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A gas chromatography–mass spectrometry multi-target method for the simultaneous analysis of three classes of metabolites in marine organisms

David M. Pereira [a], Juliana Vinholes [a], Paula Guedes de Pinho [b], Patrícia Valentão [a], Teresa Mouga [c], Natércia Teixeira [d,e], Paula B. Andrade [a,*]

[a] REQUIMTE/Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal
[b] REQUIMTE/Laboratório de Toxicologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal
[c] GIRM—Marine Resources Research Group, School of Tourism and Maritime Technology, Polytechnic Institute of Leiria, Santuário N.ª Sra. dos Remédios, Apartado 126, 2524-909 Peniche, Portugal
[d] Laboratório de Bioquímica, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal
[e] IBMC—Instituto de Biologia Molecular e Celular, Universidade do Porto, 4150-180 Porto, Portugal

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ABSTRACT

In this work a fast and simple multi-target gas chromatography–mass spectrometry (GC–MS) method for the simultaneous detection and absolute quantification of amino acids, fatty acids, sterols and lupanes in marine organisms is proposed. The methodology was applied to the characterization of the echinoderm *Marthasterias glacialis* Linnaeus spiny sea star extracts. The main factors influencing the extraction of target compounds were evaluated by using different extraction procedures, solvent systems and temperature conditions and a comparison with a reference technique was performed. The most suitable procedure, capable of successfully extract the three classes of target compounds, was ethanol as solvent at 40 °C under magnetic stirring. Good analytical parameters were obtained since calibrations curves for the 40 compounds under analysis (15 amino acids, 16 fatty acids, 6 sterols and 3 lupanes) showed regression coefficients (r^2) ranging from 0.9844 to 0.9978, with low RSD (from 0.00 to 9.45%), and detection limits varying from 0.03 to 15.40 μg/L. The RSD values for intra- and interday variations studies were also good (RSD < 13.5%, for both) and recoveries were higher than 92%. Variation in samples from different harvests and origins and their chemical composition during the year is reported. The fact that no previous treatment of samples is required can make this a useful technique for metabolite profiling in marine organisms, among others, both in biomedical and nutritional studies. Moreover, due to the fast and robust character of the proposed method it seems to be suitable for the implementation as routine analysis.

1. Introduction

Nowadays multi-target approaches have been increasingly used, thus allowing the identification of several classes of compounds in different matrices [1–3]. Gas chromatography–mass spectrometry (GC–MS) is a popular preference since it provides powerful structural and quantitative information [4]. Adequate extraction procedures are critical for obtaining a chemical profile that is representative of the original sample, both qualitatively and quantitatively. Techniques such as microwave assisted extraction [5], accelerated solvent extraction [6] and supercritical fluid extraction [7,8] have been developed and different derivatization procedures tested. Derivatizing agents are responsible for the increment of volatility of less volatile and non-volatile compounds and, consequently, a higher sensitivity and resolution can be obtained by GC–MS analysis. Currently, silylation is widely used as derivatization method for GC–MS metabolic profile studies, since it allows the determination of metabolites from different classes. Among silylation agents, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) has been increasingly used due to its ability to react with primary amines and amides, alcohols, phenols, carbohydrates and carboxylic groups [9,10].

Marine organisms have been paving their way as an interesting source of bioactive molecules for biomedical research, as well as alternative foodstuffs for nutrition. In both cases, the use of these organisms must be guided by elevated standards in quality control and hence analytical techniques for fast metabolite profiling are required. Among the metabolites present in marine species, lipids are growing in interest, especially by their content

* Corresponding author. Tel.: +351 220428654; fax: +351 226093390.
E-mail address: pandrade@ff.up.pt (P.B. Andrade).

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Table 1
Validation parameters for amino acids, fatty acids, sterols and lupanes.

Compound	Concentration range (μg/mL)	Slope (±SD)	Interception (±SD)	Correlation coefficient (±SD)	LOD (μg/mL)	LOQ (μg/mL)	RSD
Amino acids							
Alanine	12.00–120.00	0.0086 (0.0001)	0.0035 (0.0092)	0.9952 (0.0260)	0.36	1.12	0.10
Glycine	20.00–200.00	0.0130 (0.0002)	0.0193 (0.0247)	0.9974 (0.0696)	1.75	5.83	0.77
Valine	1.50–150.00	0.0631 (0.0007)	0.1612 (0.0588)	0.9978 (0.1659)	0.13	0.43	0.27
Leucine	1.50–88.00	0.0727 (0.0012)	−0.0290 (0.0581)	0.9958 (0.1544)	0.18	0.61	0.45
Isoleucine	1.50–150.00	0.0523 (0.0005)	0.0183 (0.0368)	0.9978 (0.0978)	0.22	0.75	0.38
Proline	12.00–120.00	0.0588 (0.0012)	−0.0726 (0.0892)	0.9938 (0.2135)	0.03	0.11	0.06
Serine	12.00–120.00	0.0125 (0.0002)	−0.0079 (0.0155)	0.9958 (0.0371)	2.51	8.35	1.04
Threonine	5.00–170.00	0.0375 (0.0005)	−0.0290 (0.0365)	0.9972 (0.0970)	0.58	1.94	0.73
Aspartic acid	12.50–200.00	0.0574 (0.0010)	−0.1894 (0.1217)	0.9972 (0.1100)	1.77	5.89	3.10
trans-4-Hydroxyproline	15.00–150.00	0.0180 (0.0004)	−0.0254 (0.0334)	0.9940 (0.0799)	1.67	5.55	1.00
Cysteine	28.00–140.00	0.0440 (0.0013)	0.0041 (0.1016)	0.9888 (0.2424)	2.68	8.92	3.93
Methionine	12.50–200.00	0.0661 (0.0019)	0.5070 (0.2064)	0.9898 (0.1923)	1.46	4.85	1.52
Phenylalanine	13.00–130.00	0.0330 (0.0010)	−0.1386 (0.0761)	0.9864 (0.1822)	0.54	1.81	2.19
Glutamic acid	6.25–200.00	0.0381 (0.0001)	0.1066 (0.0623)	0.9940 (0.0241)	0.61	2.03	1.75
Lysine	12.50–200.00	0.0730 (0.0016)	−0.7400 (0.1570)	0.9926 (0.0450)	0.97	3.27	2.09
Fatty acids							
Pelargonic	20.00–100.00	0.0054 (0.0001)	−0.0216 (0.0078)	0.9910 (0.0188)	4.61	15.36	0.83
Capric	40.00–200.00	0.0039 (0.0001)	−0.0123 (0.0117)	0.9902 (0.0281)	7.86	26.21	1.02
Lauric	5.00–130.00	0.0057 (0.0001)	−0.0276 (0.0115)	0.9886 (0.0275)	0.61	2.04	0.11
Myristic	25.00–500.00	0.0027 (0.0001)	0.1516 (0.0229)	0.9938 (0.0382)	2.71	9.05	0.26
Pentadecanoic	10.00–250.00	0.0052 (0.0001)	−0.0003 (0.0150)	0.9914 (0.0362)	2.27	7.58	0.38
Palmitic	60.00–300.00	0.0056 (0.0001)	0.0613 (0.0264)	0.9891 (0.0633)	7.72	25.75	1.44
Margaric	10.00–120.00	0.0106 (0.0003)	−0.0153 (0.0191)	0.9920 (0.0457)	2.02	6.73	0.71
Oleic	10.00–140.00	0.0004 (0.0000)	−0.0012 (0.0009)	0.9866 (0.0022)	0.14	0.48	0.00
Linoleic	10.00–100.00	0.0104 (0.0003)	−0.0521 (0.0180)	0.9901 (0.0430)	1.34	4.45	0.71
Linolenic	10.00–500.00	0.0003 (0.0000)	−0.0045 (0.0026)	0.9941 (0.0058)	1.77	5.91	0.02
Stearic	10.00–176.00	0.0094 (0.0003)	0.0215 (0.0373)	0.9953 (0.0892)	0.31	1.02	0.10
Arachidonic	10.00–500.00	0.0065 (0.0002)	−0.0259 (0.0541)	0.9932 (0.1294)	3.18	10.61	0.07
Eicosapentaenoic	40.00–200.00	0.0138 (0.0003)	−0.1064 (0.0413)	0.9903 (0.0989)	6.54	21.81	3.01
*cis*11-Eicosenoic	60.00–300.00	0.0002 (0.0000)	−0.0091 (0.0013)	0.9909 (0.0019)	8.35	27.84	0.06
Eicosanoic	28.00–140.00	0.0136 (0.0002)	0.0249 (0.0203)	0.9951 (0.0485)	4.68	15.59	2.12
Docosahexaenoic	30.00–180.00	0.0094 (0.0002)	−0.0642 (0.0385)	0.9947 (0.0866)	4.95	16.48	1.55
Sterols and lupanes							
Cholesta-3,5-diene	14.00–70.00	0.0170 (0.0006)	−0.0478 (0.0265)	0.9844 (0.0596)	3.22	10.73	1.82
Cholesterol	30.00–250.00	0.0108 (0.0004)	−0.0397 (0.0359)	0.9913 (0.0807)	4.17	13.88	1.54
Cholestanol	5.00–100.00	0.0265 (0.0007)	−0.0683 (0.0436)	0.9932 (0.0981)	0.27	0.89	0.23
Ergosterol	76.00–380.00	0.0103 (0.0003)	−0.1466 (0.0684)	0.9903 (0.1538)	15.40	51.34	5.29
Fucosterol	6.25–200.00	0.0146 (0.0002)	−0.0383 (0.0191)	0.9963 (0.0574)	0.95	3.17	9.45
Betuline	6.25–200.00	0.0170 (0.0003)	−0.0903 (0.0294)	0.9937 (0.0882)	0.97	3.24	5.48
Lupeol	6.25–200.00	0.0154 (0.0003)	−0.0743 (0.0269)	0.9935 (0.0854)	0.80	2.68	7.43
Lupeol acetate	6.25–200.00	0.0178 (0.0003)	−0.1000 (0.0257)	0.9956 (0.0771)	0.83	2.74	8.94
β-Sitosterol	10.40–52.00	0.0178 (0.0005)	−0.0201 (0.0168)	0.9896 (0.0377)	1.61	5.37	0.46

in polyunsaturated fatty acids that are important to human health and nutrition. The fatty acids (FA) composition of marine organisms is often characteristic for each species and genus, but also depends on environmental conditions [11–13]. Several protocols have been established for the extraction and analysis of FA, and recent reviews on this topic are available [11,14]. However, in most cases, the protocol includes extensive pre-treatment for the elimination of interferents [1,15] and quantification is not always possible. Separation of polar and apolar constituents is also common.

Other two classes of compounds with nutritional interest found in marine organisms are amino acids and sterols. The first is generally analyzed by GC–MS, GC-FID [16,17] or HPLC-UV/DAD [18,19], while techniques for sterol analysis can be performed either by HPLC-DAD/MS [20,21] or GC–MS [22]. However, pre-treatment of samples is usually required for both classes, which can result in high losses of analyte and low recovery rates.

In this work, a methodology for the simultaneous assessment of the four classes of compounds (amino acids, fatty acids, sterols and lupanes) in marine organisms was developed. The proposed method includes the derivatization of a crude extract and further identification and quantification by GC–MS, in 25 min chromatographic run, without sample pre-treatment or the use of hazard extraction solvents.

2. Experimental

2.1. Standards and reagents

Arginine (≥98%), asparagine (≥98%), aspartic acid (≥98%), cysteine (≥98%), glutamic acid (≥98%), glutamine (≥98%), histidine (≥98%), lysine (≥98%), methionine (≥98%), tryptophan (≥98%), tyrosine (≥98%), alanine (≥98%), glycine (≥99%), valine (≥98%), leucine (≥98%), isoleucine (≥98%), proline (≥99%), serine (≥99%), threonine (≥98%), *trans*-4-hydroxyproline (≥98%), phenylalanine (≥98%), norvaline (≥99%), methyl linolelaidate (≥99%), cholesta-3,5-diene (≥95%), cholesterol (≥95%), cholestanol (≥99%), ergosterol (≥95%) *β*-sitosterol (≥97%), desmosterol (≥85%), fucosterol (≥95%), betulin (≥95%), lupeol (≥95%), lupeol acetate (≥95%), *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), as well as pelargonic (≥99%), decanoic (≥99%), capric (≥99%), lauric (≥95%), myristic (≥99%), pentadecanoic (≥99%), palmitic (≥99%), margaric

(≥98%), stearic (≥99%), oleic (≥99%), linoleic (≥99%), linolenic (≥99%), arachidonic (≥99%), 5,8,11,14,17-eicosapentaenoic (EPA) (≥99%), *cis*11,14-eicosadienoic (≥98%), *cis*11-eicosaenoic (≥99%), docosahexaenoic (DHA) (≥98%) and eicosanoic acids (≥97%) and dichloromethane were from Sigma (St. Louis, MO, USA). The *n*-alkane series (C_8-C_{40}) and boron trifluoride (BF_3) 10% methanolic solution were from Supelco (Bellefonte, PA, USA). Chloroform, methanol, anhydrous sodium sulfate and isooctane were from Panreac Quimica SA (Barcelona, Spain). Potassium hydroxide was obtained from Pronalab (Lisboa, Portugal).

2.2. Preparation of standard solutions

Stock solutions of amino acids, fatty acids, sterols and lupanes and the internal standards (IS) norvaline, methyl linolelaidate and desmosterol were prepared individually in ethanol and kept at −20 °C until analysis. Calibration solutions were then prepared by mixing and diluting each stock solution in appropriate amounts with ethanol to achieve the concentration range discriminated in Table 1.

2.3. Marine organisms

Marthasterias glacialis Linnaeus individuals were collected at the rocky coast at Cabo Carvoeiro, west Portugal, in July and September of 2009 and at Praia do Baleal and Praia da Consolação in February of 2010 (Fig. 1). The organisms were placed on ice and immediately transported to the laboratory in ice-boxes. The macro-invertebrates were then cleaned and washed with sea water and kept at −20 °C, prior to their freeze-drying in a Labconco 4.5 Freezone apparatus (Kansas City. MO. USA). The dried material was powdered and sifted (<910 μm) before extraction. Each sample corresponds to a mixture of three individuals.

2.4. GC–MS system and data acquisition

2.4.1. GC–MS general conditions

In all cases, analysis was performed with a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 4000 mass selective ion trap detector (USA) and a Saturn GC–MS workstation software version 6.8. A VF-5 ms (30 m × 0.25 mm × 0.25 μm) column (VARIAN) was used. A CombiPAL automatic autosampler (Varian, Palo Alto, CA) was used for all experiments. The injector port was heated to 250 °C. Injections were performed in split mode, with a ratio of 1/40. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 ml/min. The ion trap detector was set as follows: transfer line, manifold and trap temperatures were 280, 50, and 180 °C, respectively. The mass ranged from 50 to 600 *m/z*, with a scan rate of 6 scan/s. The emission current was 50 μA and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionization time was 25.000 μs, with an ionization storage level of 35 *m/z*. The injection volume was 2 μL and the analysis was performed in Full Scan mode.

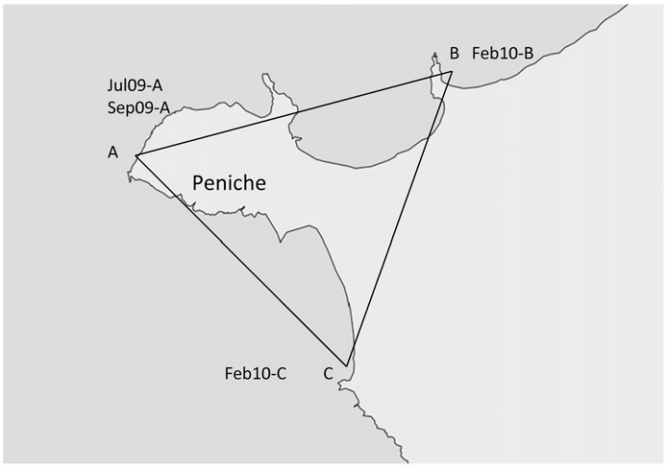

Fig. 1. Collection sites of the organisms used in this study (west Portugal) and respective sample identification.

2.4.2. GC conditions for trimethylsilyl (TMS) derivatives analysis

The oven temperature was set at 100 °C for 1 min, then increasing 20 °C/min to 250 °C and held for 2 min, 10 °C/min to 300 °C and held for 10 min. All mass spectra were acquired in electron impact (EI) mode. Ionization was maintained off during the first 4 min to avoid solvent overloading. For quantification purposes, each sample was injected in triplicate and the amount of metabolites present in samples was achieved from the calibration curves of the respective TMS derivatives. All compounds were quantified in Full Scan mode, with the exceptions of linoleic (*m/z* 262, 337 and 352), linolenic (*m/z* 191, 335 and 350), and oleic (*m/z* 264, 339 and 354) derivatives that were quantified by the area obtained from the re-processed chromatogram, using the characteristic *m/z* fragments. Identification of compounds was achieved by comparisons of their retention time and mass spectra with those of pure standards TMS derivatives prepared and injected under the same conditions, and from NIST 05 MS Library Database. In addition, the retention index (RI) was experimentally calculated and the values were compared with those reported in the literature for GC columns with 5%-Phenyl-95%-dimethylpolysiloxane (Table 6). For the RI determination, an *n*-alkanes series C_8-C_{40} was used.

2.4.3. GC conditions for fatty acids methyl esters (FAMEs) analysis

For comparison purposes, we have used chromatographic conditions published before [23]. The oven temperature was set at 40 °C for 1 min, then increasing 5 °C/min to 250 °C, 3 °C/min to 300 °C and held for 15 min. The injection volume for liquid extracts was 1 μL and the analysis was performed in Full Scan mode. Identification of compounds was achieved by comparisons

Table 2
Recovery values for representative amino acids, fatty acids and sterols. For each compound, three different concentration levels were tested.

Compounds	Concentration (μg/mL)	Recovery (%)
Isoleucine	2.00	94
	4.00	94
	20.00	99
trans-4-Hydroxyproline	10.00	96
	50.00	97
	150.00	103
Palmitic acid	15.00	95
	30.00	93
	150.00	95
Arachidonic acid	40.00	95
	80.00	96
	300.00	94
Cholesta-3,5-diene	10.00	92
	20.00	95
	80.00	98
Cholestanol	7.50	106
	15.00	95
	34.00	101

Table 3
Values of intra- and interday reproducibility for amino acids.

Compounds	Concentration (μg/mL)	Intraday (RSD)	Interday (RSD)
Alanine	12.00	4.87	12.94
	72.00	3.30	7.76
	120.00	0.49	7.30
Glycine	20.00	2.30	7.82
	120.00	1.57	5.91
	400.00	3.11	4.67
Valine	1.50	2.89	6.82
	90.00	0.06	5.49
	150.00	0.20	8.14
Leucine	1.50	0.41	6.55
	66.00	3.67	8.26
	88.00	2.27	3.66
Isoleucine	15.00	2.77	5.91
	90.00	1.86	4.83
	120.00	1.31	7.63
Proline	24.00	0.05	4.00
	72.00	2.50	7.01
	120.00	0.81	4.69
Serine	24.00	2.48	8.43
	72.00	4.17	8.70
	120.00	0.62	12.82
Threonine	17.00	1.17	5.14
	102.00	3.26	5.55
	136.00	1.04	7.22
Aspartic acid	12.50	3.10	7.17
	50.00	4.18	6.18
	200.00	1.57	8.75
trans-4-Hydroxyproline	30.00	2.17	11.65
	90.00	4.26	4.34
	150.00	0.11	6.68
Cysteine	26.00	2.19	7.27
	78.00	4.01	8.35
	130.00	0.58	8.27
Methionine	1.52	1.52	6.73
	5.06	2.73	3.69
	2.45	2.45	8.68
Phenylalanine	13.00	1.92	9.19
	78.00	2.29	10.61
	130.00	2.73	8.22
Glutamic acid	6.25	1.75	9.89
	50.00	1.05	8.15
	200.00	2.06	8.06
Lysine	6.25	2.09	4.24
	50.00	6.80	7.36
	200.00	4.99	9.22

Table 4
Values of intra- and interday reproducibility for fatty acids.

Compounds	Concentration (μg/mL)	Intraday (RSD)	Interday (RSD)
Lauric acid	26.00	3.73	9.87
	78.00	0.60	6.74
	130.00	3.72	8.16
Myristic acid	25.00	1.31	5.15
	300.00	2.46	7.80
	500.00	0.98	9.71
Pentadecanoic acid	10.00	2.87	9.32
	150.00	2.42	10.57
	250.00	2.97	10.44
Palmitic acid	60.00	3.25	3.93
	180.00	3.16	9.41
	300.00	1.96	4.74
Margaric acid	10.00	8.70	8.73
	72.00	6.64	10.12
	120.00	1.93	8.88
Linoleic acid	10.00	7.85	10.77
	40.00	5.47	7.13
	100.00	0.77	5.54
Linolenic acid	10.00	9.92	11.51
	300.00	2.76	10.83
	500.00	3.11	8.02
Oleic acid	10.00	8.64	12.53
	84.00	3.25	4.82
	140.00	2.19	4.62
Stearic acid	10.00	3.74	10.18
	132.00	3.25	9.20
	220.00	0.99	9.98
Arachidonic acid	100.00	1.85	8.62
	300.00	3.66	8.05
	500.00	2.95	6.24
Eicosapentaenoic acid	40.00	4.88	7.29
	120.00	2.66	11.93
	200.00	2.76	7.38
*cis*11-Eicosenoic acid	60.00	4.02	11.81
	180.00	3.61	9.81
	300.00	3.00	11.58
Eicosanoic acid	28.00	5.67	10.64
	84.00	2.96	3.76
	140.00	2.65	7.74
Docosahexaenoic acid	30.00	10.45	11.96
	180.00	4.86	12.37
	300.00	2.54	8.68

of their retention time and mass spectra with those from pure standards injected under the same conditions, and from NIST 05 MS Library Database.

2.5. Metabolites extraction and derivatization

2.5.1. Procedure for TMS derivatives

In order to establish the most suitable method for the extraction and quantification of metabolites present in marine samples,

Table 5
Values of intra- and interday reproducibility for sterols and lupanes.

Compounds	Concentration (μg/mL)	Intraday (RSD)	Interday (RSD)
Cholesta-3,5-diene	14.00	0.54	7.39
	42.00	3.56	10.49
	70.00	8.63	12.47
Cholesterol	30.00	1.29	9.27
	90.00	4.12	9.74
	150.00	11.48	13.67
Cholestanol	5.00	5.70	11.51
	80.00	1.84	13.39
	100.00	1.95	10.97
Ergosterol	150.00	3.46	6.90
	228.00	6.40	9.64
	380.00	2.16	8.73
Fucosterol	6.25	6.55	6.63
	50.00	3.76	4.79
	200.00	0.45	6.69
β-Sitosterol	10.40	3.87	9.30
	31.20	1.72	10.51
	52.00	2.75	10.99
Betuline	6.25	5.25	6.28
	50.00	1.63	5.42
	200.00	4.91	6.96
Lupeol	6.25	5.68	7.24
	50.00	4.50	5.90
	200.00	4.97	5.19
Lupeol acetate	6.25	8.94	9.89
	50.00	3.66	6.21
	200.00	1.50	5.30

different extracts from *M. glacialis* (sample Sep09-A) were prepared. Briefly, 100.00 ± 1.00 mg of dried sample was transferred to a glass vial and the internal standards were added: 80 μL of norvaline (0.30 mg/mL), 20 μL of methyl linolelaidate (10.00 mg/mL), and 80 μL of desmosterol (2.00 mg/mL). The volume was then completed to 2.00 mL with either ethanol or a solution of choloroform:methanol, according to the experiment being carried.

Extractions with ethanol were performed at different temperatures (40, 50 and 60 °C) by incubating for 20 min under magnetic stirring (200 rpm). Ultra-sonication with ethanol was also performed and all extracts were compared with the extraction with chloroform/methanol (2:1) [24] incubated for 20 min under magnetic stirring 200 rpm. Samples were then filtered through a 0.45 μm membrane (Millipore). Extractions were carried out in triplicate.

An aliquot of 50 μL of extract was transferred to a glass vial, the solvent was evaporated under nitrogen stream and 50 μL of the derivatization reagent (MSTFA) was added to the dried residue. The vial was capped, vortexed and heated for 20 min in a dry block heater maintained at 40 °C. All analyses were performed in triplicate.

2.5.2. Procedure for FAMES

Methyl esters derivatives were obtained as described by Ribeiro et al. [23], with some modifications: 100 mg of the powdered sample plus 20 μL of methyl linolelaidate (10.00 mg/mL) ethanol solution (internal standard) were mixed with chloroform: methanol (2:1) (2 × 2 mL) with magnetic stirring (500 rpm), for 10 min, at 40 °C. The resulting extract was filtered, concentrated to dryness under reduced pressure (40 °C) and redissolved in 2 mL of ethanol. Derivatization was assured by treatment with 1 mL of BF_3 methanolic solution (10%), at 90 °C, for 10 min. Derivatives were purified with 2 × 6 mL of isooctane and anhydrous sodium sulfate was added to assure the total absence of water. The extract was then evaporated under a stream of nitrogen and redissolved in 200 μL of isooctane.

2.6. Method validation

At least six concentration levels of compounds' trimethylsilyl (TMS) derivatives were analyzed. Each calibration solution contained norvaline, methyl linolelaidate and desmosterol as internal standards, at a final concentration of 12.00, 100.00 and 80 μg/mL, respectively. The ratios of the peak areas of compounds *versus* those of IS were plotted against the corresponding concentration to obtain the calibrations graphs. The derivatization procedure was carried out as described in 2.5.1., using 50 μL of calibration solution instead of the extract.

2.6.1. Linearity

Method linearity was determined by evaluation of the regression curve (ratio of analyte peak area/IS area *versus* analyte concentration) and expressed by the correlation coefficient. The linearity range of the method was analyzed by performing calibration curves using at least six different concentration levels of the analytes, according to the range of concentrations present in the samples (Table 1).

2.6.2. Limits of detection and of quantification

Limit of detection (LOD) and limit of quantification (LOQ) were determined from calibration curve data and were obtained by the following formula [25]:

$$\mathrm{LOD} = (3.3 \times \mathrm{SD})/b \text{ and } \mathrm{LOQ} = (10 \times \mathrm{SD})/b$$

where SD is the residual standard deviation of the linear regression, and b is the slope of the regression line.

2.6.3. Precision, accuracy and recovery tests

Precisions and accuracies were determined using intra- and interday assays at three different concentrations (low, medium and high) and are expressed as coefficients of variation (CV). Recovery tests were performed by spiking *M. glacialis* (sample Sep09-A) with two compounds representative of each class: isoleucine (2.00, 4.00 and 20.00 μg/mL), *trans*-4-hydroxyproline (10.00, 50.00 and 150.00 μg/mL), palmitic (15.00, 30.00 and 150.00 μg/mL) and arachidonic acids (40.00, 80.00 and 300.00 μg/mL), cholesta-3,5-diene (10.00, 20.00, and 80.00 μg/mL) and cholestanol (7.50, 15.00 and 34.00 μg/mL).

3. Results and discussion

3.1. Optimization of the extraction procedure

For the development of the method, sample Sep09-A was selected. A preliminary screening revealed that fatty acids were the predominant compounds in *M. glacialis*. Given the fact that fatty acids were important compounds in the samples, both from a qualitative and quantitative point of view, we applied a methodology available in the bibliography that is currently used for the analysis of this class of metabolites derivatives (Folch

Table 6
Quantification of amino acids, fatty acids and sterols in *M. glacialis* samples.

Peak	RI^{Exp}	RI^{Lit}	Compound	*Marthasterias glacialis*			
				Jul09-A	***Sep09-A***	***FeB10-B***	***Feb10-C***
Amino acids				Average mg/100 **g** (± **SD)**			
1	1096	1095 [41]	Alanine	–	279.34 (19.87)	319.46 (1.20)	956.50 (73.61)
2	1115		Glycine	957.46 (89.13)	1520.42 (85.48)	1213.07 (251.30)	1969.08 (218.50)
3	1210	1210 [42]	Valine	18.14 (0.01)	30.36 (0.11)	23.80 (3.47)	105.94 (5.22)
4	1250	1262 [43]	Leucine	40.26 (5.26)	57.20 (2.60)	52.16 (1.08)	94.30 (3.39)
5	1274	1290 [41]	Isoleucine	20.08 (0.87)	25.63 (2.33)	28.71 (0.97)	57.61 (5.21)
6	1286	1299 [42]	Proline	48.91 (1.04)	29.28 (0.20)	23.91 (6.33)	–
7	1352	1343 [43]	Serine	100.57 (1.45)	69.62 (6.01)	56.24 (16.28)	–
8	1364	1367 [43]	Threonine	21.44 (0.72)	30.30 (4.19)	21.25 (3.47)	25.10 (3.79)
9	1500		*trans*-4-Hydroxyproline	94.88 (1.43)	131.26 (9.17)	37.79 (4.64)	–
10	1619	1622 [43]	Phenylalanine	11.22 (0.16)	38.94 (4.01)	33.80 (8.02)	65.46 (15.67)
Total				**1312.96 (94.75)**	**2212.34 (133.98)**	**1810.19 (231.29)**	**3273.99 (269.27)**
Fatty acids							
11	1837	1843 [43]	Myristic	316.46 (5.53)	96.15 (1.68)	23.87 (7.89)	334.74 (9.97)
12	1938	1943 [43]	Pentadecanoic	141.37 (0.73)	71.53 (6.03)	74.64 (18.25)	147.69 (2.90)
13	2043	2040 [43]	Palmitic	538.31 (68.28)	293.98 (4.68)	180.96 (14.99)	288.06 (10.37)
14	2147		Margaric	73.78 (0.33)	48.21 (0.43)	14.36 (0.20)	26.83 (0.21)
15	2214	2212 [44]	Linoleic	< LOQ	–	< LOQ	–
16	2225	2218 [44]	Linolenic	–	–	27.35 (2.20)	27.85 (0.32)
17	2238	2248 [44]	Oleic	27.76 (0.69)	44.49 (9.19)	21.73 (2.68)	19.63 (0.38)
18	2253	2234 [44]	Stearic	251.87 (0.88)	157.56 (0.92)	62.55 (0.69)	124.04 (0.66)
19	2389	2373 [43]	Arachidonic	444.20 (10.23)	267.82 (0.35)	425.13 (16.61)	602.34 (7.05)
20	2398	2380 [44]	Eicosapentaenoic	240.48 (9.14)	144.30 (1.04)	215.26 (5.52)	303.84 (5.30)
21	2404	2413 [39]	*cis*11,14-Eicosedienoic[a]	213.42 (18.77)	144.26 (7.12)	137.86 (3.96)	239.51 (22.14)
22	2425		*cis*11-Eicosenoic isomer[a]	438.09 (38.03)	243.24 (2.74)	190.45 (11.84)	252.09 (17.61)
23	2430	2420 [39]	*cis*11-Eicosenoic	374.67 (48.09)	265.36 (11.90)	181.42 (8.67)	207.19 (6.18)
24	2585	2562 [43]	Docosahexaenoic	63.28 (3.25)	117.89 (3.89)	99.72 (3.79)	72.19 (2.58)
Total				**3132.60 (23.82)**	**1895.12 (9.94)**	**1653.33 (14.10)**	**2645.16 (6.78)**
Sterols							
25	2938		Cholesta-3,5-diene	–	34.72 (0.22)	–	51.57 (0.50)
26	3176		M^+ 458 *m/z* (73(100), 369(88), 330(82), 75(75), 95(74), 129(72), 458(70), 81(51), 146(49), 91(48))[b]	–	–	22.29 (2.25)	90.16 (0.11)
27	3181		M^+ 456 *m/z* (344(100), 73(83), 81(70), 118(68), 95(60), 69(59), 148(58), 97(54), 75(54), 256(52))[b]	–	< LOQ	27.81 (2.65)	–
28	3190		M^+ 462 *m/z* (75(100), 216(88), 217(51), 446(38), 93(30), 356(28), 148(25), 55(25), 202(25), 81(24))[b]	–		29.24 (0.02)	64.05 (2.07)
29	3197		Cholesterol	–	< LOQ	40.69 (3.40)	44.28 (2.12)
30	3207		Cholestanol	–	13.05 (0.20)		–
31	3249		M+ 460 *m/z* (460(100), 75(45), 255(45), 444(38), 460(35), 213(32), 133(25), 73(25)147(23), 145(23))[b]	89.87 (4.29)	84.65 (4.94)	169.80 (6.09)	–
32	3271	3232 [45]	Ergosterol	64.18 (2.66)	45.96 (0.29)	90.14 (5.02)	65.61 (2.06)
33	3353		M^+ 474 *m/z* (472(100), 214(45), 75(39), 55(38), 91(37), 79(34), 119(33), 57(32), 149(30), 256(29))[c]	–	42.63 (1.23)	70.16 (4.48)	97.60 (1.04)
34	3443		M^+ 488 *m/z* (344(100), 75(80), 256(58), 487(55), 387(49), 93(41), 345(41), 81(36), 55(36), 214(33))[d]	–	22.80 (0.89)	29.25 (4.42)	44.05 (1.60)
35	3454		M^+ 488 *m/z* (344(100), 387(44), 75(38), 345(37), 254(33), 69(28), 55(21), 214(18), 73(18), 93(17))[d]	–	< LOQ	25.71 (3.68)	–
Total				**152.17 (4.29)**	**241.89 (7.04)**	**505.09 (31.95)**	**457.31 (0.14)**

n.i.: not identified.

[a] Quantified as *cis*11-eicosenoic acid.
[b] Quantified as cholesterol.
[c] Quantified as ergosterol.
[d] Quantified as β-sitosterol. RI^{Exp}—Retention Index obtained in this experiments; RI^{Lit}—Retention Index described in literature.

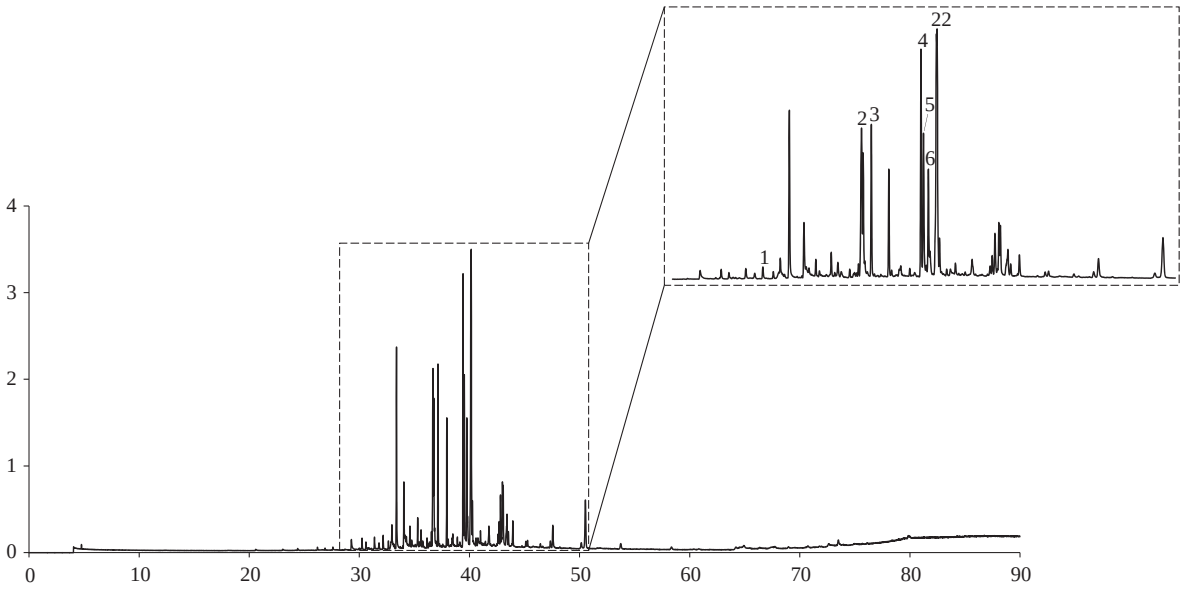

Fig. 2. GC–MS profile of *M. glacialis* using a previously described method [23]. Identity of peaks as in Table 6.

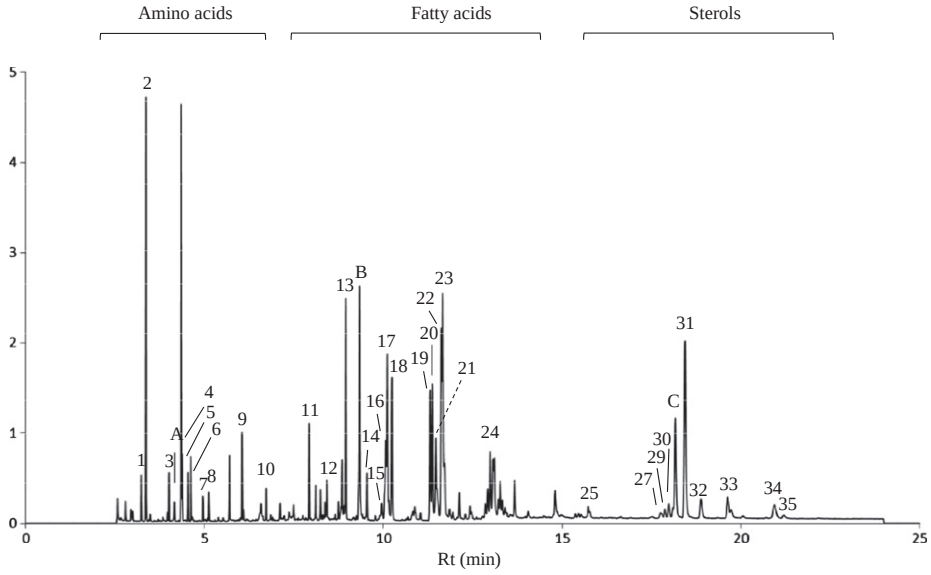

Fig. 3. GC–MS profile of *M. glacialis* using the method developed in this work. Amino acids, fatty acids and sterols are clearly separated in different areas of the chromatogram. Identity of compounds as in Table 6. IS (Internal Standard): A-norvaline, B-methyl linolelaidate, C-desmosterol.

method for the extraction and GC–MS analysis of FAME) [23]. Fig. 2 displays the resulting chromatogram, where it can be seen that, in addition to the absence of amino acids and sterols, only 6 compounds were identified. On the other hand, when the method proposed herein was used, about 16 fatty acids were determined.

Afterwards we used ethanol as extraction solvent, followed by TMS derivatization, in order to verify if the qualitative and quantitative profile would be affected. As shown in Fig. 3, ethanol effectively extracted amino acids, fatty acids and sterols. From a quantitative point of view, the amounts of fatty acids extracted exceeded those obtained with chloroform:methanol (Fig. 4). Water:-ethanol (1:1) was also tested as extraction solvent, but the high lipidic content caused insolubilization in water and, for this reason, this solvent was not used. Given the fact that our ethanol extraction and chromatographic conditions allowed the simultaneous identification of amino acids, fatty acids and sterols, we used them in subsequent studies. In addition, it has the advantage of using a cheap and non-hazard solvent (ethanol). Also, derivatization with MSTFA allows the use of mild temperatures (40 °C), while standard boron trifluoride method requires temperatures around 90 °C [23].

After this point, we studied the effect of the temperature in the extraction efficiency of ethanol: 40 °C, 50 °C and 60 °C were tested. As it can be seen in Fig. 4, extraction at 40 °C was the most effective for amino acids. The highest temperature assayed, 60 °C, resulted in high standard deviation indicating that some loss or reaction took place.

Regarding fatty acids, no major differences were noticed between different temperatures (Fig. 4). In the case of sterols, efficiency of extraction was highly affected by temperature, 40 °C being the most suitable one. After confirming that ethanol at 40 °C with magnetic stirring at 200 rpm was the most effective extraction procedure, we investigated the contribution of ultra-sonication (US) to the total amount of metabolites extracted. Although US clearly increased the level of extracted fatty acids, its low efficiency concerning both sterols and amino acids (Fig. 4) prevented its use and hence, all the

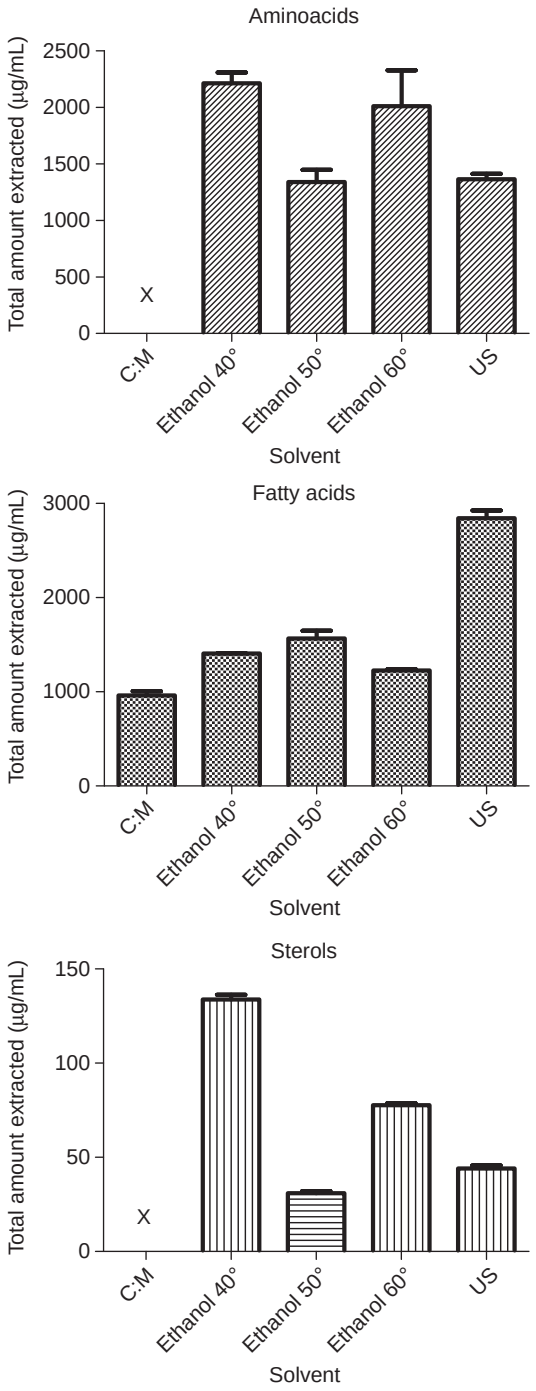

Fig. 4. Comparison of total amounts of amino acids, fatty acids and sterols extracted with different experimental conditions. C:M, chloroform:methanol (2:1); US, ultra-sonication. Data are means ± SD (n=3).

remaining analysis for validation parameters were conducted using ethanol as extraction solvent, at 40 °C, for 20 min, with a magnetic stirrer set at 200 rpm. This procedure represents an advantage when compared with other methods for fatty acids [7], amino acids [4,19] and sterols [5,23] that are time-consuming and employ high temperatures, which can result in the advent of artifacts or loss of analytes.

3.2. Method performance

In this work we developed a method for a quick screening analysis that renders both identification and quantification of three distinct classes of metabolites, useful for routine analysis and comparisons of samples. For this reason, among the several experimental conditions assayed, we selected the one that represented a compromise between the three classes. In this matter, ethanol at a temperature of 40 °C was the one that scored better results for 2 out of 3 classes.

3.2.1. Linearity

The linearity of the method was tested using calibration solutions prepared as described in the Experimental section. Internal standards for each class of compounds were used in order to account for analyte losses and matrix effect. Calibration curves were constructed by plotting the analyte/IS peak areas ratio obtained against the concentration values.

Good linearity for the concentration range studied was obtained for all compounds from the different chemical classes, as can be observed in Table 1, with correlations coefficients higher than 0.9844. Among the three classes, amino acids were those with best results (Table 1).

3.2.2. Recovery, precision and reproducibility

Ideally, all compounds addressed should be tested for recovery values. However, this becomes increasingly harder as the number of metabolites to be analyzed rises. Due to the diversity and complexity of metabolites present, in metabolomics of natural matrices it is not usual to determine the efficiency of the extraction of all of the compounds and the most abundant or more characteristic of each class are selected [26,27]. For this reason, and taking into account that we describe the identification and quantification of over 40 compounds, we decided to use two compounds of each class: isoleucine and *trans*-4-hydroxyproline for amino acids, palmitic acid and arachidonic acid for saturated and unsaturated fatty acids, respectively, and cholesta-3,5-diene and cholestanol for sterols, although we also tested other compounds, for example eicosapentaenoic acid (40 μg/ml—91% recovery; 80 μg/ml—95% recovery and 200 μg/ml—92% recovery) in order to check whether the method was acceptable.

Given the fact that over 15 fatty acids can be analyzed with this method, it is difficult to choose an internal standard that represents all compounds present equally. Furthermore, the fact that about 40 compounds are eluted in a 25 min run turns this issue even more difficult. For this reason, we choose methyl linolelaidate as internal standard for fatty acids as it was not present in the samples and did not co-elutes with any of the compounds.

Table 2 summarizes the results obtained for the analysis, in triplicate, of three concentrations levels. As can be observed, in the case of amino acids best recoveries were obtained for *trans*-4-hydroxyproline when compared to isoleucine. Similar recovery values were obtained for both palmitic and arachidonic acids (higher than 93%). Finally, sterols also showed good recoveries, varying between 92 and 106%.

Results for accuracy and precision can be found in Tables 3–5. The intraday variations (RSD) for the standard compounds ranged from 0.05 to 13.19% (n=3), being generally lower than those of the interday studies. Among the four classes, amino acids presented the smaller variations regarding both accuracy and precision.

3.2.3. Limit of detection and limit of quantification

In general, amino acids were the class yielding the lowest LOD and LOQ. LOD ranged between 0.03 and 2.68 μg/mL, for proline and cysteine, respectively. LOQ varied from 0.11 to 8.92 μg/mL for the same compounds, respectively (Table 1). Recently, a GC–MS method for the analysis of several amino acids was described [28].

That method presented very low detection limits and employed methyl chloroformate derivatization; however, quantification was obtained by applying deuterated derivatization reagents and, for this reason, it can be too expensive for routine screening analysis.

Regarding fatty acids, oleic acid was the compound with lower LOD and LOQ, 0.14 μg/mL and 0.48 μg/mL, respectively. Some recent works report the fatty acids profile of marine organisms [29–32]. Nevertheless, in most cases no complete validation data is available, which renders direct comparison difficult. Furthermore, quantification is frequently presented as percentage and not as absolute amounts [29,31,33,34].

3.3. Profiling of the echinoderm *M. glacialis*

In 2010 it was possible to collect organisms in the same month from two different sites (Praia do Baleal and Praia da Consolação, samples Fev10-B and Fev10-C, respectively) and, for this reason, direct comparison regarding the influence of geographical origin can be established. Sample Fev10-C presented ca. 80% higher amounts of amino acids than that from Baleal (Fev10-B) (Table 6). A similar trend was found for fatty acids, with organisms from Consolação having 40% more when compared to that of Baleal. Unsaturated fatty acids are major compounds in both samples (Table 6). In the case of sterols, no important changes between both locations were noticed (Fig. 5).

For the study of the influence of the season in the chemical composition, samples from the same geographical origin, Cabo Carvoeiro (samples Jul09-A and Set09-A), were used. Overall, samples from September had higher amounts of sterols and amino acids, with both classes displaying an increase of around 70% when compared to their July homologs (Fig. 5). Glycine was always the compound present in higher amounts (Table 6).

In the case of fatty acids, samples from September displayed a decrease in total amounts of around 35%. Both saturated and unsaturated free fatty acids have been described in echinoderms from Asteroidea, Holothuroidea and Echinoidea [35–37]. In the case of sea stars, palmitic acid is frequently the compound present in higher amounts, which may constitute a defense mechanism given its antifouling properties [36]. Our results show that in the case of *M. glacialis* this is only true for samples collected in July and September. As it can be seen in Table 6, in samples collected in February arachidonic acid was the major compound, far exceeding palmitic acid. It should be highlighted that among all samples studied, unsaturated fatty acids were present in higher amounts than saturated ones (Table 6).

Regarding sterols and lupanes, there was a clear distinction between samples from February and July/September, with the former displaying higher amounts of sterols and also greater diversity. Compounds 26 and 28 were found solely in organisms from February (Table 6). Ergosterol was the only compound present in all analyzed samples, while β-sitosterol, fucosterol, betulin, lupeol and lupeol acetate could not be found in any of them. However, a compound similar to β-sitosterol (34) was present in all samples, excepting in sample Jul09-A. In fact, when analyzing sterols we found several compounds whose mass spectra closely resembled those of cholesterol (compounds 26, 27 and 28), ergosterol (33) and β-sitosterol (compounds 34 and 35). We compared the mass fragmentation of these unknowns with some published MS data for sterols, such as brassicasterol, ergosta-7,22-dienol, ergosta-5,7-dienol, ergosta-7-enol [38], but no matches were found.

Apart from the interest of these metabolites for their bioactivity, the knowledge of the composition of these organisms can also be exploited from a chemico-ecological point of view. For instance, in the case of fatty acids most animals are unable to synthesize longer chain polyunsaturated fatty acids, such as EPA and DHA, obtaining them by preying on organisms from lower trophic levels, like bacteria and phytoplankton [31,39]. The same trend can be found in the case of sterols, which are synthesized by algae and plants [40].

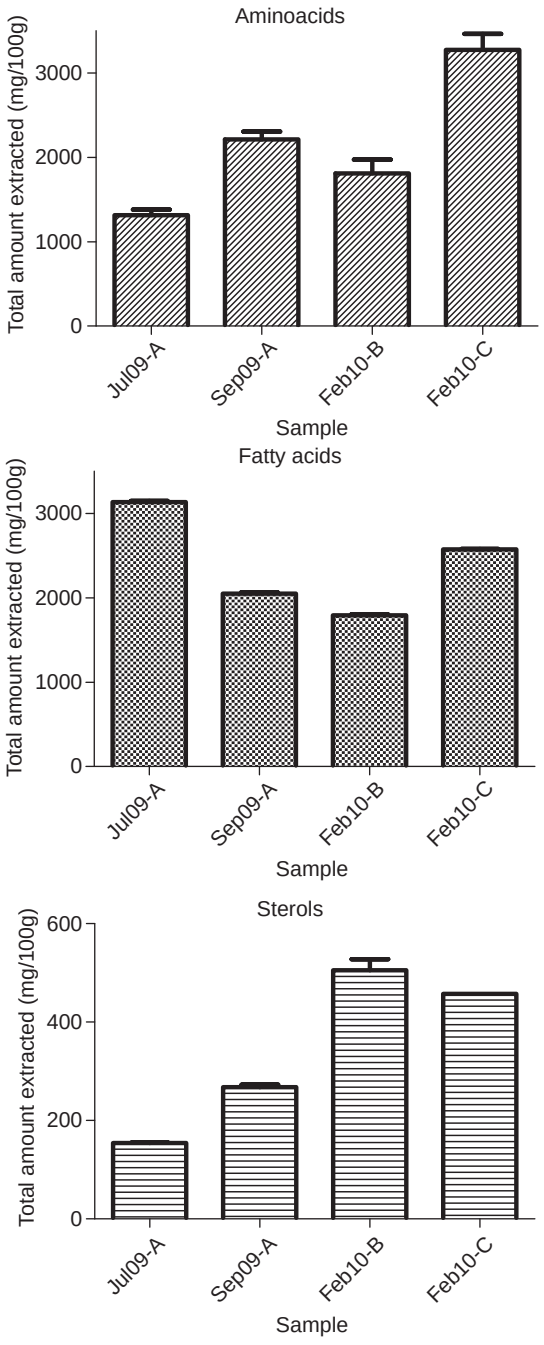

Fig. 5. Comparison of total amounts of amino acids, fatty acids and sterols in different *M. glacialis* samples. Data are means ± SD (n=3).

4. Conclusions

In this work, we report a GC–MS method for metabolite profiling of extracts of marine organisms. This approach constitutes a fast and powerful option for the identification and quantification of amino acids, fatty acids, sterols and lupanes in marine organisms. The extraction procedure is simple and

employs ethanol at mild temperatures, thus preventing degradation of sample or the production of artifacts. Since no purification or pre-treatment of samples are required the loss of analytes is minimized. The extraction method and the analytical method here developed appear to be good analytical tools available to researchers, for other matrices than marine organisms containing amino acids, fatty acids, sterols and lupanes.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.08.004.

Appendix. Supporting information

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