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Identification of Minor Fatty Acids from Mussels (*Mytilus galloprovincialis*) by GC-MS of their 2-alkenyl-4,4 dimethyloxazoline derivatives.

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ABSTRACT

The fatty acid composition of mussel lipids was determined by gas chromatography-mass spectrometry of their corresponding 2-alkenyl-4,4-dimethyloxazoline derivatives. Silver ion thin layer chromatography isolated enriched fractions of minor fatty acids extracted from mussels. Several non methylene interrupted fatty acids (NMI) were singled out, and the non methylene interrupted trienoic fatty acids (NMIT) 5,11,14-20:3 and 7,13,16-22:3 have been detected in marine mussels for the first time. This discovery strongly supports a previously suggested biosynthetic route implying the desaturation and subsequent elongation of 20:2 ω 6.

KEY WORDS

Fatty acids; Gas chromatography-mass spectrometry; Dimethyl oxazoline derivatives; *Mytilus galloprovincialis*; Non methylene interrupted fatty acids.

INTRODUCTION

Marine species are known to contain a wide variety of fatty acids. Non methylene interrupted fatty acids are usually found in invertebrates and seagrasses [1,2]. The function of NMI is not fully understood, although it has been suggested that a competitive incorporation into lipids may exist between the NMI and polyunsaturated fatty acids (PUFA) in marine organisms [3,4]. Other works have suggested that NMI in the membranes of marine bivalves may act as inhibitors of lipid peroxidation processes [5]. The accurate determination of both types of fatty acids (NMI and PUFA) may be of great importance to physiological and biochemical studies in marine molluscs [6]. However, this analysis may be hampered by low concentrations of some of these compounds and the complexity of marine lipid samples.

One of the more convenient approaches to the location of double bonds in the alkyl chain of fatty acids consists of analysing by gas chromatography-mass spectrometry (GC-MS) their corresponding derivatives containing a nitrogen atom [7]. Most analysts now prefer either picolynil or 4,4-dimethyloxazoline (DMOX) derivatives, which have proven advantages for particular types of fatty acids, and are best considered as complementary [7]. DMOX derivatives are markedly stable towards most reagents [8] and can be readily prepared and purified without great losses of PUFA [8,9]. The high yield of the derivatization procedure, usually better than 95% [10], allows the production of clear mass spectra even in the case of low concentrations of sample components.

The gas chromatographic characteristics of both derivatives are quite different. The analysis of picolynil esters requires temperatures approximately 50° higher than fatty acid methyl esters (FAMES). This fact constrained their GC analysis to the use of non-polar phases until the introduction of new polar phases stable to high column temperatures [7]. DMOX volatility is comparable to that of simple esters. In consequence, DMOX derivatives are readily separable by GC on highly polar phases such as cyanopropyl silicones with good resolutions in short analysis times and with a temperature maximum far below the recommended maximum column temperature [10, 11].

In the analysis of fatty acids from *M. galloprovincialis*, Christie et al [12] employed GC-MS of the picolynil ester derivatives separated on non-polar phases (5% phenylmethyl silicone). Considering that the use of DMOX derivatives may provide additional information on mussel fatty acid composition, we decided to undertake a complementary study employing these derivatives. However, in spite of the fact that most of the compounds in a complex fatty acid mixture can be identified and the recovery of the reaction is high, the main problem can be to achieve the suitable sample concentration range required for clear mass spectra of minor components without an overlapping of major fatty acids.

One approach to overcome this problem may be the fractionation of the FAMES by Silver Ion Chromatography (e.g. HPLC or TLC) [13]. This technique has been successfully applied to all lipid classes in order to resolve a complex lipid mixture into simpler molecular species. It allows the separation of fatty acid methyl esters on the basis of the number, configuration and to some extent the position of the double bonds [13].

In this work we report the identification of unusual fatty acids in marine mussels, such as non methylene interrupted dienoic (NMID) and NMIT fatty acids, via prior fractionation of FAMES by silver ion TLC according the number of unsaturations followed by GC-MS analysis of their corresponding DMOX derivatives.

EXPERIMENTAL

Materials. Acethyl chloride (for analysis), silver nitrate, 2,7 dichlorofluorescein , 2-amino-2-methylpropanol (for synthesis) were purchased from Merck (Darmstadt, Germany). All organic solvents employed were HPLC grade (Merck, Darmstadt, Germany). Mussels (*Mytilus galloprovincialis*) were collected from the Ria of Arosa coast (Pontevedra, Spain).

Extraction. Lipids were extracted from mussel muscle by the method of Bligh and Dyer [14].

Transmethylation of lipids. Lipids were converted into FAME by the method of Lepage and Roy [15].

Silver Ion Thin Layer Chromatography. FAMES were resolved by silver ion TLC on the basis of their number of the double bonds. Silica gel plates (20 x 20 cm) were impregnated by dipping into 10% silver nitrate in acetonitrile (w/v), dried horizontally, and activated at 100°C for one hour [16]. The total FAME solution was subsequently applied and developed twice in a solvent system of hexane:diethyl eter:acetic acid (94:4:2). After drying, plates were sprayed with a solution of 2,7-dichlorofluorescein and viewed under UV light. Zones were then carefully scraped from the plates and lipids extracted from the sorbent with a mixture of methanol-chloroform (2:1).

Derivatization. To obtain the DMOX derivatives, individual groups of FAMES according their degree of unsaturation were evaporated, redissolved in 100 µl of toluene and subjected to derivatization by the method of Fay et Richli [17] as modified by Dunstan et al [9].

GC-MS. A Hewlett-Packard Model 5890 gas chromatograph equipped with a split/splitless injection system in combination with a Hewlett-Packard 5971 mass-selective

detector was used. DMOX were analysed employing a fused silica capillary column (0.25 mm i. d. x 30 m) coated with 68% cyanopropyl silicone (SP-2330, Supelco Inc, Bellefonte, PA, USA). Helium was used as carrier gas at a column head pressure of 5 psi. The oven temperature was maintained at 80°C for 2 min, raised at 40°C/min to 150°C and then to a final temperature of 210°C (at a rate of 1°C/min).

The mass spectrometer was operated in the electron impact ionization mode (70 eV). The transfer line and ion source temperature were both 280°C. The structures of the different DMOX were elucidated from their mass spectra.

RESULTS AND DISCUSSION

The mussel *M. galloprovincialis* is known to show a very complex fatty acid composition [12]. The use of optimised injection procedures such as the programmable temperature vaporizer (PTV) [18] highly increases the sensibility of the analysis with the subsequent increase of chromatogram complexity. In mussel physiological studies undertaken using such techniques several unknown fatty acids, appearing to play an important physiological role [19], were detected in the chromatograms of the corresponding methyl ester derivatives. In order to identify these unknown components, the DMOX derivatives were obtained and analysed in a highly polar stationary phase column, but identification was difficult due to the small proportions in the sample. The FAME extract was fractionated by silver ion TLC in order to enrich the injected sample in minor components and give a simultaneous simplification of the chromatogram. All the saturated and most of the unsaturated fatty acids identified in this work (Table 1) coincide with those detected in a previous study using picolinyl derivatives [12]. The unknown fatty acids appeared as components of the di- and triunsaturated acid fractions (Figs. 1 and 2).

The chromatograms of both fractions show good resolutions, permitting clear mass spectra even for minor peaks. Both of them show the presence of significant amounts of certain fatty acids with a degree of unsaturation different to that expected (especially 16:0, Figs. 1 and 2), which can be explained by an incomplete resolution of the bands in the preparative silver ion TLC plate. The chromatogram of the trienoic fraction (Fig. 3) shows two peaks (both with intense molecular ions at m/z 331) that were tentatively identified as DMOX derivatives of diunsaturated C18 cyclic fatty acids [20]. These compounds, not detected in the FAMES chromatogram from the same sample, could have originated from

linolenic acid (very abundant in this fraction) during the preparation of DMOX derivatives, that requires prolonged high temperatures.

In the fraction of dienoic fatty acids, the use of DMOX derivatives allowed the identification of several NMID derivatives of 16, 17, 20 and 22 carbon atoms. Among them, the acid 7,13-16:2, not previously described in mussels (Fig.3). The DMOX derivative of this acid shows a molecular ion at m/z 305, and double bonds clearly assigned by the gaps of 12 mass units between 168-180 and 250-262.

In the same fraction several C20 (5,11-20:2, 5,13-20:2) and C22 (7,15-22:2) NMID fatty acids, characteristic of mollusc lipids, were also detected. These NMID fatty acids are synthesised as a result of the action of a $\Delta 5$ desaturase (C20 compounds) and subsequent elongation (C22) [21]. The biosynthetically related 7,13-22:2 acid could not be detected in our samples, and has been claimed to be only a minor component in the majority of marine bivalve molluscs [21].

In addition to other triunsaturated fatty acids previously reported [12], two NMIT derivatives were identified in the fraction corresponding to acids with three double bonds. These compounds, that appeared in proportions high enough to provide very clear mass spectra, were the acids 5,11,14-20:3 (Fig. 4) and 7,13,16-22:3 (Fig. 5). The first acid shows a clear molecular ion at m/z 359, and the position of double bonds can be identified individually. Most specifically, the double bond in position 5 is identified by the diagnostic ion at m/z 153, and the other two by the gaps of 12 units between m/z 222-234 and m/z 262-274 (Fig. 4). In the spectrum of the second NMIT acid, the molecular ion is located at m/z 387 and the positions of the double bonds are indicated by the gaps of 12 units between m/z 168-180, 250-262 and 290-302 (Fig. 5). These spectra exactly match those of the DMOX derivatives of the corresponding acids shown in the exhaustive review available on the internet by Christie [22].

Although the fatty acid composition of marine mussels (*M. edulis* and *M. galloprovincialis*) has been examined in several works [12,23,24], the occurrence of NMIT fatty acids in *M. galloprovincialis* is described here for the first time. The fact that these fatty acids have not been previously can be explained by biological (acids absent in the samples) or analytical reasons.

Until now only a few works, in which GC-MS of nitrogen-containing fragmentation-directing derivatives was applied, report the occurrence of NMIT fatty acids in other molluscs [9, 25, 26]. These results suggest that NMIT fatty acids may be found in more species if they are examined (or re-examined) using appropriate analytical methodologies. The fatty acids 5,11,14-20:3 and 7,13,16-22:3 (analysed as picolinylnyl esters) were described in the freshwater mussel *Unio tumidus* [25], where they appeared at very low concentrations. The use of pyrrolidides allowed the identification of the unusual fatty acid 7,11,15-22:3 in hydrocarbon seep mussels [26]. Using DMOX derivatives, Dunstan et al [9] detected 7,13,16-22:3 NMIT in the oyster *Crassostrea gigas*. Although they failed to identify an intermediate C20 NMIT acid, these authors suggested that this acid can be synthesized in molluscs by $\Delta 5$ desaturation and subsequent elongation of 20:2n-6. The discovery of the 5,11,14-20:3 NMIT in this work supports this biosynthetic route for NMIT fatty acids (Scheme 1). The mass spectra of picolinylnyl esters, pyrrolidides and DMOX derivatives of these and many other fatty acids can be found in [22].

Scheme 1

The combined results of this work and those of the previous study by Christie and co-workers [12] strongly support the fact that complementary information is provided by DMOX and picolinylnyl ester derivatives and the convenience of using both derivatives when studying complex fatty acid mixtures [7]. Such a double approach could be especially useful in the case of biochemical and biosynthetic studies of fatty acids in marine invertebrates, where relevant fatty acids can appear in very low proportions.

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REFERENCES

- [1] R.G. Ackman, in R.G. Ackman, Marine Biogenic Lipids, Fats and Oils, Vol. 1, CRC Press, Boca Raton, 1989, p. 103.
- [2] J.D. Joseph, in R.G. Ackman, Marine Biogenic Lipids, Fats and Oils, Vol. 2, CRC Press, Boca Raton, 1989, p. 49.
- [3] J.S. Klingensmith, *Lipids* 17 (1982) 976.
- [4] M. Abad, C. Ruiz, D. Martínez, G. Mosquera.; J. L. Sánchez, *Comp. Biochem. Phys. C* 110 (1995) 109.
- [5]: M.V. Zakhartsev; N.V. Naumenko., V.P. Chelomin, *Russ. J. Mar. Biol.* 24 (1998) 183.
- [6] U. Labarta, M.J. Fernández-Reiriz, A. Pérez-Camacho, *Comp. Biochem. Physiol. A* 123 (1999) 249.
- [7] W.W. Christie, *Lipids* 33 (1998) 343.
- [8] Q.T. Yu, B.N. Liu, J.Y. Zhang, Z.H. Zang, *Lipids* 23 (1988) 804
- [9] G.A. Dunstan, J.K. Volkman, S.M. Barrett, *Lipids* 28 (1993) 937.
- [10] V. Spitzer, *Prog. Lipid Res.* 35 (1997) 387.
- [11] J.L. Garrido, I. Medina, *J. Chromatogr. A* 673 (1994) 101.
- [12] W.W. Christie, E.Y. Brechany, K. Stefanov, *Chem. Phys. Lipids* 46 (1988) 127.
- [13] B. Nikolova-Damyanova, in W.W. Christie, *Advances in Lipid Methodology*, The Oily Press, Ayr, 1992, p. 181.
- [14] E.G. Bligh, W.J. Dyer, *Can. J. Biochem.* 37 (1959) 911.
- [15] G. Lepage, C.Roy *J. Lipid. Res.* 27 (1986) 114.
- [16] M. Kates, *Techniques of Lipidology*, 2nd Ed., Elsevier, Amsterdam, 1986.
- [17] J. Fay, U. Richli, *J. Chromatogr.* 541 (1991) 89.
- [18] I. Medina, F. Linares, J.L. Garrido, *J. Chromatogr.* 659 (1994) 472.
- [19] M.J. Fernández-Reiriz, U. Labarta, J.M.F. Babarro, *J. Shellfish Res.* 15 (1996) 349.
- [20] G. Dobson, W.W. Christie, E. Y. Brechany, J.L. Sebedio, J.L. Le Quere, *Chem Phys. Lipids* 75 (1995) 171.
- [21] N.V. Zhukova, *Comp. Biochem. Physiol. B* 100 (1991) 801.
- [22] W.W. Christie, *Mass Spectrometry of Fatty Acid Derivatives*, <http://www.lipid.co.uk/infores/masspec.html>.
- [23] A.V. Frolov, S.L. Pankov, *Comp. Biochem. Physiol. A* 112 (1995) 441.
- [24] N. Leonardos, I.A.N. Lucas, *Aquaculture* 184 (2000) 155.
- [25] K. Stefanov, K. Seizova, E.Y. Brechany, W.W. Christie, *J. Nat. Prod.* 55 (1992) 979.

- [26] J. Fang, P.A. Comet, J.M. Brooks, T.L. Wade, *Comp. Biochem. Physiol. B* 104 (1993) 287.

Table 1. Unsaturated fatty acids* identified in the different fractions of *M. galloprovincialis*

Monoenoic	Dienoic^a	Trienoic^a	Tetraenoic	Pentaenoic	Hexaenoic
14:1 ω 9 (11.1)					
14:1 ω 7 (11.7)					
14:1 ω 5 (11.9)					
15:1 ω 11 (12.3)					
15:1 ω 9 (12.7)					
16:1 ω 7 (17.7)	1.16:2ω7 (17.5)	1.16:3ω4 (21.7)	16:4ω4 (22.4)		
16:1 ω 5 (18.1)	2.16:2ω6 (17.8)				
16:1 ω 3 (18.2)	3.16:2ω4 (18.7)				
	4.7,13-16:2 (19.3)				
17:1 ω 11 (18.5)					
17:1 ω 9 (20.3)					
17:1 ω 8 (20.6)					
17:1 ω 7 (20.9)					
17:1 ω 5 (21.4)					
18:1 ω 13 (23.3)	5.18:2ω6 (26.3)	2.18:3ω6 (28.5)	18:4ω4 (34.1)		
18:1 ω 11 (24.4)	6.18:2ω4 (27.3)	3.18:3ω4 (31.2)	18:4ω3 (34.5)		
18:1 ω 9 (25.3)		4.18:3ω3 (36.1)	18:4ω2 (34.7)		
18:1 ω 7 (26.0)					
18:1 ω 5 (26.1)					
19:1 ω 8 (29.4)					
20:1 ω 13 (33.7)	7.5,11-20:2 (33.6)	5.5,11,14-20:3 (38.2)	20:4ω6 (40.4)	20:5ω3 (46.6)	
20:1 ω 11 (34.7)	8.5,13-20:2 (34.6)	6.20:3ω6 (39.2)			
20:1 ω 9 (35.8)	9.20:2ω6 (36.6)	7.20:3ω3 (41.6)			
20:1 ω 7 (36.1)					
	10.7,15-22:2 (46.7)	8.7,13,16-22:3 (50.5)	22:4ω6 (52.1)	22:5ω6 (58.2)	22:6ω3 (59.3)

* Retention times (min) are given in brackets. ^a Numbers in bold correspond to peaks in Figures 1 and 2.

Figure captions

Fig. 1. Total ion chromatogram of diene fraction of DMOX derivatives obtained from fatty acids of *M. galloprovincialis*. Peak numbers as in Table I. The asterisk (*) designates a non-lipidic contaminant.

Fig. 2. Total ion chromatogram of triene fraction of DMOX derivatives obtained from fatty acids of *M. galloprovincialis*. Peak numbers as in Table I. Peaks marked with an asterisk (*) correspond to DMOX derivatives of diunsaturated C₁₈ cyclic fatty acids (tentative identification, see text)

Fig. 3.- The mass spectrum of the DMOX derivative of 7,13 hexadecadienoic acid (7,13-16:2)

Fig. 4.- The mass spectrum of the DMOX derivative of 5,11,14 eicosatrienoic acid (5,11,14-20:3)

Fig. 5.- The mass spectrum of the DMOX derivative of 7,13,16 docosatrienoic acid (7,13,16-22:3)