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Detection of short-chain aldehydes in marine organisms: the diatom *Thalassiosira rotula*

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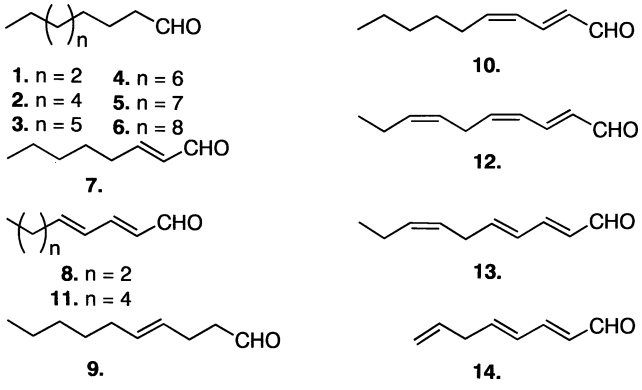
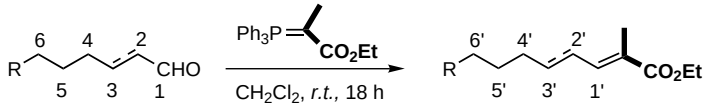
Abstract—Short-chain aldehydes are analysed by GC–MS and NMR after their transformation into the corresponding carboxyethylethylidene (CET) derivatives via Wittig reaction. The procedure implies the treatment of the aldehyde with (carbetoxyethylidene)-triphenylphosphorane under very mild conditions. The method is suitable for the detection of short and medium chain aldehydes. CET derivatives are easily prepared and can be utilised for the analysis of raw biological samples. The efficacy of the method has been tested in the identification of biologically active aldehydes in the marine diatom *Thalassiosira rotula*. At least two compounds, *trans*,*trans*-octadienal and 2-*trans*-4-*trans*-2,4,7-octatrienal, that have not been revealed in previous papers are unambiguously identified in the microalga. © 2002 Published by Elsevier Science Ltd.

Enzymatic oxidation of polyunsaturated fatty acids produces a plethora of bioactive metabolites including linear aldehydes with 6–10 carbon atoms. The biochemical steps leading to short-chain aldehydes (SCA) have been the subject of much research and these molecules have been related to tumour and arteriosclerosis progress in mammalian cells,[1,2] as well as to development and response to stress in plants.[3] Recently, diatom-produced SCAs have been found to reduce egg viability of copepods, the dominant constituents of the zooplankton. At sea, this activity is related to a control on copepod populations during marine diatom-dominated bloom. The diatom–copepod interaction has no equivalent in other marine or terrestrial systems and may represent one of the major topics for the oceanographic communities in the next years, since diatoms and copepods constitute the first source of organic material in the marine food chain.[4] Therefore, it can be anticipated that increased attention will be devoted to find fast and facile methodologies to address large-scale purification and analysis of SCAs in marine samples, by keeping in mind that: (a) low amounts of SCAs are present in cells, (b) SCAs are very unstable and (c) they may have structures that have not been encountered in terrestrial sources so far. In this article we present a novel approach for monitoring SCAs in marine samples, that fulfils the prerequisites mentioned above. The procedure has been tested on the marine diatom *Thalassiosira rotula*, from which Miralto and co-workers have already described three C10 aldehydes responsible of the abortive effects on copepod eggs.[5] In analogy with





Scheme 1. Derivatisation procedure with 1.1 equiv. of CET-triphenylphosphorane.

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the mechanism acting in other plants, these aldehydes are very likely derived from oxidation of fatty acids,[6] although very little is known on their chemical composition, role and biogenesis in marine microalgae. Interestingly, these metabolites seem to have a general inhibitory activity on cell growth since they are also able to arrest in vitro cleavage of sea urchin embryos and development of human tumours.[5]

Aldehyde-containing compounds are generally very unstable and prompt to decompose, thus they are usually analysed after transformation into products that are easier to handle.[7,8] In particular, to tackle the inherent difficulties involved with the isolation of aldehydes from marine diatoms, we have worked out an alternative procedure based on the conversion of aldehyde into ethyl ester by Wittig reaction with (**C**arbetoxy**ET**hylidene)-triphenylphosphorane [**CET**-triphenylphosphorane] (Scheme 1). The method was designed to allow the GC–MS detection of compounds with an alkyl chain ranging from 8 to 16 carbon atoms, but the procedure is also suitable for NMR and HPLC applications. The effect of temperature, solvent and reaction time was carefully evaluated in order to establish the most advantageous conditions for the derivatisation.[9] Reaction with 1.1 equiv. of derivatising agent in CH_2Cl_2 at room temperature for 18 hours was found as a good balance between yield and product alteration. Under these conditions, CET derivatives were formed almost quantitatively (about 98% of yield) with unconjugated aldehydes (**1**–**5** and **9**), whereas lower yields (87% of yield) were found with α,β-unsaturated homologues (**7**–**8** and **10**–**12**). The new generated double bond showed almost exclusively *E* configuration (95–98%), even if the fraction of *Z* isomers were to raise with the increase of the amount of derivatising agent and reaction temperature.

For GC–MS analysis,[10] the reaction mixture was evaporated to dryness and dissolved again in *n*-hexane or CH_2Cl_2 in order to obtain a final concentration of 0.1 or 1 μg/μL. These solutions were directly analysed by GC–MS, thus preventing any loss of material due to further sample manipulation. Alternatively, the sample was applied onto a SepPack SiO_2-cartridge and the derivatised compounds were purified with petroleum ether/Et_2O 95:5. An average recovery of 90% was obtained in this latter case. Gas chromatographic elution of CET derivatives required a slow temperature gradient that led to separate saturated compounds at intervals of 2–3 min (Table 2). On the other hand, in agreement with the number of double bonds, derivatives of conjugated aldehydes were eluted later than the saturated homologues (Fig. 1). Unambiguous characterisation of each component was facilitated by the very characteristic fragmentation pattern in the high mass range of the MS spectra (Table 1). These included the intense molecular ion (M^+) and ion formed by loss of $CH_3CH_2O^\bullet$ ($M-45^+$), as well as ions at m/z 115 and ($M-29^+$) in the saturated and conjugated series, respectively. However, the analysis of the MS spectra proved to be rather simple as most of the peaks could be explained by fragmentation of the ($M-45^+$) ions.

It is noteworthy that we did not find any double bond isomerisation as a consequence of the derivatisation procedure. The method was totally faithful even with very unstable compounds, such as *trans*,*cis*,*cis*-2,4,7-decatrienal (**12**) and *trans*,*cis*-2,4-decadienal (**10**), that have an intrinsic instability of the double bond geometry.[11] In this regard, an unambiguous determination of the double bond configuration of CET derivatives was obtained by NMR spectroscopy, since introduction of the carboxyethylidene group produced a strong regiodifferentiation of the hydrogens in α, β and γ to the carboxylic group (see Table 2). Clear evidence of this effect is offered by the CET derivatives of *trans*,*cis*-2,4-decadienal (**10a**) and *trans*,*trans*-2,4-decadienal (**11a**) that differ significantly for the chemical shift of H-1′, H-2′ and H-3′ [respectively, δ 7.27, 6.48 and 6.84 in **10a** and at δ 7.20, 6.38, and 6.50 in **11a**] (Table 2). Obviously, the NMR properties of the CET derivatives may

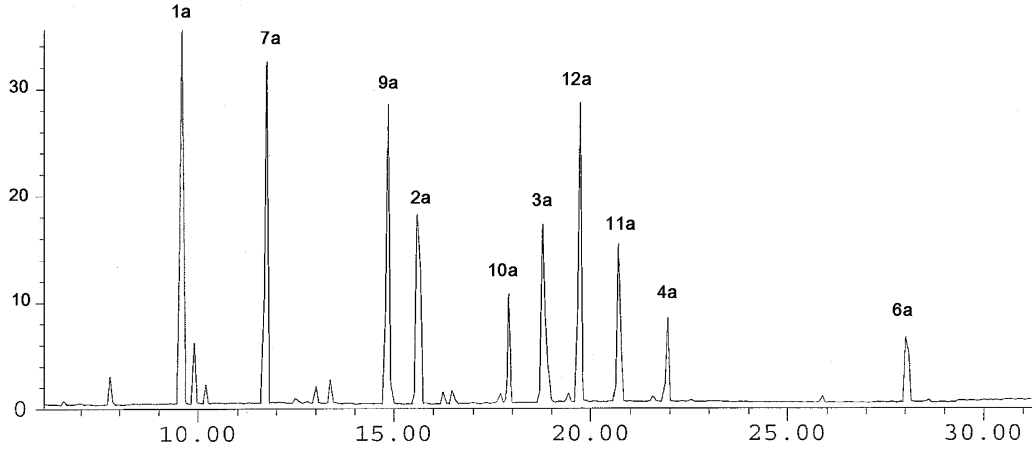

Figure 1. GC profile of CET derivatives of octanal (**1a**), 2-*trans*-octenal (**7a**), 4-*trans*-decenal (**9a**), decanal (**2a**), 2,4-*trans*,*cis*-decadienal (**10a**), undecanal (**3a**), 2,4,7-*trans*,*cis*,*cis*-decatrienal (**12a**), 2,4-*trans*,*trans*-decadienal (**11a**), dodecanal (**4a**), tetradecanal (**6a**).

Table 1. GC–MS (EI, 70 eV) data of selected CET derivatives

	Parent aldehyde	Time (min)	*m*/*z*
1a	C8:0	9.6	212 (25), 167 (45), 141 (30), 115 (80), 102 (100), 87 (65)
2a	C10:0	15.6	240 (15), 195 (35), 141 (15), 115 (85), 102 (100), 87 (50)
4a	C12:0	21.9	268 (15), 223 (25), 141 (15), 167 (60) 115 (80), 102 (100)
5a	C13:0	25.5	282 (15), 237 (40), 171 (15), 141 (20), 117 (75), 102 (100)
7a	C8:1 (2*E*)	11.7	210 (45), 165 (25), 139 (100), 111 (70), 81 (50), 79 (55)
8a	C8:2 (2*E*,4*E*)	13.9	208 (65), 179 (50), 163 (20), 133 (75), 105 (70), 93 (100)
9a	C10:1 (4*E*)	14.8	238 (10), 193 (15), 128 (100), 100 (45), 69 (65)
10a	C10:2 (2*E*,4*Z*)	17.9	236 (30), 207 (25), 291 (65), 290 (70), 161 (70), 109 (80), 93 (100)
11a	C10:2 (2*E*,4*E*)	20.8	236 (70), 207 (35), 191 (15), 137 (40), 133 (65), 93 (100), 79 (55)
12a	C10:3 (2*E*,4*Z*,7*Z*)	19.8	234 (60), 205 (15), 189 (25), 159 (40), 131 (65), 91 (100), 79 (90)
13a	C10:3 (2*E*,4*E*,7*Z*)	21.0	234 (30), 205 (10), 189 (15), 159 (25), 131 (55), 91 (100), 79 (80)

Table 2. ^{1}H NMR (400 MHz) data of standard CET derivatives of C_{10} aldehydes

	2a	**9a**	**10a**	**11a**	**12a**
1′	6.75, dt	6.75, dt	7.27, bd	7.20, dd	7.27, bd
2′	2.16, bq	1.97, bq	6.48, dd	6.38, dd	6.48, dd
3′	1.33–1.20, m	2.21, bt	6.84, dd	6.50, dd	6.84, dd
4′	1.23, m	5.42, m	6.14, t	6.18, dd	6.14, t
5′	1.23, m	5.42, m	5.58, dd	5.88, dt	5.60, dd
6′	1.23, m	2.13, bt	2.09, t	2.16, bqt	2.99, t
7′	1.23, m	1.23, m	1.42, m	1.42, m	5.35, m
8′	1.23, m	1.23, m	1.29, m	1.30, m	5.41, m
9′	1.23, m	1.23, m	1.29, m	1.30, m	2.09, t
10′	0.88, t	0.88, t	0.98, t	0.90, t	0.99, t

Spectra were recorded in $CDCl_3$ and are referenced to $CHCl_3$ (δ 7.26) as internal standard.

be particularly useful for the characterisation of unknown SCAs, when standard compounds are not commercially available.

The CET derivatisation described above was applied to detect the production of short-chain aldehydes in *T. rotula*.[12] A very limited amount of the microalga (3 g) was sonicated as described by Miralto et al.[5] and the resulting water suspension was diluted with acetone and extracted with CH_2Cl_2. Direct derivatisation of the organic extracts gave an enriched pool of CET derivatives that were examined after purification on mini-columns of silica gel. As expected, GC–MS analysis of this mixture (Fig. 2) showed the presence of unsaturated C_{10} compounds (**12a**, **13a** and trace of **11a**), that were unambiguously characterised by comparison of

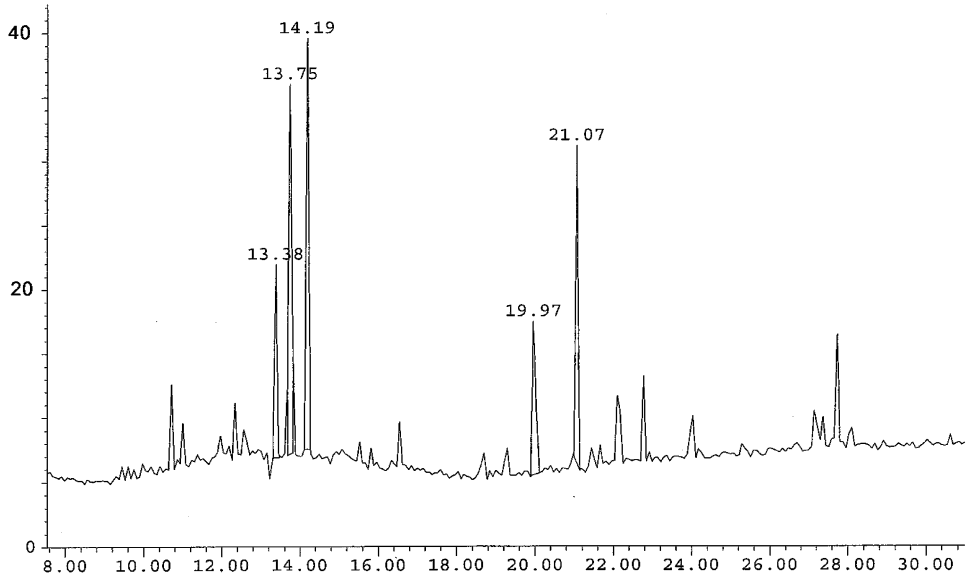

Figure 2. GC profile of CET-aldehydes from *T. rotula*. Derivatives of 2-*trans*-4-*trans*-2,4,7-octatrienal (**14a**), *trans*,*trans*-2,4-octadienal (**8a**), *trans*,*cis*,*cis*-2,4,7-decatrienal (**12a**), *trans*,*trans*-2,4-decadienal (**11a**), *trans*,*trans*,*cis*-2,4,7-decatrienal (**13a**).

their spectroscopic data with those of standard products. This finding confirmed the results previously reported by Miralto et al.,[5] although under the experimental conditions described here *T. rotula* extracts also contained high levels of other aldehydes, including *trans*,*trans*-2,4-octadienal (**8**), and 2-*trans*-4-*trans*-2,4,7-octatrienal (**14**). The role and origin of the last compounds are uncertain although it is plausible that they may derive by oxidation of fatty acids, as already suggested for **11**–**13**.[6] Interestingly, 2-*trans*-4-*trans* isomers were significantly predominant over the corresponding 2-*trans*-4-*cis* analogues, thus suggesting that some extent of Z/E isomerisation occurs during the extraction of the sample. No aldehyde was detected when microalgae were extracted without treatment (sonication) of the cell pellets.

In conclusion, this procedure is sensitive (detection limit is approximately 100–200 μg of aldehyde in the starting sample) and specific, since only molecules containing aldehydic groups react satisfactorily with the derivatising agent. The MS fragmentation patterns of CET derivatives (mainly, M^+, $M-29^+$ and $M-45^+$) also make possible the use of GC–MS analysis of unknown samples in the selected ion monitoring (SIM) mode, thus allowing detection of very low amounts of SCAs with confidence. Furthermore, the combination of GC–MS and NMR provides a useful tool to allow a complete structural determination of unknown SCAs from biological samples. The identification of polyunsaturated C_{10} and C_8 aldehydes in stressed cells of *T. rotula* is an evident demonstration of the potentiality of this analytical procedure. It is interesting to note that the presence of other polyunsaturated compounds, closely related to the previously described decanals **11**–**13**, suggests the existence of a family of polyunsaturated aldehydes involved in the chemical interactions within planktonic communities. Furthermore, the absence of these compounds in non-disrupted cells seems to imply activation of phospholipases in order to make the unsaturated fatty acids available for the lipoxygenase/hydroperoxide lyases oxidation. The procedure described above is a first analytical proposal to tackle the many questions that have been opened by the identification of polyunsaturated aldehydes as 'indirect regulators' of the grazing pressure by herbivores on phytoplankton populations. We are only now approaching this new and exciting area of marine science, a higher investment of technologies and energies may be foreseen.

Acknowledgements

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9. *Preparation of CET derivatives*. In a typical procedure, 10 mg of aldehyde was dissolved in 2 mL of dry CH_2Cl_2 and then treated with 1.1 equiv. of CET-triphenylphosphorane. The reaction was stirred at room temperature for 18 h in CH_2Cl_2 and then the organic solvent was evaporated at reduced pressure. CET derivatives were purified on silica gel by eluting with petroleum ether/Et_2O 95:5.
10. *GC–MS analysis of CET-aldehydes*. CET-aldehydes were dissolved in *n*-hexane or CH_2Cl_2 and analysed by a temperature gradient from 130 to 220°C at 3°C/min (injector temperature 240°C, detector temperature 260°C, nitrogen flow 1 mL/min). Electron voltage was set at 70 eV. GC–MS data of CET derivatives of **1**–**14** are reported in Table 1.
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12. *Analysis of T. rotula extract*. A frozen sample of *T. rotula* was obtained and treated in agreement with Miralto and co-workers.[5] Briefly, the pelletted cells (3 g wet weight) were sonicated in distilled water (3 ml) for 1 min and left on the bench for 30 min. Acetone (3 ml) was then added to the suspension and the resulting solution was centrifuged three times at 4000 rpm for 10 min. The supernatant was transferred to a separatory funnel and then extracted with CH_2Cl_2 three times. The organic layers were combined, dried over dry Na_2SO_4 and then evaporated at reduced pressure. The resulting oil (about 32 mg) was re-dissolved in 5 mL of CH_2Cl_2 and treated with 16 mg of (carbetoxyethylidene)-triphenylphosphorane. The reaction was stirred at rt for 18 h and then evaporated to dryness. The residue was then fractionated by SiO_2 column to give 1.7 mg of CET derivatives.