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Long-chain (C_{19}–C_{29}) 1-chloro-*n*-alkanes in leaf waxes of halophytes of the Chenopodiaceae

Vincent Grossi*, Danielle Raphel

Laboratoire d'Océanographie et de Biogéochimie (UMR CNRS 6535), Centre d'Océanologie de Marseille (OSU), Campus de Luminy, case 901, F-13288 Marseille, France

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Abstract

The hydrocarbon fraction of leaf waxes of three halophytes of the Chenopodiaceae common to Mediterranean salt marshes (*Suaeda vera*, *Sarcocornia fruticosa* and *Halimione portulacoides*) revealed the presence of a minor series of odd and even chains 1-chloro-*n*-alkanes ranging from C_{19} to C_{29}. The identification of these new chlorinated plant constituents was based on a combination of mass spectrometry data with selective chlorine detection (CPG-AED) and was confirmed by comparison with authentic standards. The qualitative and quantitative distributions of these 1-chloro-*n*-alkanes varied inter-specifically. Homologues with an odd carbon-chain were predominant in all species but maximised at C_{25} and C_{27} in *S. vera* and *S. fruticosa*, and at C_{27} and C_{29} in *H. portulacoides*. Remarkably, 1-chloro-nonacosane was an abundant homologue only in this latter species. Leaves of *S. vera* contained 4 to 7 times more of total chloroalkanes than leaves of the other two species. These compounds accounted for 10, 4 and 1% of the hydrocarbon fraction of leaf waxes of *S. vera*, *S. fruticosa* and *H. portulacoides*, respectively. Attempts to link the occurrence of these chloroalkanes with other classes of leaf waxes (*n*-alkenes, *n*-aldehydes and *n*-alcohols) did not allowed a clear precursor–product relationship to be established. The biological functions as well as the mode of synthesis of alkylchlorides in (halophyte) plants remain unknown but undoubtedly deserve further attention.

Keywords: *Suaeda vera*; *Sarcocornia fruticosa*; *Halimione portulacoides*; Chenopodiaceae; Halophytes; Leaf waxes; Chloro-alkanes; Hydrocarbon fractions

1. Introduction

Cuticular and epicuticular plant waxes (also called surface lipids) play an important role in all physical and physiological processes that occur within plant tissues. Especially, they constitute the first physico-chemical barrier to the entry of pathogens and to adverse environmental factors. These waxes occur primarily on the surface of leaves, sheaths, stems and fruits although variable waxy material has been found in other plant organs such as seeds (Bianchi, 1995).

Plant surface lipids are composed of numerous chemical classes of compounds which differ from one another in the number, relative percentage and composition of homologues. Surface lipids mostly comprise alicyclic and long-chain aliphatic components that can be further classified according to the structure, functional groups and distribution of their dominant homologues. Hydrocarbons are one of the most ubiquitous wax class being present in almost all plant surface lipids in percentages varying from traces to over 50% of the whole wax (Maffei, 1996 and references therein).

Unlike marine plants, relatively few higher plant metabolites contain halogens (Gribble, 1996a, 2003). Moreover, most of the halogenated compounds detected in plants have a (poly)cyclic chlorinated structure (e.g. chloroindoles, chlorohydrins; Gribble, 1996a, 2003). A notable exception is chloromethane produced by evergreen trees, potato tubers, cabbage and the ice plant (Gribble, 1996a, 1996b, 2003; Harper, 2000). In the present study, we report the occurrence of longer chains (C_{19} to C_{29}) 1-chloro-*n*-alkanes in the hydrocarbon fraction of leaf waxes of three halophytes members of the Chenopodiaceae: *Suaeda vera* Forsskal ex

* Corresponding author. Tel.: +33-491-829-651; fax: +33-491-826-548.
E-mail address: grossi@com.univ-mrs.fr (V. Grossi).

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J.F. Gmelin, *Sarcocornia fruticosa* (L.) A.J. Scott and *Halimione portulacoides* (L.) Aellen. These species are common halophytes growing on marine coastlines and are particularly abundant in Mediterranean salt marshes (Pujol et al., 2000; Álvarez Rogel et al., 2001).

2. Results and discussion

2.1. Characterization and distribution of 1-chloro-n-alkanes in hydrocarbon fractions

The amount of total hydrocarbons in leaves of the halophytes varied (on a dry weight basis) from 75 μg/g in *S. fruticosa* up to *ca.* 460 μg/g in *H. portulacoides* (Table 1). The chromatograms of the three hydrocarbon fractions classically revealed a dominance of *n*-alkanes with a strong odd-over-even carbon-chain length predominance maximising in the C_{25}–C_{29} range (Figs. 1A and 2A). These linear hydrocarbons were sometimes accompanied by small proportions of *iso*- (2-methyl-) and *anteiso*- (3-methyl-) alkanes and of some monounsaturated hydrocarbons, common to many plants (Bianchi, 1995).

Intriguingly, the chromatograms of the three hydrocarbon fractions further revealed the presence of another minor series of compounds eluting between *n*-alkanes and methyl-branched alkanes (Fig. 1A). These compounds exhibited EI-mass spectra resembling those of hydrocarbons but with an extra series of low intensity ions at m/z 91 + 14n (n = 0, 1, 2, 3, etc.) accompanied by "isotopic clusters" characteristic of *n*-alkyl chlorides containing more than five carbon atoms (Fig. 3; McLafferty and Tureček, 1993). The presence of chlorine in these compounds was confirmed unambiguously by analysing the hydrocarbon fractions with a GC coupled to an atomic emission detector set on the wavelength of one characteristic emission line of chlorine (Fig. 1B). In the EI-mass spectra of these organochlorines (Fig. 3), the molecular ions and the proportion (*ca.* 30%) of "isotopic peaks" two mass units higher of all the chlorine-containing fragments (m/z 91 + 14n as well as $M^{+}\bullet$) indicated the presence of one atom of chlorine. Moreover, the quasi-absence of $(M-HCl)^{+}\bullet$ and $(M-Cl)^{+}$ ions was indicative for primary chlorides. These compounds were thus identified as linear 1-chloro-alkanes; this was confirmed by the comparison of their retention times and mass spectra with those of synthetic standards. The ion series at m/z 91 + 14n (together with the lower "isotopic peaks" at m/z 93 + 14n) in the EI-mass spectra of these long-chain chloroalkanes comes from intramolecular cyclizations of the chlorine radical to form divalent chloronium ions ($C_nH_{2n}Cl^{+}$ with $n \geqslant 4$; Fig. 3) with displacement of alkyl groups (McLafferty and Tureček, 1993).

Odd and even carbon-chain homologues of 1-chloro-*n*-alkanes ranging from C_{19} to C_{29} were observed in *S. fruticosa* whereas all but the C_{19} homologue were detected in *S. vera* and *H. portulacoides* (Table 1 and Fig. 1B). It is noteworthy that similar chloroalkanes were not detected in another halophyte of the Umbelli-

Table 1
Amounts of individual and total 1-chloro-*n*-alkanes and of total hydrocarbons, *n*-aldehydes, *n*-alkanols and fatty acids in leaves of three halophylic members of the Chenopodiaceae

	Suaeda vera		*Sarcocornia fruticosa*		*Halimione portulacoides*	
Compounds	μg/g dry wt	μg/mg HC	μg/g dry wt	μg/mg HC	μg/g dry wt	μg/mg HC
1-Cl-C_{19}	n.d.[a]	–	tr	–	n.d.	–
1-Cl-C_{20}	tr[b]	–	tr	–	tr	–
1-Cl-C_{21}	0.2	1.0	0.1	1.0	tr	–
1-Cl-C_{22}	0.9	4.1	0.2	3.1	0.2	0.5
1-Cl-C_{23}	2.0	9.0	0.5	6.1	0.2	0.5
1-Cl-C_{24}	3.5	16.0	0.5	6.1	0.4	0.9
1-Cl-C_{25}	5.6	25.8	1.1	14.3	0.8	1.8
1-Cl-C_{26}	2.0	9.0	0.2	3.1	0.4	0.9
1-Cl-C_{27}	6.9	31.4	0.7	9.2	1.7	3.6
1-Cl-C_{28}	0.2	1.0	tr	–	0.2	0.5
1-Cl-C_{29}	0.3	1.3	tr	–	1.0	2.3
Σ1-Cl-HC	21.6	98.6	3.3	42.9	4.9	11.0
ΣHC[c]	218	–	75	–	462	–
Σ*n*-aldehydes[c]	475	–	50	–	546	–
Σ*n*-alkanols[c]	1306	–	2622	–	462	–
Σfatty acids[c,d]	2766	–	5539	–	11325	–

[a] n.d. = not detected.
[b] tr = traces (unreliable quantification).
[c] GC quantifiable compounds.
[d] The major fatty acids of the three species are the C_{16} and C_{18} saturated, mono- and di-unsaturated homologues, with palmitic ($C_{16:0}$) and linoleic ($C_{18:2}$) acids as dominant components.

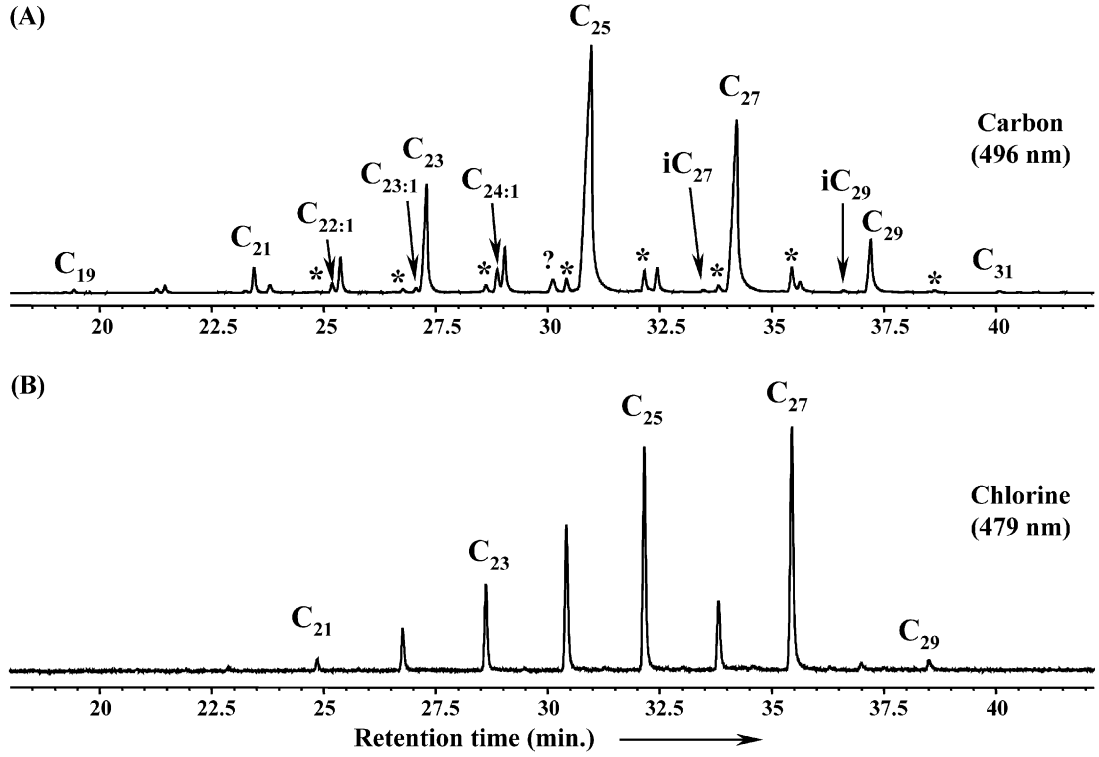

Fig. 1. Partial selective chromatograms (CPG-AED) of the hydrocarbon fraction of leaf waxes of *S. vera* analysed simultaneously for A) carbon (496 nm) and B) chlorine (479 nm). In the chromatogram of carbon, 1-chloro-*n*-alkanes are marked with an asterisk.

ferae (*Crithmum maritimum* L.) growing in the same salt marshes. Quantitatively, significant differences were observed for the chloroalkanes of the three Chenopodiaceae. Leaves of *S. vera* contained 4 to 7 times more of chloroalkanes (sum of all homologues on a dry weight basis) than leaves of the other two species (Table 1). Moreover, chloroalkanes accounted for about 10, 4 and 1% of the hydrocarbon fraction of leaf waxes of *S. vera*, *S. fruticosa* and *H. portulacoides*, respectively. Although homologues with an odd number carbon-chain were predominant in all species, alkyl chlorides maximised at C_{25} and C_{27} in *S. vera* and *S. fruticosa*, and at C_{27} and C_{29} in *H. portulacoides* (Fig. 1B and Table 1). Remarkably, 1-chloro-*n*-nonacosane was an abundant homologue only in this latter species.

2.2. Possible origin and biosynthetic relationships of 1-chloro-n-alkanes

As far as we know, the identification of C_{19} to C_{29} 1-chloro-*n*-alkanes in plants, and more generally in any biological sample, is unprecedented. C_{12} to C_{18} chlorinated hydrocarbons have been detected in carbonaceous chondrites and these compounds are supposed to have formed in the solar nebula (Studier et al., 1965), whereas C_6–C_{18} alkyl chlorides are commonly used in manufacturing of metal working fluid and in the building industry (NICNAS Public Chemical Assessment Report, 2001). The chain-length distribution of chloroalkanes observed in the examined halophytes clearly rules out an anthropogenic contamination.

The mode of synthesis of these long-chain chloroalkanes is still unknown. The incorporation of halogen atoms into organic compounds by living organisms is an enzyme-catalyzed reaction (van Pée, 1999). Most halogenating enzymes characterized so far (called halogenases) require hydrogen peroxide and halides for the halogenation of organic substrates, although some halogenases requiring NADH instead of hydrogen peroxide have been detected in bacteria (van Pée, 1999). The biosynthesis of chloroalkanes by the halophytes might involve the halogenation of organic precursors containing long hydrocarbon chains and suitable for electrophilic attack. Alkenes, *n*-aldehydes and *n*-alkanols could constitute such precursors but no evidence for a precursor-product relationship between the 1-chloro-*n*-alkanes and these other classes of compounds could be established. Indeed, only few *n*-alkenes were detected in the C_{19}–C_{29} range (see example in Fig. 1A) and these were essentially even-chain homologues. Odd and even-chains *n*-aldehydes (C_{20} to C_{30}) and *n*-alkanols (C_{16} to C_{30}) were present in the leaves of the three plants but their distribution, maximising between C_{22} and C_{28}, showed a strong even-over-odd chain-length distribution and, thus, did not match chloroalkanes distribution (Fig. 2). It is noteworthy, however, that the C_{30} *n*-aldehyde (triacontanal) was an abundant homologue only in *H. portulacoides*, as was 1-chloro-*n*-nonacosane.

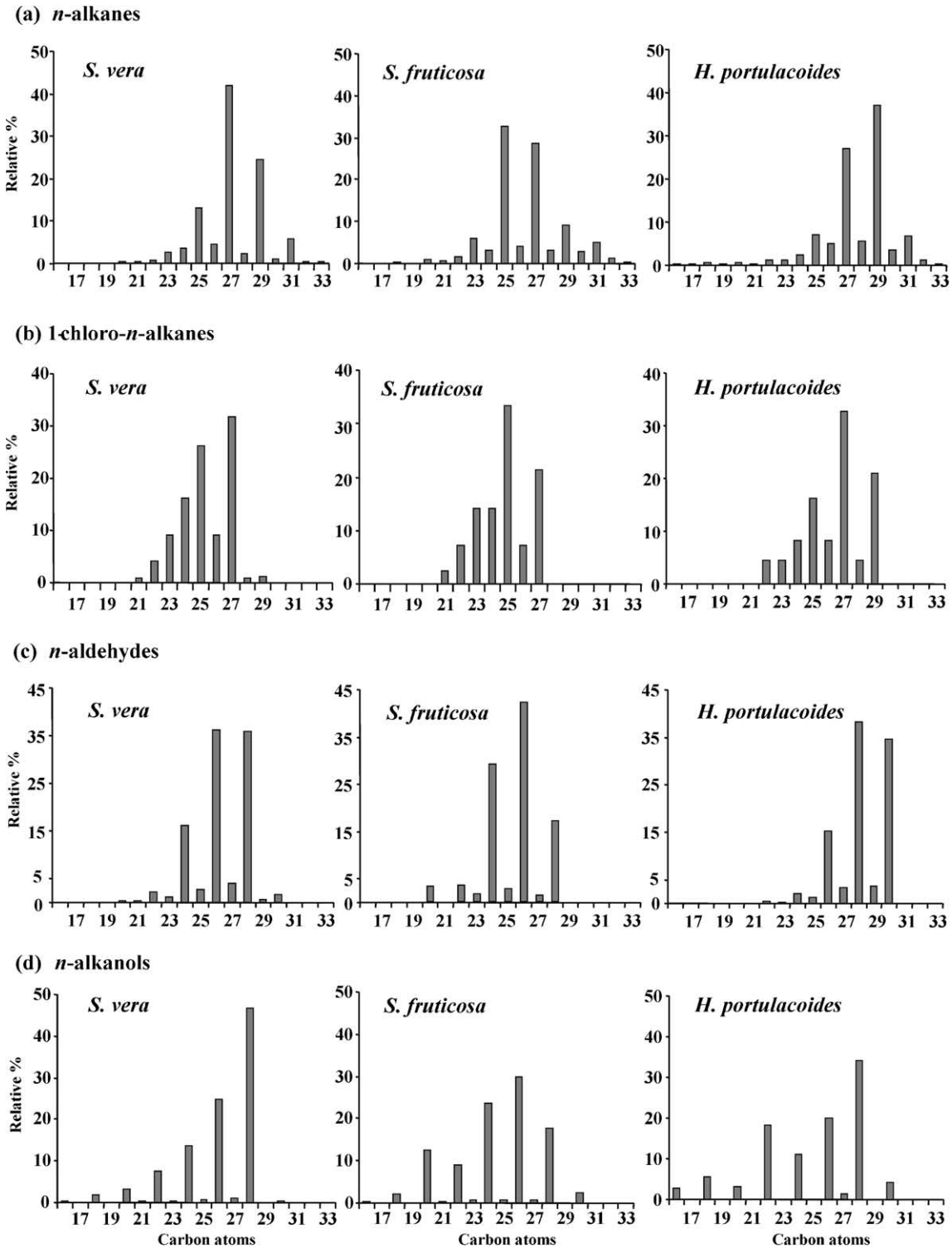

Fig. 2. Distribution of *n*-alkanes A), 1-chloro-*n*-alkanes B), *n*-aldehydes C) and *n*-alkanols D) in *S. vera*, *S. fruticosa* and *H. portulacoides* sampled in September in salt marshes from Camargue (Southern France).

3. Concluding remarks

The characterization of long-chain 1-chloro-*n*-alkanes in leaves of halophylic Chenopodiaceae increases the number and the diversity of chlorine-containing compounds synthesized within the plant kingdom (Gribble, 1996a, 1996b, 2003; Harper, 2000). The paucity of reports of chloroalkanes in plants seems surprising, given the numerous reports of hydrocarbons (see Bianchi, 1995 for an overview). It is possible that such com-

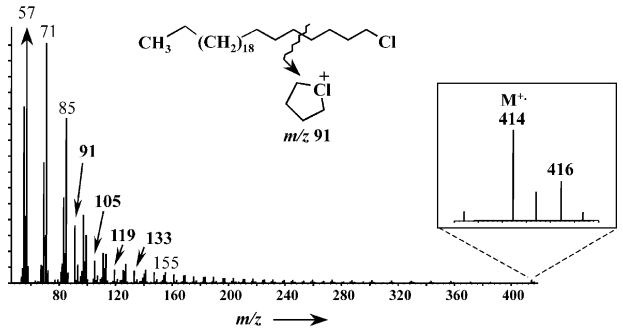

Fig. 3. Electron impact mass spectrum of 1-chloro-*n*-heptacosane detected in three halophylic Chenopodiaceae.

pounds have been missed in previous studies due to their low concentrations and to their mass spectra resembling those of *n*-alkanes, and that they may be relatively common. In addition to the species-dependent occurrence of chloroalkanes observed, the overall distribution of these waxes might vary intra-specifically with developmental stages and/or environmental conditions. These components might thus have potential value as chemotaxonomic and/or environmental markers. Undoubtedly, the occurrence, the biological functions and the biosynthetic pathways of 1-chloro-*n*-alkanes in (halophyte) plants deserve further attention.

4. Experimental

4.1. Plant sampling and preparation

Plants were collected in September in the saltern of Salins-de-Giraud (Camargue; SE France). Species nomenclature follows Tutin et al. (1964–1983). Fresh leaves were separated from the stems and rinsed thoroughly with distillated water before lipid extraction.

4.2. Lipid extraction and separation

A known amount (ca. 20 g) of fresh leaves was extracted (7 times) in a mortar with 40 ml of *n*-hexane–acetone (1:1, v/v). Following filtration, the combined extracts were concentrated by rotary evaporation. Half of the total organic extract was chromatographed over a wet packed (*n*-hexane) column of silica gel (38 g; Merck, kieselgel 60, 3% H_2O). The hydrocarbon fraction and a fraction containing *n*-aldehydes were eluted with *n*-hexane (200 ml) and toluene (200 ml), respectively. The second half of the total organic extract was saponified (reflux 2 h) with 100 ml of 1N KOH in MeOH/H_2O (1:1, v/v). After cooling, unsaponifiable polar lipids (e.g. *n*-alkanols) were extracted from the basic filtrate (*n*-hexane, 3×30 ml) whereas saponifiable acidic compounds were extracted with DCM (3×30 ml) following addition of 2N HCl (pH = 2). Extracts were dried over Na_2SO_4, concentrated by rotary evaporation and evaporated to dryness under a stream of nitrogen. Hydroxylated compounds were transformed into TMSi ethers by reaction with 300 μl of pyridine/BSTFA (1:1 v/v, 20 min at 60 °C) before GC–MS analyses.

4.3. Standards

n-C_{22} and *n*-C_{27} 1-chloro-*n*-alkanes were synthesized from docosanoic (behenic) and heptacosanoic acids (Sigma), respectively. The synthesis involved two steps: the acid (ca. 0.5 mmol) was first quantitatively reduced (30 min at room temperature) to its corresponding alcohol in 60 ml Et_2O–DCM (5:1, v/v) by excess $LiAlH_4$. The use of DCM increased the solubility of the acid which increased the yield of the reaction. After reduction, 10 ml of a saturated solution of ammonium chloride were added cautiously, the mixture was shaken and extracted with *n*-hexane. The combined extracts were then dried over Na_2SO_4. The alcohol obtained was then transformed to the corresponding 1-chloro-*n*-alkane by reaction with thionyl chloride in the presence of pyridine (Vogel et al., 1989). Thionyl chloride (1 mmol) was slowly added under stirring to an equimolar solution of alcohol and pure pyridine (0.5 mmol each), and the mixture was refluxed for 3 h. After cooling, the solution was cautiously washed with $NaHCO_3$ (5%) and water and was extracted with DCM. The combined extracts were dried over Na_2SO_4, concentrated by rotary evaporation and chromatographed over a wet packed column of silica gel as described above; a fraction eluted with *n*-hexane/DCM (4:1, v/v; 200 ml) yielded the 1-chloro-*n*-alkane (*ca.* 30–40%).

4.4. Gas chromatography–atomic emission detection

Alkylchlorides were quantified using a HP 6990 series plus gas chromatograph connected to a HP G2350A atomic emission detector. The gas chromatograph was equipped with a splitless injector (290 °C), and a HP-1 bonded phase capillary column (25 m; 320 μm i.d.; 0.17 μm film thickness) with helium as the carrier gas (constant flow 2 ml min^{-1}). Following injection, the oven temperature was maintained at 60 °C for 1 min and then programmed to 130 °C at 20 °C min^{-1}, from 130 °C to 300 °C at 4 °C min^{-1}, and held at this temperature for 10 min. The temperatures of the transfer line and of the plasma cavity of the AED were 275 °C and 260 °C, respectively; helium pressure in the cavity was 0.7 bar. The photodiode array was set on wavelengths of characteristic emission lines for the simultaneous analysis of carbon (496 nm) and chlorine (479 nm). Oxygen (1.3 bar) was used as the reagent gas and the make-up gas flow was 40 ml min^{-1}. Quantitative determinations of alkylchlorides were based on the response factor of the chlorine emission line of external standard solutions.

4.5. Gas chromatography–mass spectrometry

EI GC–MS analyses were performed on a HP 5890 series II plus gas chromatograph coupled with a HP 5972 mass spectrometer operated at 70 eV with a mass range m/z 50–700 (source temperature 170 °C; cycle time 1.5 s). The gas chromatograph was equipped with a splitless injector (290 °C) and a HP-1MS bonded phase capillary column (30 m; 250 μm i.d.; 0.25 μm film thickness). Helium, used as the carrier gas, was maintained at 1.04 bar until the end of the temperature program and then increased to 1.5 bar at 0.04 bar min^{-1}. The oven temperature was programmed as for the GC-AED analyses.

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