) selected for GC/MS SIM and retention times.

Analytes	MW	MW-$(TMS)_n$	Target m/z (ab. %)	Qualifier Ions (ab. %)		t_R
Arabitol	152	512	**217**=[M-295] (100)	**307**=[M-205](39)	**319**=[M-193] (32)	5.3
^{13}C-Mannitol (ISTD)	*188*	*620*	*323=[M-297] (100)*	*220=[M-400](41)*	*205=[M-118] (83)*	*5.9*
Mannitol	182	614	**319**=[M-295] (100)	**217**=[M-397] (50)	**205**=[M-409] (69)	5.9
Dehydroco (ISTD)	*384*	*456*	*351=[M-105] (100)*	*456=[M] (3)*		*11.2*
Ergosterol	396	468	**363**=[M-105] (100)	**337**=[M-131](68)	**468**=[M] (11)	11.7

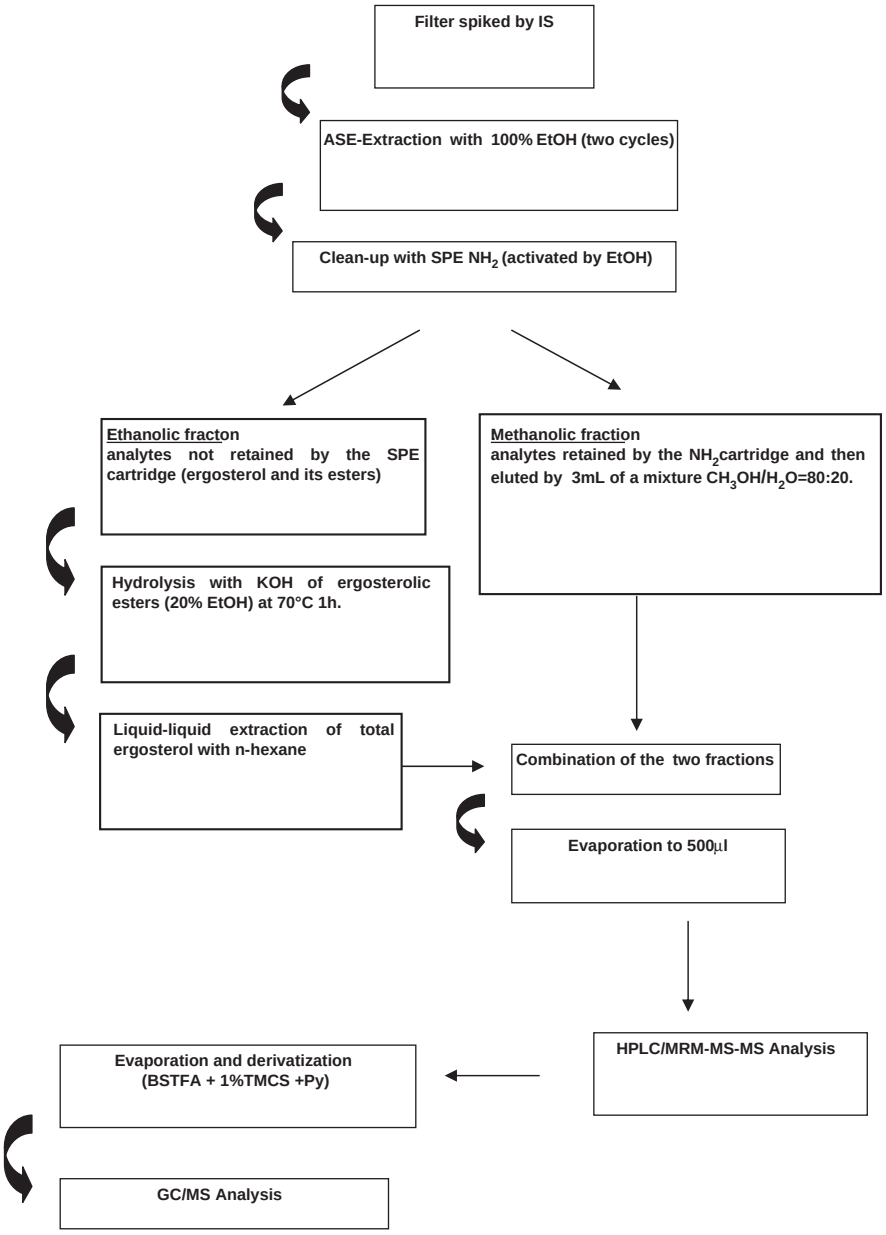

Fig. 1. Block diagram of the complete procedure for environmental sample analyses.

The injector was a Rheodyne 8125 system with a 5 μL sample loop. Chromatographic separations were performed on the following columns:

CN column 165 mm × 4.6 mm I.D., slurry packed in our lab with size particles of 5 μm (Spherisorb, Deeside Ind. Est. Queensferry, Cluey. UK).

NH_2 column 150 mm × 4.6 mm I.D., 5 μm (Kromasil Phenomenex Castel Maggiore—BO-Italy).

C_8 column 150 mm × 4.6 mm I.D., 5 μm (Phenomenex Castel Maggiore—BO-Italy).

Luna Hilic column 150 mm × 4.6 mm I.D. (Phenomenex Castel Maggiore—BO-Italy).

NH_2 column 30 mm × 2.1 mm I.D., 5 μm (Restek Chebios s.r.l. Rome-Italy).

C_{18} column 50 mm × 2.1 mm I.D., 5 μm (Restek Chebios s.r.l. Rome-Italy).

Several isocratic and gradient elutions were tried with methanol/water and ACN/water.

Table 4
Range of concentration and calibration curve in HPLC-MS/MS in MRM mode.

Compounds	Concentration ranges (μg mL^{-1})	Equations of calibration curves	R^2
Dehydrocholesterol (ISTD)	0.050		
Ergosterol	0.031–2.99	$Y=2.0061x-0.034$	0.9996
^{13}C-Mannitol (ISTD)	1.0		
Arabitol	0.21–3.0	$Y=1.2262x-0.0427$	0.9997
Mannitol	0.22–3.4	$Y=2.0061x-0.034$	0.9996

Table 5
Range of concentration and calibration curve in GC/MS in SIM mode.

Compounds	Concentration ranges μg mL^{-1}	Equations of calibration curves	R^2
Dehydrocholesterol (ISTD)	0.50		
Ergosterol	0.05–0.6	$Y=0.9074x-0.0205$	0.9989
^{13}C-Mannitol (ISTD)	0.1		
Arabitol	0.09–0.50	$Y=2.783x-0.2009$	0.9981
Mannitol	0.09–0.50	$Y=1.024x-0.0164$	0.9989

Preliminary experiments were made with UV MICROUVIS20RS (Carlo Erba Instruments Milan-Italy) and RI detectors (Well-Chrom K-2301 Knauer Berlin Germany).

2.5. Mass spectrometry equipment and conditions

An Applied Biosystems API 365 triple-quadrupole mass spectrometer (ABI Sciex Instruments, Foster City, CA, USA), equipped with Turbo Ion Spray (TIS) and Atmospheric Pressure Chemical Ionization (APCI) interfaces, was used to perform MS and MS/MS analyses. Acquisition parameters were optimized by direct continuous pump infusion of standard working solutions of the analytes (10 ng/μL in MeOH/H_2O) at a flow rate of 10 μL/min, in full scan (mass range m/z 50–500) and product ion mode. APCI was preferred and the instrument was operating in negative ion mode for mannitol and arabitol, and in positive ion mode for ergosterol. Air was used as nebulizing gas, and nitrogen was both curtain gas and collisional gas. The settings for the nebulizer, curtain and collision gases were 12, 8 and 3 respectively (arbitrary units). The orifice potential (OR) and the ring potential (RNG) were optimized for the various compounds. The collision energy was adjusted by variation of the voltage difference between the high pressure entrance quadrupole (Q_0) and the potential of collision cell quadrupole (RO_2, collision cell rod offset) and was optimized to give the highest sensitivity for the analytes. The source probe heater temperature was set at 400 °C. Finally, all the analyses were carried out by LC/MS/MS in multiple reaction monitoring (MRM) mode, acquiring two or more diagnostic product ions from the chosen precursors to obtain high specificity and sensitivity. Table 1 shows all the optimized electrical parameters. Table 2 summarizes retention times, precursors and fragments chosen for MRM analysis. First acquisition period (exp A) was

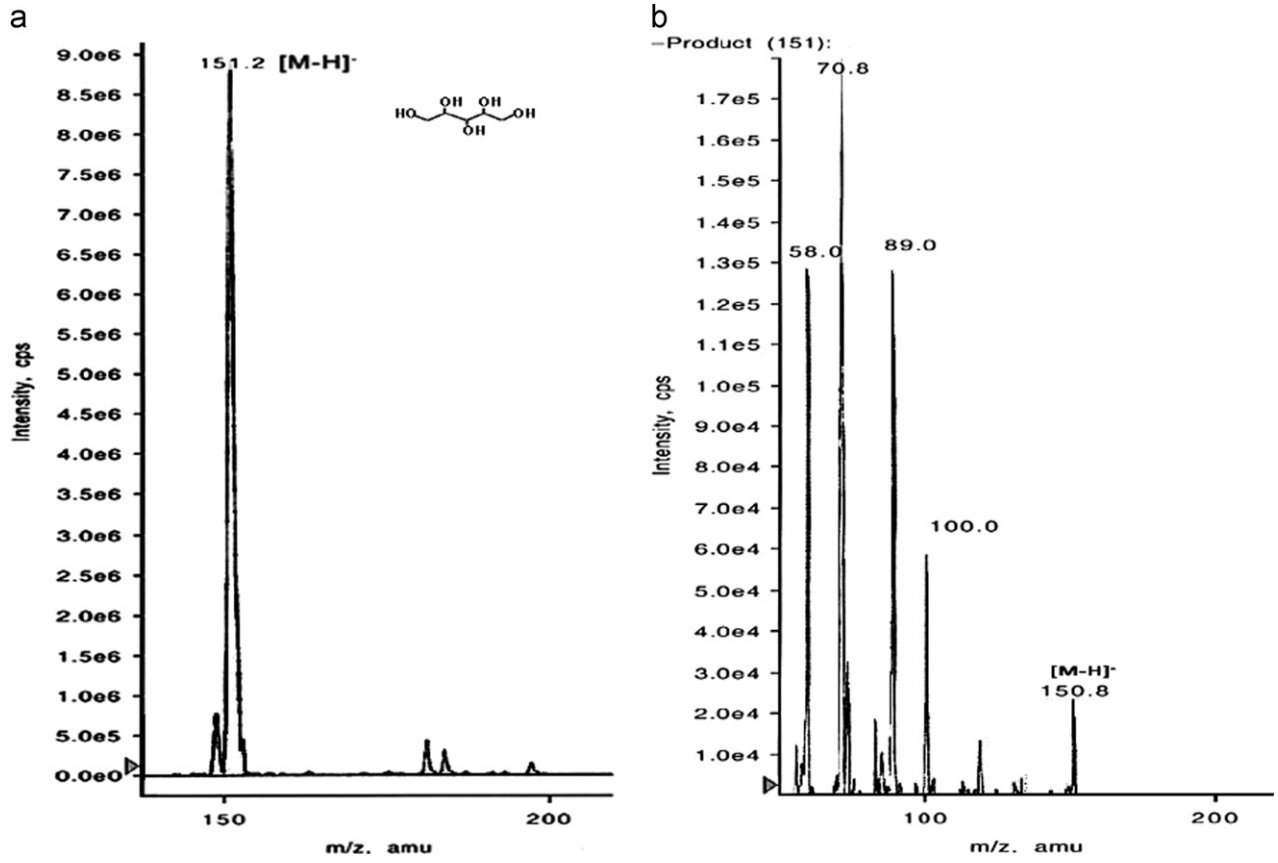

Fig. 2. (a):Q1 full scan negative APCI ionization of arabitol (10 ng/μL), working flow 10 μL/min. (b) MS\MS full scan spectrum of arabitol, precursor ion m/z 151 amu. Parameters as in Table 1.

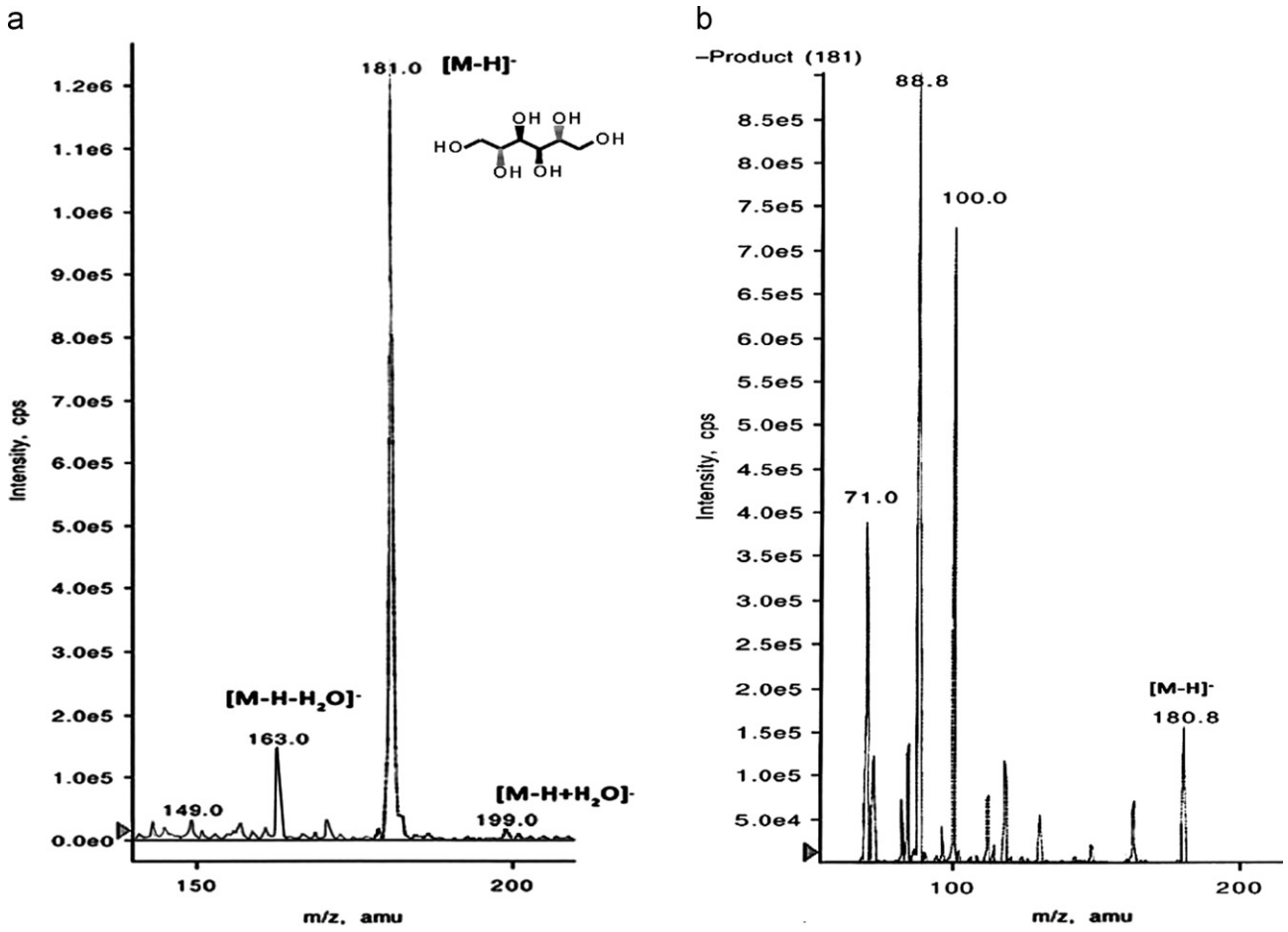

Fig. 3. (a):Q1 full scan negative APCI ionization of mannitol (10 ng/μL), working flow 10 μL/min. (b) MS\MS full scan spectrum of mannitol, precursor ion m/z 181 amu. Parameters as in Table 1.

set from 0 to 6.5 min for sugars and their IS in negative ionization, while second acquisition period (exp B) was set for ergosterol and its IS in positive ionization, up to 11 min.

2.6. GC/MS equipment and conditions.

An HP 6890 gas chromatograph fitted with an HP 7683 autosampler and connected to an HP 5973A single quadrupole mass-selective detector (Agilent Technologies) was used for EIGC/MS analysis. GC separation was achieved in 12 min on a DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 mm film thickness; J&W Scientific). GC analyses were performed after analyte derivatization (1 h 70 °C) by a mixture of N,O-bis(trimethylsilyl) trifluoroacetamide, BSTFA and 1% of TMCS (trimethylchlorosilane).

The temperature program was: 100 °C initial temperature, ramped at 25 °C/min to 180 °C, then ramped at 40 °C/min to 300 °C and held for 8 min. Samples (1 μL) were injected in splitless mode. The injector temperature was set at 280 °C. The helium carrier gas was maintained at a constant flow of 1.0 ml min^{-1}. The quadrupole and ion source temperatures were set at 150 °C and 230 °C, respectively. Acquisitions were performed in selected ion monitoring (SIM) mode according to Table 3. The mass spectrometer was operated in the EI mode at 70 eV, using the Agilent MSD ChemStation D.01.00 software.

2.7. Sample preparation

After particulate sampling, each filter was sealed, and stored at −18 °C. Filters were weighed before and after sampling, spiked with IS and ASE extracted with ethanol by two static cycles, at temperature of 100 °C and pressure of 1500 psi.

The extracted volume (about 20 mL) was evaporated to 1 ml and loaded onto a Strata NH_2 SPE column (previously activated by EtOH), linked to a vacuum system. The sugars and polar compounds, retained from the cartridge, were eluted by 3 mL of a mixture methanol–water=80:20 (methanolic fraction), while ergosterol and other apolar compounds were not retained by the SPE (ethanolic fraction). The two fractions, ethanolic (containing ergosterol) and methanolic (containing sugars) were handled in two different ways (see Fig. 1) and finally joined together and reduced to a suitable volume (500 μL) by nitrogen stream for HPLC–MS–MS analysis. The same solution, after dryness, was derivatized for 1 h at 70 °C, by adding 50 μl of BSTFA [N,O-bis(trimethylsilyl)trifluoroacetamide] at 1% of trimethylchlorosilane and 10 μl of pyridine and injected to GC–MS before and after a dilution to 5 mL respectively for ergosterol and sugar analysis.

Ethanolic fraction containing ergosterol and its esters, was hydrolyzed with 2 ml of KOH (20% in Ethanol) at the temperature of 70 °C for 1 h, and then extracted twice by a mixture of n-hexane-water (1:1), in ultrasonic bath at the temperature of 40 °C for 30 min. Water was added to reduce the solubility of ergosterol in the polar solvent. After centrifugation (for 3 min at 4000 rpm) the upper organic phase was separated by the aqueous phase. Such step for the hydrolysis of esterified ergosterol was afterward considered negligible because of the small percentage of esterified ergosterol (10%).

The whole procedure is summarized in the block diagram of Fig. 1.

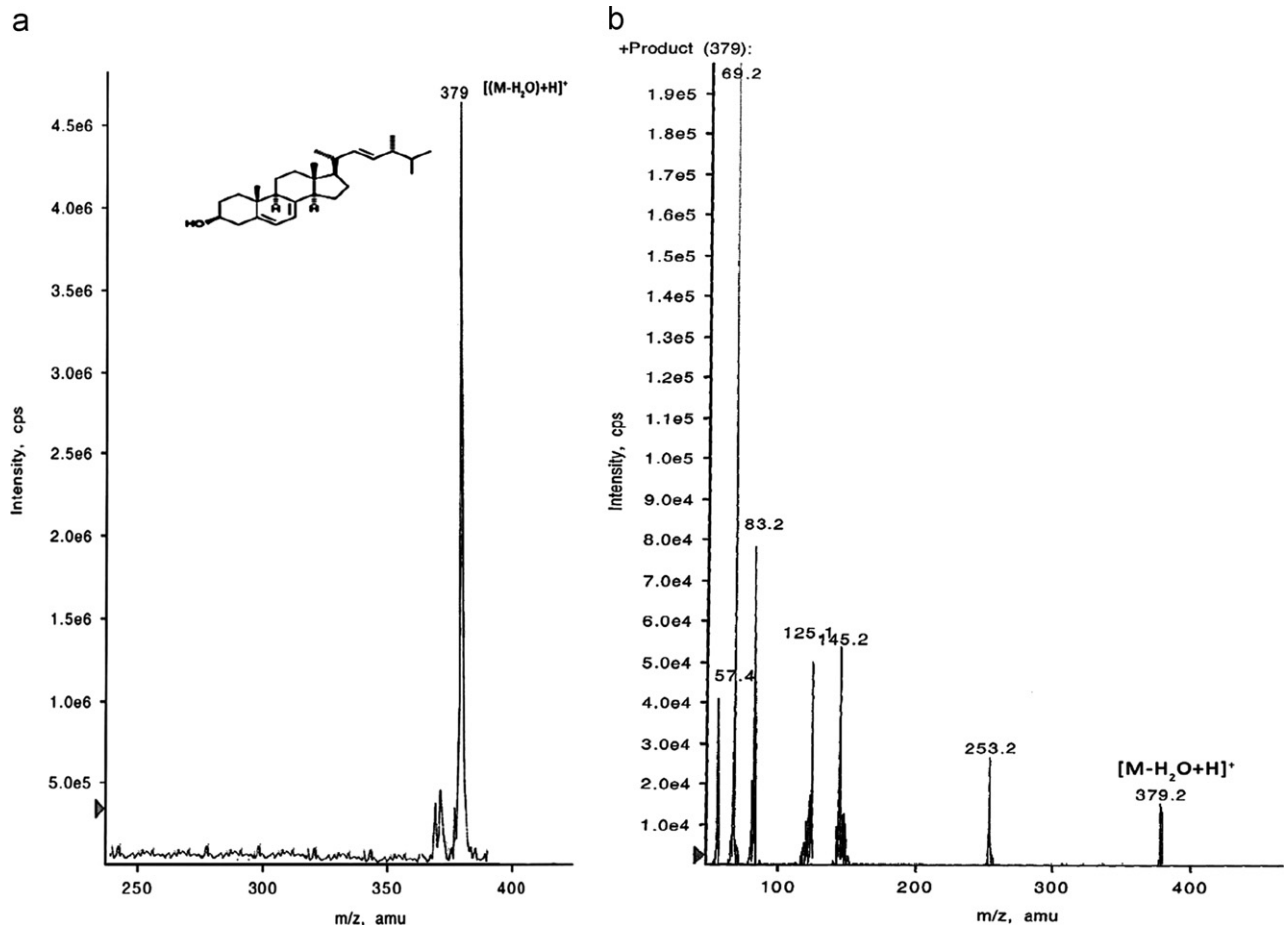

Fig. 4. (a):Q1 full scan negative APCI ionization of ergosterol (10 ng/μL), working flow 10 μL/min. (b) MS\MS full scan spectrum of ergosterol, precursor ion m/z 379 amu. Parameters as in Table 1.

2.8. Quantitative analysis

Calibration curves were built adding standard solutions on PTFE filters, and following the whole procedures with both techniques to take account of any matrix effects. The standard solutions were prepared diluting the stock solutions with methanol, according to Tables 4–5. Six calibration levels in triplicate were prepared and each solution was injected three times in HPLC/MS–MS and GC–MS systems.

Different calibration ranges were due to different sensitivity of the techniques and environmental samples were diluted accordingly.

2.9. Recovery

The recovery of the analytes was determined after ASE extraction, after ASE+SPE purification and after the whole procedures, using filters spiked with standard solutions and adding the IS just before injection. The solutions were analyzed in triplicate by both LC–MS/MS in MRM mode and GC–SIM–MS and quantized through the corresponding linear calibration curves, built adding, to blank filters processed, all the standard solutions just before injections.

3. Results and discussion

The different chemical nature of the three analytes has greatly complicated the choice of the conditions to detect them in a single step of extraction, purification and injection. Polyols are very hydrophilic, while the steroid ring structure of ergosterol makes it very hydrophobic. Several experimental conditions for the best resolution of our analytes were tested.

3.1. Chromatographic conditions

Preliminary experiments were performed to select the most suitable column for our purpose. Five columns with different stationary phases, length and diameters were tried, in particular: CN, C_{18}, C_8, NH_2 and HILIC. The columns showed, as expected, different selectivity, and the best choice was NH_2 stationary phase for sugars and C_{18} column for ergosterol. In order to analyze our three analytes in a single run, the two columns were coupled in series, so that sugar alcohols were retained by NH_2 column and ergosterol by C_{18} column. The total length of the two columns was 80 mm and all the dead volumes were minimized.

A step elution, changing sharply (in 5′) the mobile phase composition from 100% *Pump A* (H_2O–CH_3CN=15:85), to 100% Pump B (CH_3CN), was used to perform the analysis at working flow of 250 μl/min.

Under these conditions all the compounds and IS were eluted in less than 11 min with a good separation.

3.2. LC/MS/MS analysis

The development of a MRM LC/MS/MS method requires experiments carried out by infusion mass spectrometry on standard solutions to determine suitable source parameters for

the best sensitivity and S/N ratio, as well as the molecule-related ions. The first issue to address was the selection of the ionization mode (ESI or APCI) that provides and enhances most the formation of protonated or deprotonated ions of the target compounds. Experiments were carried out under positive and negative polarity with different mobile phase mixtures and additives. In order to select the ionization technique and polarity, all the instrumental parameters, and lens potentials were optimized. After maximizing the molecular ion intensities on Q1, the MS–MS transitions were optimized. Two characteristic product ions of each compound with high intensities were selected and the collision pressure of N_2 and the collision energy of each transition were optimized (see Tables 1 and 2), to be used in the following MRM experiments.

The acquisition with a heated nebulizer (HN)–APCI source in positive mode for the ergosterol was the most suitable choice, because ESI ionization gave an up front source fragmentation with loss of fragment m/z $[C_6H_7O]^+$ due to the breakage of A ring. Because of their character, sugars are better analyzed in negative ion mode, since they are easily deprotonated both in ESI and in APCI ionization. As precursors for collision-induced dissociation (CID) in MS/MS the most abundant ions $[M–H]^-$ and $[M–H_2O+H]^+$ were respectively chosen for sugars and ergosterol.

The mass spectra of the analytes were first acquired on quadrupole 1 (Q1) in full scan mode using continuous infusion (10 μL/min) of standard solutions (10 ng/μL). The spectra shown in Figs. 2 and 3a, are dominated by the deprotonated molecules $[M-H]^-$, which easily provide molecular weight for sugar alcohols. MS–MS spectra of the sugar alcohol analytes are shown in Figs. 2 and 3b. Fig. 4a displays Q1 mass spectrum of ergosterol and Fig. 4b shows its MS\MS full scan spectrum, choosing as precursor ion m/z 379 amu.

Fig. 5 shows the MRM chromatogram of a standard mixture (TIC and the extracted ion currents XICs). The XICs refer respectively to the most intense chosen transitions for polyols and their C_{13}-IS in the experiment A, and for ergosterol and its dehydrocholesterol-IS in the experiment B. As shown, all the investigated compounds were well separated and detected, with high S/N.

The presence of signal suppression due to any coeluting interferences was excluded by analyzing (data not shown) blank field filters, i.e. filters located in the sampler housing, during all the sampling period and handled as environmental samples.

3.3. GC–MS analysis

During the development of the GC/MS method, derivatization with different agents was tested. N,O-bis(trimethylsilyl)trifluoroacetamide, BSTFA, with 1% of TMCS (trimethylchlorosilane) gave better results for our analytes, according to literature data [29–32]. The most abundant ions were chosen from GC/MS full scan spectra and used in selected ion monitoring (SIM) analysis according to Table 3.

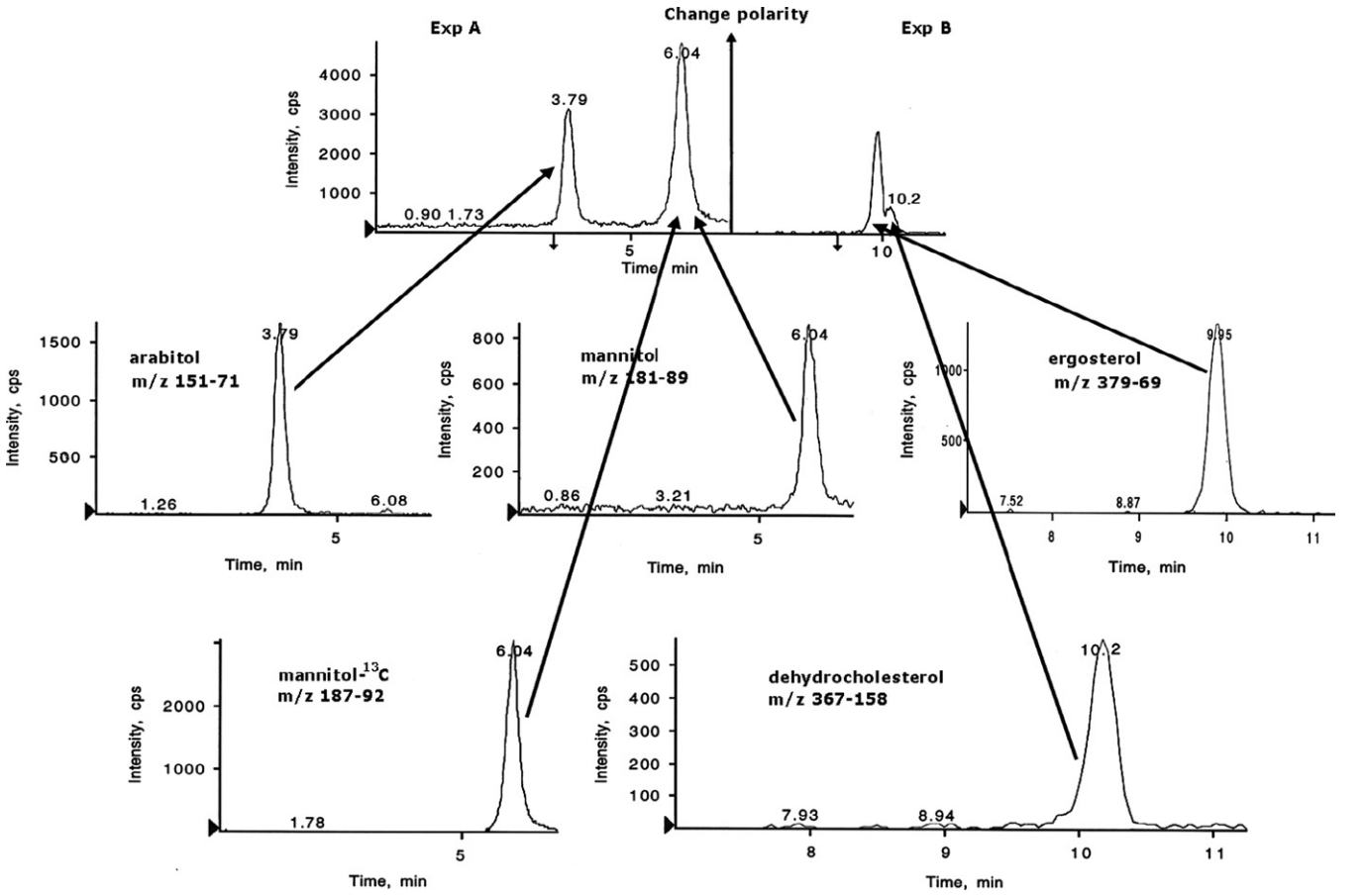

Fig. 5. HPLC–APCI–MS/MS in MRM mode of a standard mixture (2 μg/mL polyols and 0.05 μg/mL ergosterol). The upper part shows the TIC, while the lower part the extracted ion currents (XICs) of the most intense transitions (see Table 2) for polyols, their C_{13} ISTD in experiment A (negative ionization), and ergosterol and dehydrocholesterol ISTD in experiment B (positive ionization). Column and condition as in the text.

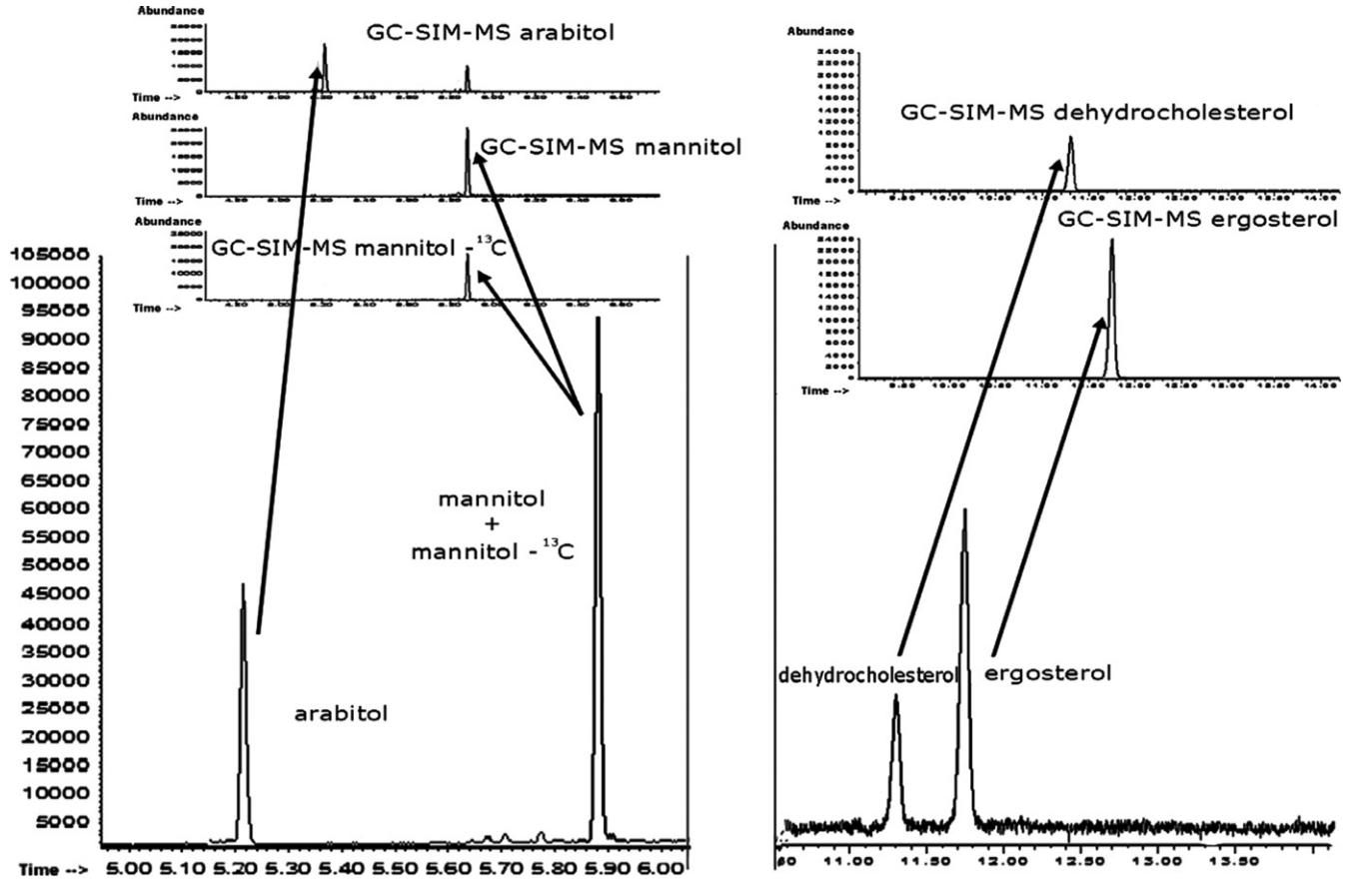

Fig. 6. TIC in GC–MS in SIM (lower part) and extract ion (upper part) of a standard mixture (arabitol and mannitol 140 ng/mL, ergosterol 600 ng/mL) Chromatographic conditions as in the text, selected ions as in Table 3.

The lower part of Fig. 6 shows the GC–SIM–MS TIC chromatogram of a standard mixture, and the upper part illustrates the extracted ion currents of the most intense selected ions.

3.4. Extraction efficiency

Different solvents were tried to check the extraction efficiency. Experiments were carried out by methanol, ethanol and n-hexane with two cycles of ASE-extraction so as to enhance the effectiveness and give higher yields for all the analytes.

Differently from n-hexane there are no significant differences between the other two solvents, but ethanol resulted more suitable for the retention in the SPE NH_2 cartridge. While ergosterol and its esters were eluted with the ethanolic fraction and afterwards extracted twice by n-hexane at 40 °C for 30′, retained sugar alcohols were recovered from the SPE cartridge, by elution with methanol–water 80:20 (see Fig. 1). The choice of NH_2 cartridge was due to the nature of the analytes. On one hand polarity of sugars make them suitable to be retained from this kind of cartridge, while the hydrophobic ergosterol is soon released and collected.

The partial recovery (analytes recovery after ASE extraction, after ASE extraction and SPE NH_2 purification), total recovery % and on the whole procedure of Fig. 1 are summarized in Table 6 and expressed as average of three different samples.

3.5. Application to environmental samples

Atmospheric particulate matter was collected in late autumn in a suburban/rural site nearby Rome. Three sampled filters (samples 1, 2 and 3) were weighed to determine PM_{10} concentration in air, resulting to be 30 μg/Nm^3 in average. After conditioning, filters were added with IS in the same concentration as calibration curve and processed, according to our procedure (see Fig. 1). Analyses were carried out by both techniques. Next, to correlate amount of mannitol, arabitol and ergosterol to the number of fungal spores, we used literature conversion factors [11,34,35]. Three blank field filters, processed and analyzed according to the same procedure, showed no interfering peaks at the retention times of the three analytes both in HPLC and in GC (data not shown).

Fig. 7 displays a sample analysis by HPLC-MS/MS in MRM mode. All the analytes are detected with a good ratio S/N. Fig. 8a–c presents the concentrations (ng/Nm^3) of the three biomarkers extracted from the three PM_{10} samples. These results were obtained taking into account the total volume of air sampled on each filter of about 50 m^3. The standard deviation associated to each measure is relatively low, making the method precise.

As illustrated in Fig. 8, mannitol and arabitol show higher concentrations than ergosterol, and their HPLC–MS values are in good agreement with those obtained by GC–MS. Finally we tried to correlate concentration of biomarkers to fungal spore biomass using literature conversion factors of Table 7, obtaining the number of fungal spores/μg of particulate. By considering the average weight of a spore equal to 65 pg [36] we can obtain the contribute w/w of fungi to PM_{10} as shown in Table 8.

As we can see, mannitol and arabitol give comparable results in terms of fungal biomass, while ergosterol shows a percentage of fungal spores in PM_{10} lower of about 80%. The great variability of results, in terms of fungal contribute to PM, demonstrate that there are still some aspects that need to be deeply studied. It is well known from literature that there are other not always

Table 6
Analyte recoveries after ASE extraction, after ASE extraction and SPE NH_2 purification and after the whole procedure.

	Ergosterol recovery (%)	**Arabitol recovery** (%)	**Mannitol recovery** (%)
ASE-extraction			
Methanol	97 ± 12	93 ± 15	96 ± 11
n-hexane	98 ± 9	–	–
Ethanol	$\mathbf{94 \pm 6}$	$\mathbf{95 \pm 3}$	$\mathbf{92 \pm 3}$
Ethanol ASE-extraction and SPE NH_2 purification (elution by CH_3OH/H_2O=80:20)			
	$\mathbf{81 \pm 1}$	$\mathbf{96 \pm 2}$	$\mathbf{89 \pm 2}$
Total recovery by HPLC–MS/MS			
	$\mathbf{90 \pm 4}$	$\mathbf{85 \pm 4}$	$\mathbf{82 \pm 6}$
Total recovery by GC–MS			
	$\mathbf{81 \pm 4}$	$\mathbf{80 \pm 8}$	$\mathbf{78 \pm 8}$

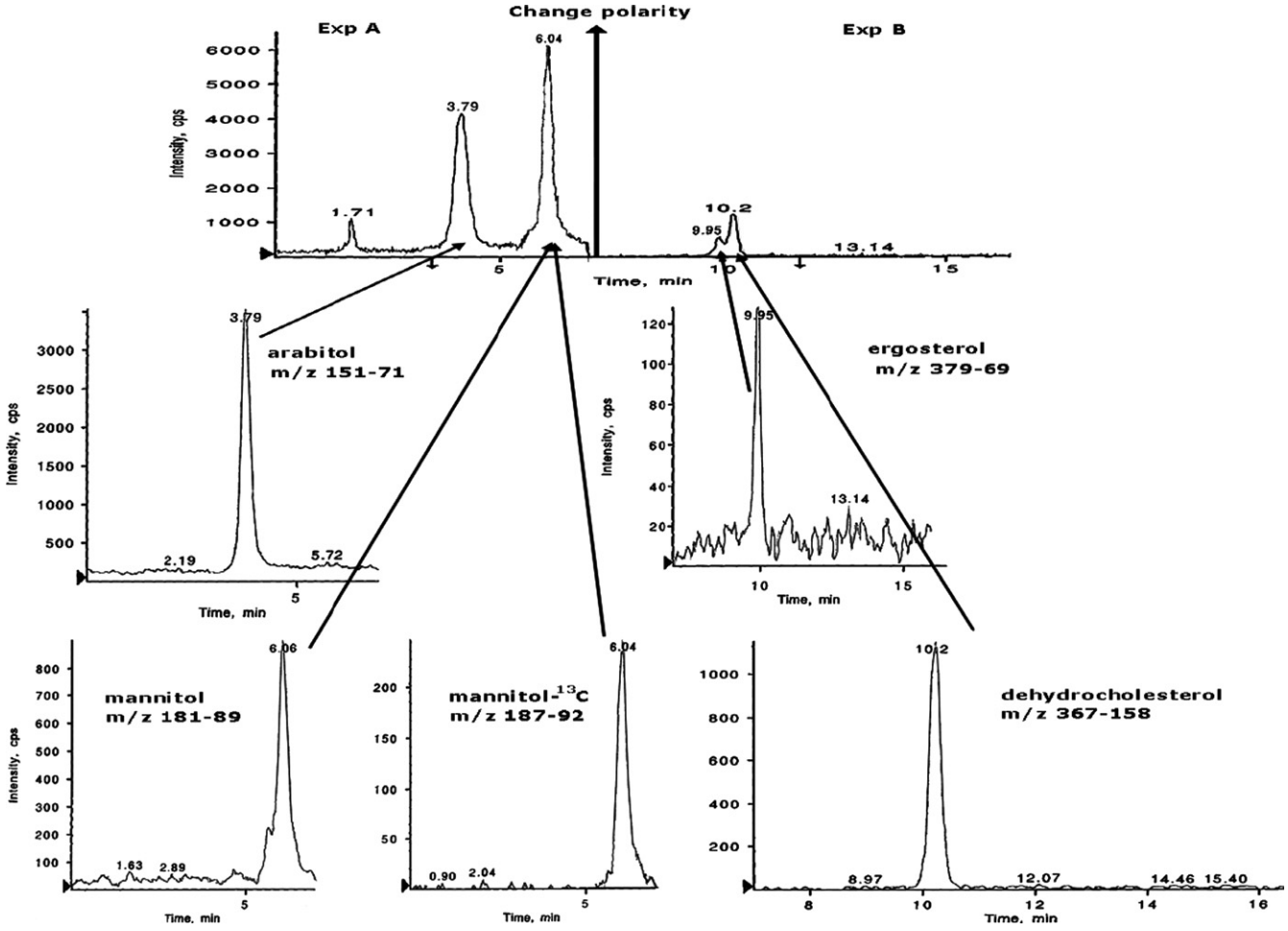

Fig. 7. HPLC–MS/MS in MRM of environmental sample 2. Upper part TIC with two acquisition periods (exp A and exp B). Exp A XIC specific transitions for polyols and there IS. Exp B XIC specific transitions for ergosterol and its IS.

negligible sources of mannitol and arabitol depending on biogenic emissions [15,26]. Secondarily it is quite difficult to establish what is the best and more reliable conversion factor. With this aim we have measured our internal conversion factors, after choosing the most representative fungal species in atmosphere, reaching a good agreement with Bauer and Cheng literature data for mannitol and ergosterol [11,34,35,37].

4. Conclusion

A new methodology for the extraction and identification of ergosterol, mannitol and arabitol, as biomarkers of fungal spores in particulate matter, was developed.

The method resulted precise, effective, and quick.

First, ethanol proved to be the best solvent for the extraction of all the three analytes and, after the extraction, SPE purification was optimized to ensure the removal of impurities.

A RP-HPLC analytical methodology, using two different columns in series, was optimized for the separation of the three compounds in less than 11 min.

Investigated analytes were then identified and quantified by HPLC–MS–MS in MRM mode in three airborne particulate matter samples collected in suburban/rural site.

A comparison between these results and those obtained, after derivatization, by GC–MS in SIM mode, was made and no significant differences were observed, proving the HPLC method to be effective.

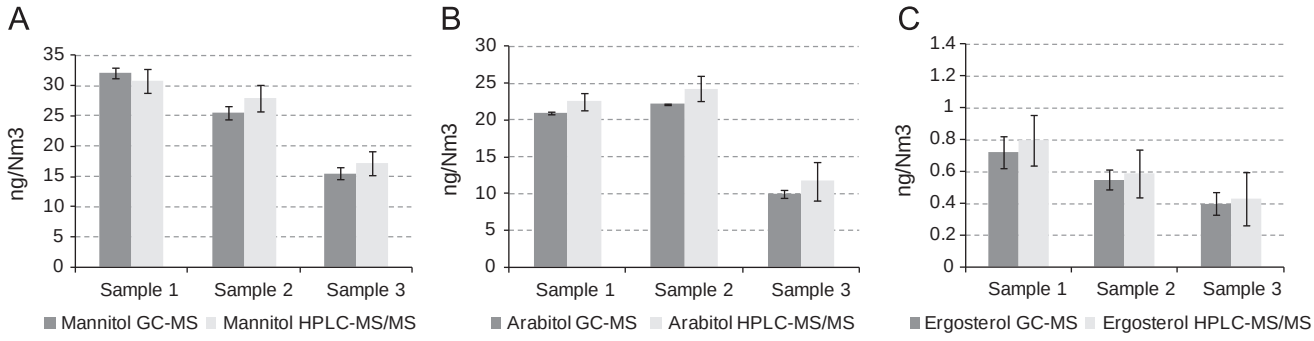

Fig. 8. (a) Mannitol concentration comparison (ng/Nm)^3 obtained by HPLC–MS/MS (white) and GC–MS (gray), (b) arabitol concentration comparison (ng/Nm)^3 obtained by HPLC–MS/MS (white) and GC–MS (gray) and (c) ergosterol concentration comparison (ng/Nm)^3 obtained by HPLC–MS/MS (white) and GC–MS (gray).

Table 7
Literature conversion factors for the three biomarkers [11,34,35].

Conversion factors (pg/spore)	
Mannitol	1.7
Arabitol	1.2
Ergosterol	0.191

Table 8
μg fungal spores/μg particulate in three different samples.

	Sample 1 μg fungal spores/μg PM10	Sample 2 μg fungal spores/μg PM10	Sample 3 μg fungal spores/μg PM10
Arabitol	0.0142	0.0175	0.0201
Mannitol	0.0154	0.0142	0.0220
Ergosterol	0.0031	0.0027	0.0050

In addition, the HPLC method proved to be quicker and less expensive than the GC method, saving the time of evaporation and derivatization.

As these biomarkers allow estimation of atmospheric fungal biomass, thanks to the use of proper conversion factors, the present methodology can be used as support tool for epidemiological and risk assessments and to increase the information about atmospheric concentrations of bioparticles collected in different sites.

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