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Importance of the α-amino group in the selective purification of synthetic histidine peptides by immobilised metal ion affinity chromatography

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Abstract

The retention behaviour of some histidine containing peptides on Cu^{2+}- and Ni^{2+}-loaded immobilised metal ion affinity chromatography (IMAC) supports has been investigated and compared with that observed for the corresponding compounds lacking the free α-amino group and/or the imidazole function.

On immobilised Cu^{2+} all histidine-containing peptides, including those with a blocked α-amino function, were strongly retained above pH 5. The presence of a free α-amino group increased the retention marginally.

On immobilised Ni^{2+} histidine peptides with a free α-amino group were strongly bound with a maximal retention at pH 8.5. Blocking of the amino group or removal of the imidazole moiety reduced the maximal retention by a factor 5 to 10, with no retention observed for peptides lacking both histidine and a free α-amino group. These observations indicate the involvement of two equipotent attachment points in the binding.

It seems that IMAC on a Ni^{2+}-loaded support can be used for the purification of histidine containing peptides synthesised by the solid-phase method. Inclusion of a capping protocol in the synthesis ensures that a free α-amino group, which can be used as an affinity handle, will be present only on the target peptide.

1. Introduction

We have earlier shown that synthetic peptides lacking histidine can be purified by selective binding via the α-amino group to immobilised Cu^{2+} and Ni^{2+} ions [1–3]. The inclusion of a capping protocol in the solid-phase synthesis ensures that a free α-amino group is present only on the target peptide, whereas failure peptides resulting from incomplete couplings have blocked (acetylated) N-termini. Thus, the α-amino group can be used as a built-in affinity handle.

Tryptophan and cysteine residues are considered the most important, next to histidine, for the retention of peptides and proteins in immobilised metal ion affinity chromatography (IMAC) [4], and so might be expected to impair the selectivity of the purification procedure. However, it was found that the indole group in itself was not sufficient for binding to Cu^{2+}- or Ni^{2+}-loaded IMAC supports although it could contribute to the binding when the peptide was

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first anchored to the immobilised metal ion via the α-amino group [2]. The presence of a thiol group did not affect the retention, but partial oxidation to the dimer was observed [3]. The lack of influence in this case may be ascribed to metal ion transfer (MIT) from the support to the peptide where the metal ion is scavenged by the thiol, alone or in combination with neighbouring amide nitrogens.

For the inclusion of histidine containing peptides in the purification protocol based on interaction of the α-amino group with metal ions, a less favourable situation can be anticipated. The interaction of histidine with transition series metals is well documented [5], and the presence of histidine is a major factor in the binding of proteins [4,6,7] and peptides [8–11] in IMAC. Single N-terminal histidine residues have also been exploited for the purification of synthetic peptides by IMAC [12]. If peptides containing histidine are already tightly bound to the IMAC adsorbent, it is not clear whether additional retention due to the presence of a free α-amino group will be sufficient to achieve adequate separation of the blocked and unblocked peptides. In order to clarify this point in an unambiguous way, a set of histidine-containing peptides with free or protected α-amino groups were synthesised and their retention on Cu^{2+}- and Ni^{2+}-loaded supports investigated. The corresponding peptides without histidine were included as references.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade and used as purchased. *tert*.-Butyloxycarbonyl (Boc) amino acids were obtained from Peninsula Labs. Europe (St. Helens, UK) or Novabiochem (Läufelfingen, Switzerland) and 9-fluorenylmethyloxycarbonyl (Fmoc) amino acids and resins from were Millipore (Sundbyberg, Sweden). Boc-Amino acyl resins were prepared according to Horiki et al. [13]. Chelating Superose was obtained from Pharmacia (Uppsala, Sweden).

2.2. Buffers

The chromatographic buffers (pH range 5–11) contained 50 m*M* sodium dihydrogenphosphate, 50 m*M* boric acid and 1 *M* sodium chloride and were prepared as described previously [1].

2.3. Peptide synthesis

Solid-phase synthesis of peptides was performed on either an Applied Biosystems 430A instrument as described earlier [1] or a Protein Technologies Symphony instrument, using Fmoc chemistry and a capping protocol similar to that previously used [1].

The peptides are listed in Table 1 and will hereafter be referred to in bold numbers with

Table 1
Structure of peptides used in this investigation

Peptide	Structure	Ref.
1A	Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala	[1]
1B	Ac–Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala	[1]
2A	Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–**His**–Ile–Tyr–Ala	
2B	Ac–Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–**His**–Ile–Tyr–Ala	
3A	Leu–Glu–Leu–Arg–Ser–Arg–Tyr–Val–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2	[2]
3B	Ac–Leu–Glu–Leu–Arg–Ser–Arg–Tyr–Val–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2	[2]
4A	Leu–Glu–Leu–Arg–Ser–Arg–Tyr–**His**–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2	
4B	Ac–Leu–Glu–Leu–Arg–Ser–Arg–Tyr–**His**–Ala–Ile–Arg–Thr–Arg–Ser–Gly–NH_2	

indication of a free (**A**) or blocked (**B**) α-amino group.

2.4. IMAC

All chromatography was performed with a fast protein liquid chromatography (FPLC) system (Pharmacia) and the conditions for IMAC were as described earlier [1]. The peptides (100 nmol in 100 μl of the chromatographic buffer) were applied to the column with isocratic elution and the capacity factor (k) determined according to

$$k = V_e/V_0 - 1$$

where V_e is the retention volume for the peptide on a metal-loaded column and V_0 the retention volume on a metal-free column.

2.5. Mass spectrometry

Synthetic products and chromatographic fractions were analysed by plasma desorption mass spectrometry (PD-MS) using a BioIon 20 instrument (Applied Biosystems, Uppsala, Sweden) as described earlier [1].

3. Results and discussion

Peptides **2** and **4** were derived from the reference peptides **1** and **3** by a substitution of His for Val in position 9 and 8, respectively.

The retention behaviour of the peptides on a Cu^{2+}-loaded IMAC column was investigated (Fig. 1). The reference peptides **1A** and **3A** have been described earlier [1,2] and show increasing retention as the pH is raised due to deprotonation of the free α-amino group. Increasing the pH above 7.5 results in decreased retention caused by metal ion transfer from the chromatographic support to the peptide. The lysine-containing peptides **1A** and **1B** are retained above pH 9 as a result of the deprotonation of the ϵ-amino group. Peptides **1B** and **3B** with blocked N-termini do not show any retention between pH 5 and 11, other than the lysine effect observed with peptide **1B**.

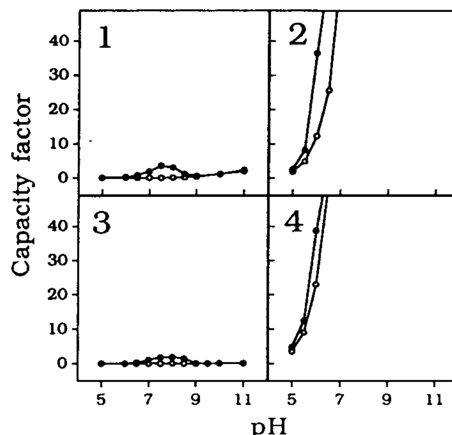

Fig. 1. Capacity factors vs. pH of peptides **1–4** on immobilised Cu^{2+}. The number in each square corresponds to the number of the peptide investigated (Table 1). Peptides with free α-amino groups are indicated with **O** and blocked with O. Column: Chelating Superose (1.8 × 1 cm I.D.) charged with Cu^{2+}. Elution: isocratic with 50 m*M* sodium phosphate–borate, 1 *M* NaCl at 1 ml/min. Sample: 100 nmol of peptide dissolved in 100 μl of chromatographic buffer. Detection: UV at 280 nm.

The imidazole group of the histidine residue has a lower pK_a value (ca. 6.5) than the α-amino group (ca. 8). Consequently, deprotonation and binding of histidine peptides is expected to occur at lower pH values than for peptides without histidine. Indeed, both the N-protected and free peptides **2** and **4** bind strongly even at pH 5.5, and above pH 6.5 they all have capacity factors greater than 50 (Fig. 1). Obviously, the α-amino group contributes to the binding. The capacity factors for the unblocked peptides are higher than for the acetylated ones. However, because of the strong interaction between histidine and Cu^{2+}, the relative importance of this contribution is small.

The peptides were also analysed on a Ni^{2+}-loaded column (Fig. 2). The behaviour of the reference peptides **1** and **3** is similar to that observed on Cu^{2+}, but maximal retention occurs at pH ≈ 8.5 and binding via the lysine ϵ-amino

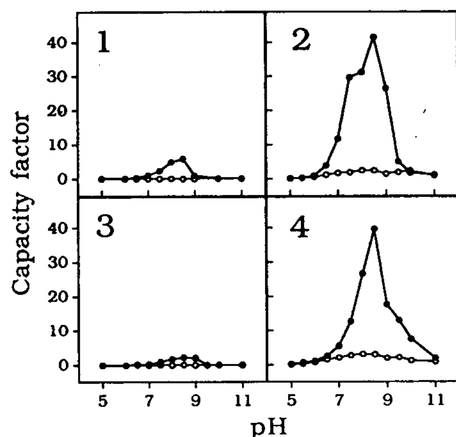

Fig. 2. Capacity factors vs. pH of peptides **1–4** on immobilised Ni^{2+}. Indications and chromatographic conditions as in Fig. 1.

group is not observed [1,2]. However, the influence of the histidine residue, as seen in the binding of peptides **2** and **4**, is considerably less on Ni^{2+} than on Cu^{2+}. The N-terminally blocked histidine peptides **2B** and **4B** are only weakly bound, having even lower capacity factors than the unblocked reference peptides **1A** and **3A**. An entirely different behaviour is observed for the histidine peptides with free α-amino groups (**2A** and **4A**) which are strongly retained. The capacity factors are 5–10 times higher than for the corresponding peptides lacking the α-amino or the imidazole group suggesting the involvement of two equipotent attachment points in the binding.

The use of IMAC for separation of histidine-containing peptides with free α-amino groups from those with blocked N-termini is illustrated in Fig. 3. On a Cu^{2+}-loaded IMAC column, separation of the peptides **2A** and **2B** can be accomplished by elution with a decreasing pH gradient (Fig. 3A), but with peptides **4A** and **4B** baseline separation is not achieved (Fig. 3B). As can be expected from the results in Fig. 1, the contribution by the α-amino group to the binding of the peptide, although evident, is not always sufficient to allow separation on this support. However, on a Ni^{2+}-loaded column separation is easily achieved either by elution

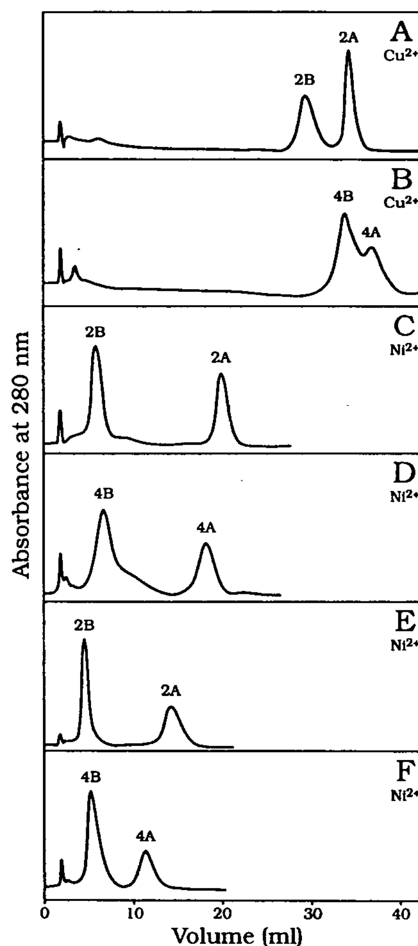

Fig. 3. Chromatography of mixtures of peptides with and without free N-termini on immobilised Cu^{2+} (A and B) and Ni^{2+} (C–F). One mixture contained the peptides **2A** and **2B** (A, C and E) and the other **4A** and **4B** (B, D and F). Column: Chelating Superose (1.8 × 1 cm I.D.). Sample: 100 nmol of each peptide dissolved in 200 μl of 50 m*M* sodium phosphate–borate, 1 *M* NaCl, pH 7.5 (Cu^{2+}) or pH 8.5 (Ni^{2+}). Elution: 30 min linear gradient from pH 7.5 to 5.0 (A and B), pH 8.5 to 5.0 (C and D) or 0 to 0.5 *M* NH_4Cl in the pH 8.5 buffer (E and F) at 1 ml/min.

with a decreasing pH gradient or with an increasing NH_4Cl gradient (Fig. 3C–F).

In other affinity methods designed for purification of synthetic peptides, special affinity handles are introduced on the α-amino group of the target peptide while this is still being attached to the resin (e.g. Refs. [14–19]). The decision of whether or not to use an affinity handle has to be

made prior to cleavage. After the peptide has been cleaved from the resin, there is no easy way to selectively introduce an N-terminal affinity handle since functional groups in the side chains are no longer protected from attack by the derivatisation agent. On the other hand, it might be an unnecessary effort to introduce an affinity handle in cases where standard purification methods would suffice. In this method the separation is based on the interaction of the free α-amino group with immobilised metal ions and such considerations need not be taken in account. In addition, material losses associated with the introduction and removal of an additional affinity handle can be avoided and the solubility of the peptide is not affected. Since no modification of the target peptide is required, the method complements standard purification procedures such as RP-HPLC and ion-exchange chromatography in a straightforward way.

There may be cases where selectivity is not achievable. Truncated peptides with two or more histidines may be retained too strongly to allow their separation from the target peptide. Combinations of histidine and tryptophan may also cause problems. As on previous occasions, impurities resulting from post-synthetic modifications (alkylation, oxidation etc.) or incomplete deprotection are not amenable to separation by this method. However, their removal in a subsequent RP-HPLC step should be facilitated.

4. Conclusions

Earlier work [1–3] has established that selective purification based on the affinity of the α-amino group for Cu^{2+} and Ni^{2+} ions can be achieved for peptides lacking histidine provided a capping procedure is included in their solid-phase synthesis. We can now conclude that the method is also useful for the purification of synthetic peptides containing histidine. On a Ni^{2+}-loaded IMAC support, the target peptide with its free α-amino group will be strongly retained whereas truncated peptides with blocked α-amino functions will show very low or, if they contain histidine, moderate retention. A free α-amino group seems to be at least equally important for the binding to Ni^{2+} as an imidazole function. On immobilised Cu^{2+}, baseline separation is not always achievable.

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High-performance liquid chromatographic study of alkaline treatment of chlorophyll

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Abstract

The degradation products of chlorophyll *a* and *b* obtained by alkaline treatment in the presence of atmospheric oxygen were studied. Reversed-phase ion-pair high-performance liquid chromatography, normal- and reversed-phase thin-layer chromatography and diode-array detection were used for the separation, isolation and preliminary identification of the following degradation products: Mg-rhodin g_7, Mg-chlorin e_6, MeO-lactone-chlorophyllide, Mg-purpurin, Mg-phytyl-rhodin g_7, Mg-phytyl-chlorin e_6, MeO-lactone-chlorophyll, Mg-phytyl-purpurin and some of their diasteroisomers. MeO-chlorophyll and OH-chlorophyll were tentatively identified. The alkaline treatment did not induce the specific de-esterification of phytol in any of the cases studied and oxidation of the isocyclic ring was simultaneously observed.

1. Introduction

Chlorophylls are the most important pigments in photosynthesis, but their interest with regard to food technology derives from their involvement in the green colour of fruit and vegetables. Chlorophylls are very stable in their natural environment inside the chloroplast, but whenever this organ becomes damaged the chlorophylls are very susceptible to undergo a wide range of chemical transformations as a consequence of the intervention of different factors such as high temperature, extreme pH values, enzymatic action, molecular oxygen and light. All these factors may combine during food processing, causing changes in the colour of the finished product [1,2]. The most appealing change is the separation under acidic conditions of the central atom of magnesium (pheophytinization) (Fig. 1), causing a drastic change in colour from bright green to olive brown [3,4]. Other possible changes are the formation of epimer at C-13^2 (chlorophyll *a*′ and *b*′), decarbomethoxylation of C-13^2 leading to pyropheophytins [5,6], de-esterification of phytol at C-17^3 [7–11], oxidation of the isocyclic ring [12], insertion of divalent metals in the porphyrin ring (re-greening) [13,14] and oxidative opening of the porphyrin chromophore group causing decoloration [15,16].

The oxidation of the chlorophyll molecule by molecular triplet oxygen in alcoholic solutions causes the replacement of the atom of hydrogen of C-13^2 located in the isocyclic ring by oxygen or an oxygen-containing species. This reaction, named "allomerization", may occur by both

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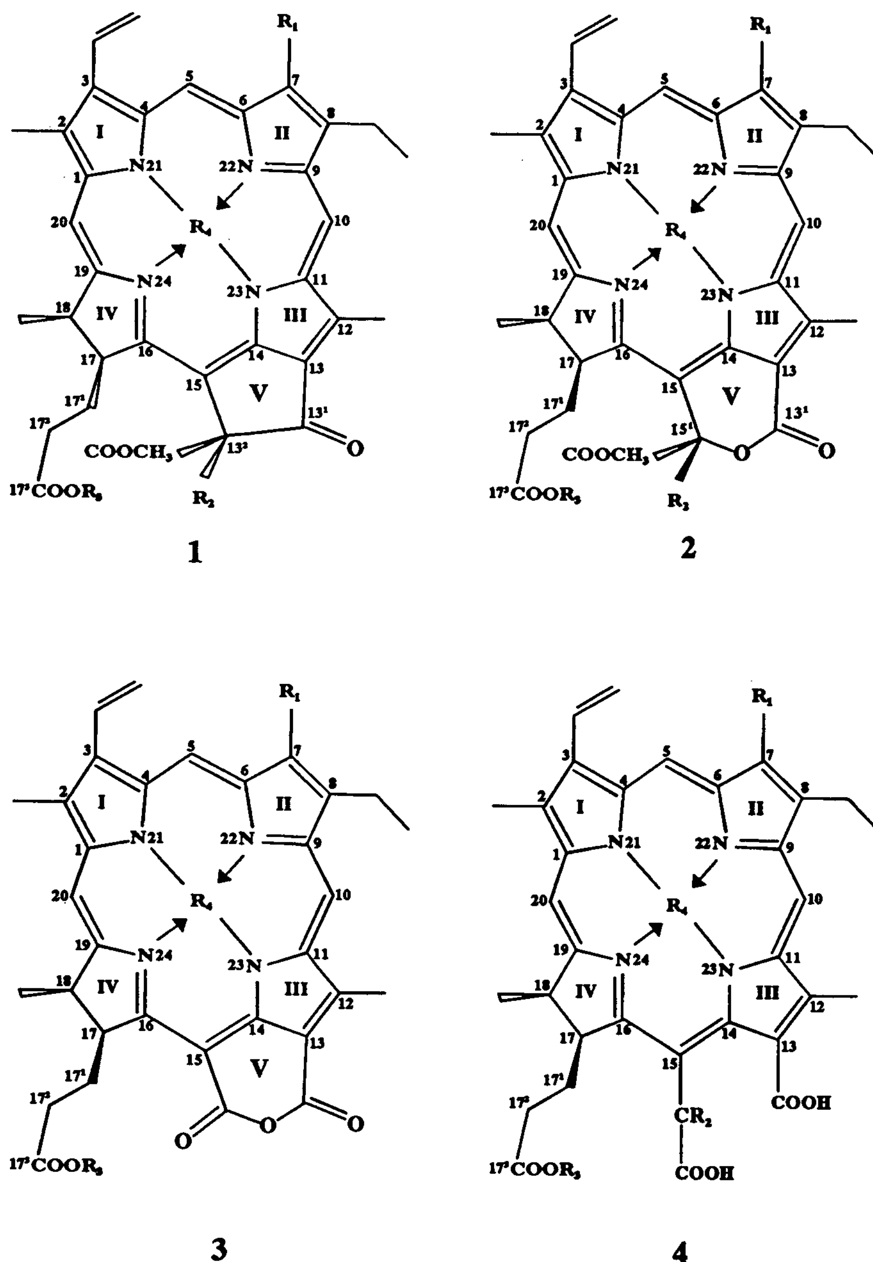

No.	Trivial name	R_1	R_2	R_3	R_4	Abbreviation
1.1a	Chlorophyll *a*	CH_3	H	Phytyl	Mg	chl *a*
1.2a	Pheophytin *a*	CH_3	H	Phytyl	2H	phy *a*
1.3a	Chlorophyllide *a*	CH_3	H	H	Mg	chld *a*
1.4a	Pheophorbide *a*	CH_3	H	H	2H	pho *a*

Fig. 1. Structures, trivial names and numbering system for chlorophylls and derivatives. The replacement of the CH_3 group in R_1 by a CHO group forms chlorophyll *b* derivatives. The replacement of the CO_2CH_3 group at C^{13} by H forms pyro derivatives.

1.5a	13^2-Hydroxychlorophyll *a*	CH_3	OH	Phytyl	Mg	OH-chl *a*
1.6a	13^2-Methoxychlorophyll *a*	CH_3	OCH_3	Phytyl	Mg	MeO-chl *a*
2.1a	15^1-Methoxylactone chlorophyll *a*	CH_3	OCH_3	Phytyl	Mg	MeO-lactone-chl *a*
2.2a	15^1-Methoxylactone pheophytin *a*	CH_3	OCH_3	Phytyl	2H	MeO-lactone-phy *a*
2.3a	15^1-Methoxylactone chlorophyllide *a*	CH_3	OCH_3	H	Mg	MeO-lactone-chld *a*
2.4a	15^1-Methoxylactone pheophorbide *a*	CH_3	OCH_3	H	2H	MeO-lactone-pho *a*
3.1a	Mg-Phytyl-purpurin *a*	CH_3		Phytyl	Mg	Mg-phyt-purp *a*
3.2a	Mg-Purpurin *a*	CH_3		H	Mg	Mg-purp *a*
3.3a	Phytyl-purpurin *a*	CH_3		Phytyl	2H	phyt-purp *a*
3.4a	Purpurin *a*	CH_3		H	2H	purp *a*
4.1a	Mg-Phytyl-chlorin e_6	CH_3	2H	Phytyl	Mg	Mg-phyt-chlr
4.2a	Mg-Chlorin e_6	CH_3	2H	H	Mg	Mg-chlr
4.3a	Phytyl-chlorin e_6	CH_3	2H	Phytyl	2H	phyt-chlr
4.4a	Chlorin e_6	CH_3	2H	H	2H	chlr
4.5a	Mg-Purpurin *a* 7-dimethyl phytyl ester	CH_3	O	phytyl	Mg	Mg-phyt-purp 7
4.1b	Mg-Phytyl-rhodin g_7	CHO	2H	phytyl	Mg	Mg-phyt-rhd
4.2b	Mg-Rhodin g_7	CHO	2H	H	Mg	Mg-rhd
4.3b	Phytyl-rhodin g_7	CHO	2H	Phytyl	2H	phyt-rhd
4.4b	Rhodin g_7	CHO	2H	H	2H	rhd

Fig. 1 *(Continued)*

enzymatic and chemical pathways [17], forming MeO-lactone chl *a* (**2.1a**), OH-chl *a* (**1.5a**) and MeO-chl *a* (**1.6a**) [18–20] as major products. The acid hydrolysis of the phytol alcohol in the chlorophyll molecule is accompanied by the loss of Mg and produces pheophorbides (**1.4**). The excision of phytol without separation from Mg is a specific reaction catalysed by the endogenous enzyme chlorophyllase and which results in chlorophyllides (**1.3**). This de-esterification may also occur under mild alkaline conditions but the hydrolysis must be carried out in an inert atmosphere to avoid the possibility of other simultaneous oxidation reactions at C-13^2 [21].

Generally, the chlorophyllic derivatives have a very different chemical behaviour as a result of differences in their molecular structure, but their separation, isolation and identification entail important difficulties. Research in this field has advanced considerably in recent years owing to the high resolution of high-performance thin-layer chromatographic plates (HPTLC) and high-performance liquid chromatographic (HPLC) columns [22]. Schaber et al. [19] were the first to isolate by semi-preparative reversed-phase (RP) HPLC the products of the allomerization of chlorophyll *a* and to confirm the structure of the allomerized products 13^2-(*R*)- and 13^2-(*S*)-OH chl *a* (**1.5a**) and 15^2-(*R*)- and 15^2-(*S*)-MeO-lactone-chl *a* (**2.1a**) by NMR and ^{254}Cf plasma desorption mass spectrometry. Recently, Kuronen et al. [20] were successful in obtaining the analytical separation and preparative isolation by normal-phase (NP) HPLC of seven products of allomerization of chlorophyll *a* including, together with those mentioned above, 13^2-(*R*)- and 13^2-(*S*)-MeO-chl *a* (**1.6a**) and Mg-purpurin *a* 7-dimethyl-phytyl ester (**4.5a**). Recently in our laboratory we have investigated different conditions of chromatographic separation for most of the oxidized derivatives of the pheophorbides (**1.4**), including pyropheophorbides, chlorin e_6 (**4.4a**), rhodin g_7 (**4.4b**) and purpurin (**3.4**) [12].

The fruits of the olive tree processed for their direct consumption as table olives are submitted to an alkaline treatment to hydrolyse the bitter glucoside called oleuropeina and later, after several washes with water, they are placed in brine whereupon they experience natural lactic fermentation. This process causes the total degradation of the chlorophylls initially present in the fresh fruit to pheophytins and pheophorbides [23,24]. Recent innovations introduced in this system of processing (addition of culture initiators with recirculation, reusage of brine, etc.) seem to have modified the natural transformation of the chlorophylls and other minority

oxidative products have being detected but not identified [12,25]. We have therefore investigated the possible oxidation products of chlorophyll caused by alkaline treatment. We used normal- and reversed-phase thin-layer chromatography (NP and RP-TLC), ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) and diode-array detection (DAD) for the separation, isolation and preliminary identification of most of the oxidized derivatives of chlorophylls obtained under alkaline conditions and in the presence of atmospheric oxygen.

2. Experimental

2.1. Thin-layer chromatography

The TLC plates for normal-phase operation were prepared by spreading a layer of a mixture of silica gel GF_{254} (Merck, Darmstadt, Germany) and water (1:2, w/v) over 20 × 20 cm glass plates. A Unoplen thin-layer spreader (Shandon Southern, Runcorn, UK) was used and the thickness was adjusted to 0.7 mm. Reversed-phase TLC was performed using silica gel C_{18} plates (Nano SI F_{254} C_{18}-100; Sharlau, Barcelona, Spain) (10 × 10 cm, thickness 0.2 mm). As mobile phases we used light petroleum (b.p. 65–95°C)–acetone–diethylamine (10:4:1) for NP-TLC [25] and methanol–acetone–water (20:4:3) for RP-TLC [12]. Chromatography was performed in a normal saturation tank and for detection a Dessaga UV–Vis lamp provided white light and UV radiation of 254 and 366 nm.

2.2. Ion-pair reversed-phase high-performance liquid chromatography

HPLC was performed using a Waters Model 600E liquid chromatograph fitted with an injection valve (Rheodyne Model 7125) and a Waters Model 994 photodiode-array detector. Chromatograms were recorded on a register-integrator (Waters Model 5200). A stainless-steel column (25 × 0.4 cm I.D.), packed with 5-μm C_{18} Spherisorb ODS-2 (Teknokroma, Barcelona, Spain) was used. The column was protected by a cartridge (5 × 0.4 cm I.D.) packed with the same material. The pigments dissolved in acetone were centrifuged at 13 000 *g* (MSE Micro Centaur centrifuge) prior to injection into the chromatograph (20 μl) and were separated using an elution gradient (flow-rate 2 ml/min) with the mobile phases (A) water–ion pair reagent–methanol (1:1:8, v/v/v) and (B) acetone–methanol (1:1, v/v). The ion pair reagent was 0.05 *M* tetrabutylammonium acetate (Fluka, Buchs, Switzerland) and 1 *M* ammonium acetate (Fluka) in water. The gradient scheme has been described in detail in a previous paper [25].

2.3. Reagents

Acetone and methanol were of HPLC grade (Tecknokroma) and the remainder were of analytical-reagent grade. Water was deionized and filtered through a 0.45-μm nylon membrane (Supelco, Bellefonte, PA, USA).

2.4. Standards

Chlorophylls were isolated from fresh spinach leaves by pigment extraction with acetone [26] followed by TLC separation on silica gel GF_{254} (20 × 20 cm plates, thickness 0.7 mm) (Merck) using light petroleum (b.p. 65–95°C)–acetone–diethylamine (10:4:1) as eluent [25]. Chlorophyll C-13^2 epimers were prepared by treatment with chloroform according to Watanabe et al. [27]. Pheophytin *a* and *b* were prepared from the respective pure solutions of chlorophylls in diethyl ether by acidification with 2–3 drops of HCl (13%, v/v) [28]. Pheophorbides *a* and *b* were formed by enzymatic de-esterification of pheophytins; a protein precipitate of *Ailanthus altissima* (Mill.) leaves was extracted with sodium phosphate buffer (pH 7) containing 50 m*M* KCl and 0.24% Triton-X 100 and incubated with Tris buffer (pH 8.5) containing 0.24% Triton X-100 and the substrate dissolved in acetone in a 5:5:1 ratio [29]. The pheophorbides formed were transferred to diethyl ether by addition of water saturated with sodium chloride. The chlorophyll allomerization procedure was performed in the presence of methanol according to Schaber et al.

[19]. Solid chlorophyll *a* or chlorophyll *b* (1–2 μmol) was dissolved in 5 ml of methanol and exposed to atmospheric oxygen in darkness. The course of the reaction was followed by HPLC. Methyl esters of chlorine e_6 and rhodin g_7 were formed by treatment of pheophorbide *a* and pheophorbide *b*, respectively, with 0.5% KOH in methanol. Free chlorin e_6 and rhodin g_7 were obtained from their respective methyl esters in methanolic alkaline medium (30% KOH) at ambient temperature and under a nitrogen atmosphere. Purpurin *a* was obtained by alkaline oxidation of pheophorbide *a* with KOH (30%) in methanol in the presence of atmospheric oxygen, and purpurin *b* from pheophorbide *b* using the same procedure [30]. All standards were purified by NP- and RP-TLC [12,25].

2.5. Alkaline treatment of the chlorophyll in aqueous media

As oxidizing agents 0.5% aqueous solutions of sodium hydroxide and potassium hydroxide were employed. The chlorophyll (*a* or *b*), solid and chromatographically pure (1–2 μmol), was dissolved in 75 ml of acetone and mixed with 150 ml of the aqueous alkaline solution. This mixture was divided into three parts, each of which was placed in a decantation funnel. The three mixtures were exposed to atmospheric oxygen at room temperature for 10, 30 and 60 min respectively. On completion, 50 ml of diethyl ether and 200 ml of distilled water saturated with sodium chloride were added to induce separation into phases in each of the funnels. Although most of the products obtained are transferred to the organic phase, in the aqueous phase an important part of the coloration was retained. The respective ether phases were collected and washed three more times with water and another three times with 2% (w/v) sodium sulphate. The solvent was removed under reduced pressure at 30°C by means of a rotary evaporator and the residue was stored dry in a nitrogen atmosphere at −20°C until it was used (fraction I). The chlorophyll derivatives that were retained in the initial aqueous phase were recovered by incorporating a new portion of 50 ml of diethyl ether and adding 1 ml of 18% (v/v) HCl to force the transfer of pigments to the organic phase. The ether phases were washed repeatedly with water until the washings were neutral and finally they were passed through anhydrous sodium sulphate, the solvent was evaporated and the dry residue was stored in a nitrogen atmosphere at −20°C until required for use (fraction II).

2.6. Pigment identification

Pigments were identified by co-chromatography with authentic samples (TLC and HPLC) and from their spectral characteristics. The online UV–Vis spectra were recorded from 350 to 800 nm with the photodiode-array detector. The presence of phytol in the compounds was determined according to Bacon and Holden [31]: a solution of each pigment in 2.5% (w/v) methanolic KOH (3.5 ml) was incubated at 70°C for 30 min. After cooling in ice, 0.75 ml of light petroleum (b.p. 40–60°C) was added, and the phases were separated by addition of water (5 ml). The phytol was separated by TLC on silica gel GF_{254} with benzene–ethyl acetate (19:1, v/v) as solvent system and detected at 254 nm. The presence of magnesium in the compounds was checked by shaking an ethereal solution of the compound with 18% (v/v) HCl for 5 min [28]. A change in the UV–Vis spectrum indicates a positive test.

3. Results and discussion

3.1. Separation and isolation of the products of the alkaline treatment of chlorophylls

Ion-pair reversed-phase HPLC

As a result of the different alkaline treatments carried out, we obtained a total of ten degradation compounds of chlorophyll *a* and another nine derivatives of chlorophyll *b*. Fig. 2 shows the HPLC traces corresponding to the separation of the chlorophyll *a* derivatives which were obtained using the DAD technique at 430 nm and Fig. 3 those corresponding to the products obtained with chlorophyll *b* and detected at 460

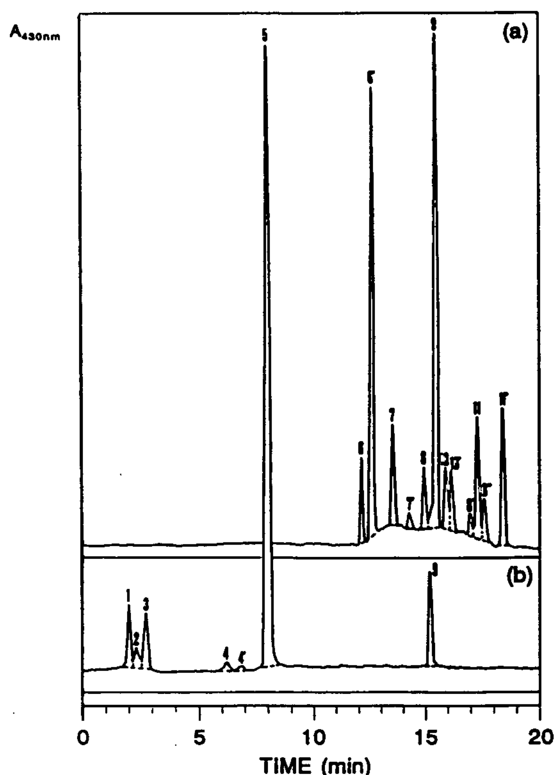

Fig. 2. HPLC separation of the alkaline treatment products from chlorophyll *a* using DAD at 430 nm. (a) Fraction I and (b) fraction II. Peaks: 1 = chlorin e_6; 2 = MeO-lactone chld *a*; 3 = Mg-purpurin *a*; 4 = MeO-lactone-pho *a*; 4′ = MeO-lactone-pho *a* isomer; 5 = purpurin *a*; 6 = Mg-phytyl-chlorin e_6; 6′ = Mg-phytyl-chlorin e_6 isomer, 7 = MeO-chl *a*; 7′ = MeO-chl *a*′; 8 = MeO-lactone-chl *a*; 8′ and 8″ = MeO-lactone-chl *a* isomers; 9 = Mg-phytyl-purpurin *a*; 10 = chl *a*; 10 = chl *a*′; 11 = OH-chl *a*; 11′ = OH-chl *a*′.

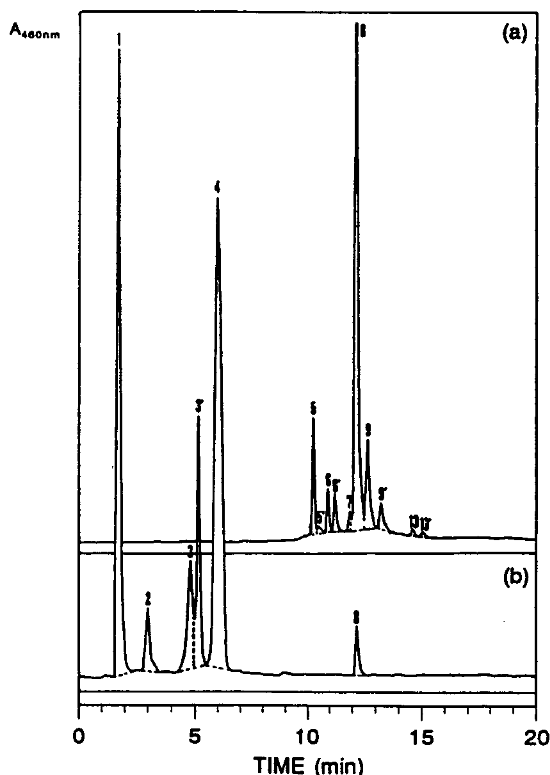

Fig. 3. HPLC separation of the alkaline treatment products from chlorophyll *b* using DAD at 460 nm. (a) Fraction I and (b) fraction II. Peaks: 1 = rhodin g_7; 2 = Mg-purpurin *b*; 3 = MeO-lactone-pho *b*; 3′ = MeO-lactone-pho *b* isomer; 4 = purpurin *b*; 5 = Mg-phytyl-rhodin g_7; 5′ = Mg-phytyl-rhodin g_7 isomer; 6 = MeO-lactone-chl *b*; 6′ = MeO-lactone-chl *b* isomer; 7 = MeO-chl *b*?; 8 = Mg-phytyl-purpurin *b*; 9 = chl *b*; 9′ = chl *b*′; 10 = OH-chl *b*?; 10′ = OH-chl *b*′?.

nm. Their chromatographic and spectroscopic characteristics are given in Tables 1 and 2, respectively. These tables include retention factors, electronic absorption maxima measured by DAD and the relationship between the absorbance of the Soret band and that of each absorption maximum that complete the UV–Vis spectral characteristics of these compounds. As the data show, the location of the absorption maxima of most of the compounds in each series derived from the chlorophylls is very different to that shown by the corresponding chlorophyll. Only two of the compounds in each series (peaks 7 and 11 for chlorophyll *a* and peaks 7 and 10 for chlorophyll *b*) have electronic absorption maxima located at exactly the same wavelengths as the chlorophyll from which they are derived. All components retained in fraction I, both in series *a* (Fig. 2a) and in series *b* (Fig. 3a), are resolved after 10–20 min of chromatographic analysis. Prior studies carried out by our group in which chlorophylls and derivatives were separated using the same gradient of chromatographic solvents as in this work guide their preliminary identification towards structures of polarity similar to those of the chlorophylls [25]. On the other hand, most of the peaks that constitute

Table 1
Chromatographic and spectroscopic properties of the alkaline treatment products from chlorophyll *a*

Peak No.	Pigment[a]	k_c	Spectral data in the IR-RP-HPLC eluent[b]													
			Soret	I		II		III		IV		V		VI	
				M	R	M	R	M	R	M	R	M	R	M	R
1	Chlorin e_6	0.27	400					500	12.25	(530)	61.25	606	35.00	660	2.92
2	MeO-Lactone-chld *a*	0.45	420					(524)	30.75	(570)	12.57	(614)	8.79	656	1.77
3	Mg-Purpurin *a*	0.72	428	(378)	2.30					584	8.79	646	4.64	686	1.91
4	MeO-Lactone-pho *a*	3.11	402					500	10.52	532	12.74	612	18.62	668	3.36
		3.49													
5	Purpurin *a*	4.21	410	360	2.52			506	21.55	546	5.39	(638)	19.75	698	2.66
6	Mg-Phytol-chlorin e_6	6.62	416					(524)	30.80	(600)	12.35			646	2.60
		6.91													
7	MeO-chl *a*	7.50	432	394	1.76	420	1.20	534	21.73	590	11.30	616	5.38	666	1.06
		7.98													
8	MeO-Lactone-chl *a*	8.40	420					(524)	30.75	(570)	12.57	(614)	8.79	656	1.77
		9.69													
		10.08													
9	Mg-Phytol-purpurin *a*	8.70	428	(378)	2.30					584	8.79	646	4.64	686	1.91
10	chl *a*	9.18	432	(394)	1.49	(420)	1.10	(534)	25.10	(590)	8.37	616	4.48	666	1.27
10′	chl *a*′	9.57	432	(394)	1.56	(420)	1.10	(534)	20.92	(590)	8.10	616	4.48	666	1.14
11	OH-chl *a*	9.89	432	(394)	1.88	(420)	1.15	(534)	24.80	(576)	11.27	616	6.20	666	1.20
		10.60													

Retention factor $k_c = (t_R - t_M)/t_M$, where t_R = retention time of the pigment peak and t_M = retention time of an unretained component; M = position maximum (nm) and R = ratio of absorbance at Soret band to absorbance at wavelength indicated.

[a] Abbreviations as in Fig. 1.

[b] The values in parentheses indicate inflection points in the absorption spectrum.

Table 2
Chromatographic and spectroscopic properties of the alkaline treatment products from chlorophyll *b*

Peak No	Pigment[a]	k_c	Spectral data in the IR-RP-HPLC eluent[b]												
			Soret	I		II		III		IV		V		VI	
				M	R	M	R	M	R	M	R	M	R	M	R
1	Rhodin g_7	0.09	426	350	7.00	(408)	2.85	(530)	15.31	(570)	14.41	(596)	17.50	648	6.45
2	Mg-Purpurin *b*	1.09	454	350	4.02	(430)	4.42					620	7.03	656	4.64
3	MeO-Lactone-pho *b*	2.39	432	366	5.44	(414)	1.78	520	16.60	556	20.75	600	31.13	650	7.78
		2.69													
4	Purpurin *b*	3.29	436			(420)	2.02	(518)	23.60	(560)	21.50	(616)	18.15	674	5.02
5	Mg-Phytol-rhodin g_7	6.09	450	350	4.28					578	9.79			624	7.83
		6.35													
6	MeO-Lactone-chl *b*	6.54	454							586	10.50			634	4.42
		6.77													
7	MeO-chl *b*	7.21	462	350	3.51					(570)	15.38	600	8.79	650	2.86
8	Mg-Phytol-purpurin *b*	7.36	454	350	4.02	(430)	4.42					620	7.03	656	4.64
9	chl *b*	7.88	466	350	3.15					(570)	16.80	600	8.69	650	2.80
9′	chl *b*′	8.24	466	350	3.32					(570)	15.75	600	8.40	650	2.83
10	OH-chl *b*	9.08	462	350	3.67					(570)	14.53	600	7.97	650	2.94
		9.41													

[a,b] See Table 1.

fraction II (Figs. 2b and 3b) were resolved in less than 9 min; this serves to indicate that these derivatives are significantly more polar than their corresponding chlorophylls.

Fundamentally, the effect of the treatment of chlorophyll with aqueous KOH or NaOH is the same although an inferior percentage transformation is obtained after treatment with KOH for the same concentration and time of reaction. As the time of contact between the aqueous alkaline solution and the organic solution of the chlorophyll increases, a higher percentage transformation of chlorophyll is obtained. When the time of reaction is just 10 min we obtained a mixture of products in which ca. 20% is unreacted chlorophyll. If the time of contact is increased to 30 or 60 min, the alkaline treatment is observed to be more effective (10% or 2% of undegraded chlorophyll maintained, respectively).

Normal-phase and reversed-phase TLC

In order to obtain a sufficient amount of each compound to allow the performance of additional chemical analyses, for the presence of both Mg and phytol, we proceeded with the separation of all the mixtures of the products of the alkaline treatment by TLC. Fig. 4 shows schematically the representation of the thin-layer chromatograms in both normal- and reversed-phase modes obtained with the different fractions of the products of degradation of chlorophyll *a* and chlorophyll *b* separated under the chromatographic conditions specified under Experimental. Table 3 details the chromatographic characteristics of the different bands isolated, including the R_F values and the colour on the TLC plate under daylight and UV radiation of 366 nm. Table 3 also shows the correspondence between the compounds developed by TLC and their location in the HPLC traces. The NP-TLC of the two fractions I showed five well defined bands in each. All of them have colours similar to or identical with the corresponding chlorophyll pattern, blue-green for series *a* and yellow-green for series *b*. Only band E has a darker coloration in both cases. It is important to note

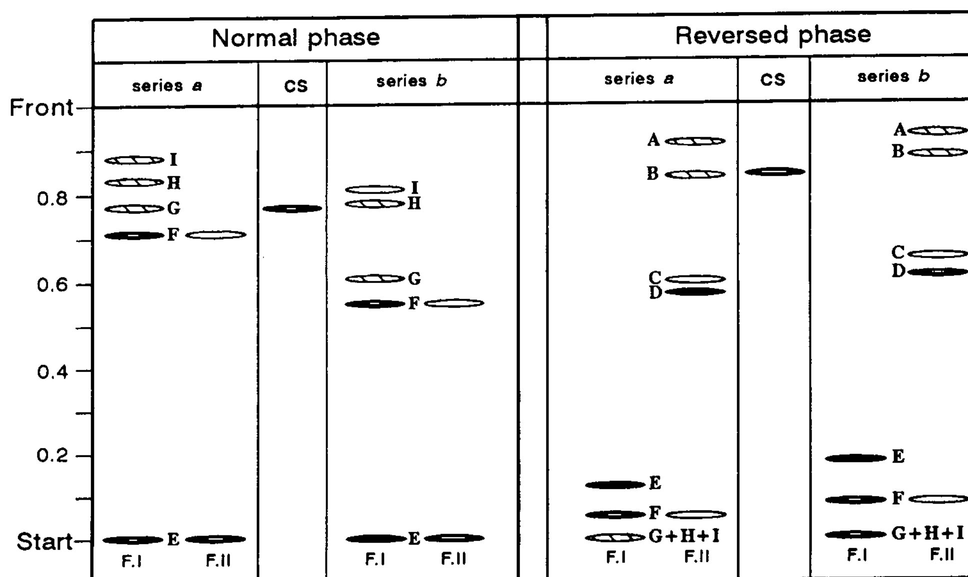

Fig. 4. TLC separation of alkaline treatment products from chlorophyll *a* and *b* and UV–Vis detection (daylight and 366 nm). NP-TLC was performed on silica gel GF_{254} plates (20 × 20 cm) eluted with light petroleum (b.p. 65–95°C)–acetone–diethylamine (10:4:1) and RP-TLC on silica gel C_{18} (10 × 10 cm) eluted with methanol–acetone–water (20:4:3). CS = chromatographic standard; chl *a* and chlorine e_6 were used as CS for NP and RP-TLC, respectively. Spots: black = major pigment; hatched = minor pigment; white = trace.

Table 3
Chromatographic characteristics of the alkaline treatment products from chlorophyll *a* and *b* isolated by TLC and location of peaks in the HPLC trace

Compound location			TLC characteristics			
TLC band		HPLC peak No.	R_F		Colour on plate	
Fraction I[a]	Fraction II		Reversed-phase	Normal-phase	Daylight	366-nm UV irradiation
Chl a derivatives						
	A	3	0.91	0.00	Blue-green	NF[b]
	B	1, 2	0.84	0.00	Grey-green	RF
	C	4, 4′	0.60	0.00	Grey	RF
	D	5	0.57	0.00	Grey-brown	NF
E		6, 6′	0.18	0.00	Brown-blue-green	IPF
F	F	9	0.05	0.71	Blue-green	NF
G, H, I[c]		7, 7′, 8, 8′, 8″, 10, 10′, 11, 11′	0.00	0.77, 0.83, 0.88	Blue-green	RF
Chl b derivatives						
	A	2	0.93	0.00	Yellow-green	NF
	B	1	0.88	0.00	Brown-green	RF
	C	3, 3′	0.65	0.00	Brown-green	RF
	D	4	0.61	0.00	Brown-green	NF
E		5, 5′	0.22	0.00	Brown-yellow-green	IPF
F	F	8	0.08	0.55	Yellow-green	NF
G, H, I[c]		6, 6′, 7, 9, 9′, 10, 10′	0.00	0.61, 0.78, 0.81	Yellow-green	RF

[a] Pigments fraction transferred to diethyl ether phase by addition of water saturated with NaCl (fraction I) or by addition of 18% (v/v) HCl (fraction II).
[b] NF = no fluorescence; RF = red fluorescence; IPF = intense pink fluorescence.
[c] Bands G, H and I were recovered together.

that the characteristic red fluorescence under 366-nm UV irradiation of chlorophyll and derivatives changes to a strong pink colour in band E while band F lacks this fluorescence. Band E is retained at the start of the chromatogram in NP-TLC and is very difficult to elute from the adsorbent. Under the conditions of RP-TLC the retention of this band was significantly lower and we recovered a larger amount of this compound, which permitted the performance of additional identification tests. The remaining pigments that constitute fraction I were accumulated without any problems from separations via NP-TLC. The fractions of pigments recovered in the organic phase by acidification (fraction II) remained at the start of the chromatogram in NP-TLC and only a light band was separated whose chromatographic features coincide with band F. RP-TLC allows the separation of, apart from this, another four bands. It is important to note that in this fraction of derivatives, as happened with fraction I, a major compound appears both in series *a* and in series *b* that does not show the characteristic red fluorescence under 366-nm UV irradiation (band D). All pigments that constitute this fraction were accumulated from separations via NP-TLC.

3.2. Identification of the products of the alkaline treatment in chlorophylls isolated by TLC

Fraction I

Band E from series *a* was shown by HPLC to consist of two isomers in the ratio 15:85 (Fig. 2a, peaks 6 and 6′) which were not separated by RP-TLC and show identical UV–Vis absorption spectra. This compound was submitted to a chemical treatment of de-esterification of the phytol alcohol group by incubation with 2.5% (w/v) methanolic KOH at 70°C. At the same time chlorophyll *a* standard was subjected to the same treatment. After de-esterification, the components recovered with light petroleum were separated by TLC over silica gel GF using benzene–ethyl acetate (19:1). In both instances a single component was separated that became visible with a blue-violet coloration under 254-nm UV irradiation and had an R_F value of 0.28. The treatment of this with dilute HCl to obtain the Mg-free derivative gave rise to a compound with an UV–Vis absorption spectrum identical (location of maxima and relationship between peaks) with that of chlorin e_6 standard. Nevertheless, the values of the retention factor (k_c) in HPLC and of R_F in RP-TLC were very different, which indicates that it is a compound with more apolar character than chlorin e_6 as a result of the presence in the molecule of the large monounsaturated chain of phytol alcohol. The set of all these chromatographic and spectroscopic characteristics together with the positive result of the test for the presence of the Mg ion and of phytol alcohol allows the preliminary identification of this pigments as Mg-phytyl-chlorin e_6 (**4.1a**). In series *b*, this band was shown to consist of two isomers with a ratio of 90:10 (Fig. 2b, peaks 5 and 5′) with identical UV–Vis absorption spectra. The form of these spectra resembles that of chlorophyll *b* but the absorption maxima in the red region is displaced 26 nm to the left and the absorption ratio of the Soret band to this band is 7.83, significantly higher than that for chlorophyll *b* (Table 2). The UV–Vis absorption spectra of the corresponding Mg-free derivative is identical with that of the rhodin g_7 standard but their chromatographic characteristics (k_c and R_F) indicate that it is a more apolar compound than rhodin g_7. The positive result of the test for the presence of phytol alcohol completed the preliminary identification of this pigment as Mg-phytyl-rhodin g_7 (**4.4b**).

The HPLC of band F from series *a* did not show isomers (Fig. 2a, peak 9). The UV–Vis absorption spectrum is very characteristic; although its form resembles that of chlorophyll *a* the absorption maximum in the red region shows a bathochromic displacement of 20 nm. This compound does not show red fluorescence under 366-nm UV irradiation and the Mg-free derivative obtained by acidification presents spectroscopic characteristics identical with those of standard purpurin *a*. However, its chromatographic properties indicate that it is a more apolar pigment. Finally, the positive result of the test for the presence of phytol allows the pre-

liminary identification of this compound as Mg-phytyl-purpuin *a* (**3.1a**). In series *b*, the UV–Vis absorption spectrum of the only peak that constitutes this band (peak 8, Fig. 2b) is characterized by the Soret band showing a hypsochromic displacement of 12 nm in relation to that of chlorophyll *b*, a shoulder appears at 430 nm and the Soret/maximum VI ratio (4.64) is higher than that for chlorophyll *b*. The spectroscopic characteristics of the Mg-free derivative are identical with those of the purpurin *b* standard but show values of k_c and R_F characteristic of a more apolar compound. Finally, the positive result of the test for presence of the phytol molecule allows the preliminary identification of this compound as Mg-phytyl-purpurin *b* (**3.1b**).

The small amounts of bands G, H and I did not allow their individual separation by NP-TLC and they were recovered from the adsorbent together. The HPLC of series *a* shows a mixture of four components spectroscopically different that were resolved into two or more isomers (Fig. 1a, peaks 7, 7′, 8, 8′, 8″, 10, 10′, 11 and 11′). The UV–Vis spectra of peak 8 and their isomers 8′ and 8″ are appreciably different from those of the rest of the components. Although its form is different to that chlorophyll *a*, in this case the point of inflection of the spectrum of the chlorophyll *a* to the left of the Soret band, located at the 394 and 420 mm, was not present. The remaining absorption maxima experience a hypsochromic displacement of ca. 10 nm and the ratio of the absorbence of the Soret band and the absorption maximum in the red region was very high (1.77). This value is very close to the 1.82 observed by Pennington et al. [17] for the allomerized derivative MeO-lactone-chl *a* (**2.1a**).

To confirm the identification of this compound, we performed a general process of allomerization of chlorophyll *a* in the presence of methanol and atmospheric oxygen, employing the technique described by Schaber et al. [19]. The course of the reaction was followed by HPLC. After 16 h (Fig. 5b), 20% of the initial chlorophyll *a* was transformed into three other compounds. According to the derivatives allomerized, mostly found by Schaber et al. [19] and

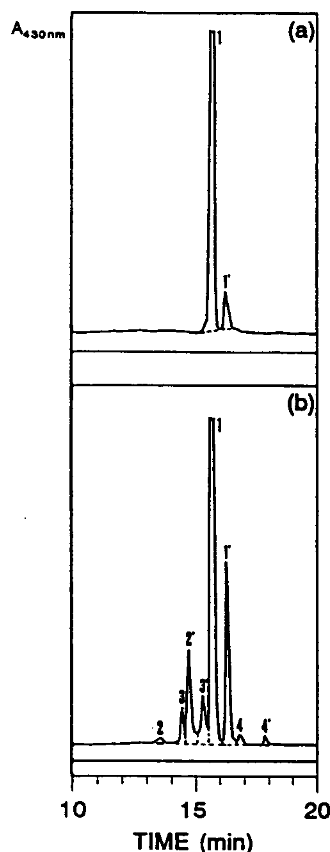

Fig. 5. HPLC separation of the allomerization products of chlorophyll *a* in presence of methanol and exposure to atmospheric oxygen. (a) Before exposure and (b) 16 h after exposure. Peaks: 1 = chl *a*; 1′ = chl *a*′; 2 = MeO-chl *a*; 2′ = MeO-chl *a*′; 3 = MeO-lactone-chl *a*; 3′ = MeO-lactone-chl *a* isomer; 4 = OH-chl *a*; 4′ = OH-chl *a* isomer.

Kuronen et al. [20], and in accordance with the absorption maxima and the relation of the peaks measured by Pennington et al. [17], compound 3 (peaks 3 and 3′) was identified as MeO-lactone-chl *a* (**2.1a**) and showed chromatographic and spectroscopic characteristics identical with those of the product of alkaline treatment of chlorophyll *a* numbered 8 in Table 1. The other two components of the set of derivatives formed in the allomerization reaction (Fig. 5b, peaks 2, 2′, 4, 4′) show UV–Vis absorption spectra identical

both in shape and in the location of the maxima with those of the chlorophyll *a* pattern. They show only slight differences in some of the ratios between the different peaks, especially between the Soret band and the maximum I that increases from 1.49 for chlorophyll *a* to 1.76 for compound 2 and 1.88 for compound 3 (10, 7 and 11 in Table 1). According to the allomerized derivatives found by Kuronen et al. [20] and their corresponding values of k_c it might be made up of a MeO-chl *a* (**1.6a**) and OH-chl *a* (**1.5a**), respectively. Also, the spectroscopic characteristics of these components are identical with those of the derivatives obtained in the alkaline treatment of chlorophyll *a* and numbered 7 and 11, but lacking other data the identification of 7 and 11 as MeO-chl *a* (**1.6a**) and OH-chl *a* (**1.5a**), respectively, can only be tentative. The identification of the peaks 10 and 10′ as chl *a* and chl *a*′, respectively, was confirmed by co-chromatography with the corresponding pure patterns.

As with the derivatives of series *a*, in series *b* bands G, H and I were recovered together and HLPC showed a mixture of four spectroscopically different components, three of them resolved into pairs of isomers (Fig. 2b, peaks 6, 6′, 7, 9, 9′, 10 and 10′). Although the shape of the UV–Vis spectrum of peak 6 was almost identical with that of chlorophyll *b*, the three absorption maxima showed a hypsochromic displacement of 12–14 nm. The ratio of absorbance between the Soret band and the absorption maximum in the red region reached a value of 4.42, significantly higher than that for chlorophyll *b* (2.80), and coincided with the value of 4.42 found by Hynninen and Ellfolk [32] for the allomerized derivative MeO-lactone-chl *b* (**2.1b**). We also performed a treatment of allomerization with chlorophyll *b* and the results of following the reaction by HPLC are shown in Fig. 6. Although we could not find recent references reporting the allomerization reaction of chlorophyll *b*, by similarity with the results found for chlorophyll *a* and according to the absorption maximum and relationship of the peaks recorded by Pennington et al. [17] and Hynninen and Ellfolk [32], we assume that peak 2 (Fig. 6b) is MeO-lactone-chl

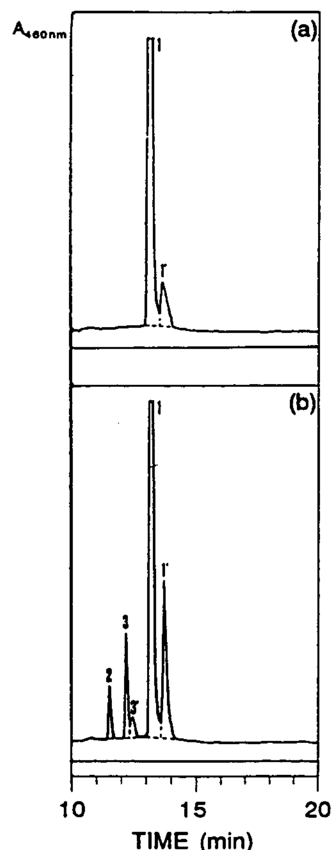

Fig. 6. HPLC separation of the allomerization products of chlorophyll *b* in presence of methanol and exposure to atmospheric oxygen. (a) Before exposure and (b) 16 h after exposure. Peaks: 1 = chl *b*; 1′ = chl *b*′; 2 = MeO-chl *b*; 3 = MeO-lactone-chl *b*; 3′ = OH-chl *b* isomer?.

b (**2.1b**); it shows chromatographic and spectrographic characteristics identical with those of the product of alkaline treatment of chlorophyll *b* numbered 6 in Table 2. The absorption spectrum of the other derivative resulting from the allomerization of chlorophyll *b* (Fig. 6b, peaks 3 and 3′) differs from that of chlorophyll *b* only in that the Soret band showed a small hypsochromic displacement of 4 nm and the ratio of absorbance of this band and that of the maximum IV is slightly larger (2.86). It could possibly be MeO-chl *b* (**1.6b**) or OH-chl *b* (**1.5b**). Lack of

further conclusive data leads to the identification of derivatives 7 and 10 obtained from the alkaline treatment of chlorophyll *b* as MeO-Chl *b* (**1.6b**) and OH-Chl *b* (**1.5b**), respectively, but only tentatively.

Fraction II

The addition of dilute HCl to this fraction of derivatives to reinforce their transfer from the aqueous to the organic phase required its identification from the corresponding magnesium-free derivatives. The UV–Vis absorption spectrum of band A from series *a* coincides exactly with that of Mg-phytyl-purpurin *a* (**3.1a**); nevertheless, under the conditions of HPLC it was resolved in less than 3 min (peak 3, Fig. 2b), which indicates that this is a compound of high polarity. The negative result of the phytol test and the positive result of the Mg test confirmed its preliminary identification as Mg-purpurin *a* (**3.3a**). In series *b* the UV–Vis absorption spectrum was identical with that of Mg-phytyl-purpurin *b* and the negative result of the test for phytol confirmed its preliminary identification as Mg-purpurin *b* (**3.2b**) (Fig. 3b, peak 2). The HPLC of band B from series *a* showed that it is formed by two peaks with a ratio of 4:1 (Fig. 2b, 1 and 2). The set of chromatographic and spectrographic properties of peak 1 is identical with that of chlorin e_6 standard (**4.4a**) and its definitive identification was confirmed by co-chromatography with the corresponding pure standard. The UV–Vis absorption spectrum of the other component coincides with that of MeO-lactone-chl *a* (**2.1a**) and its high polarity and chromatographic characteristics indicate that it may be the corresponding de-esterified derivative MeO-lactone-chld *a* (**2.3a**). In series *b*, band B is made up of a single peak (Fig. 3b, peak 1) whose chromatographic and spectrographic properties are identical with those of rhodin g_7 (**4.4b**). Its definitive identification was confirmed by co-chromatography with the corresponding pure standard. The small amounts of band C from series *a* did not allow sufficient accumulation to perform additional identification tests. The spectroscopic characteristics of the two isomers that comprise it (Fig. 2b, peaks 4 and 4′) are identical with those of the Mg-free derivative of MeO-lactone-chl *a* (**2.2a**) obtained by acid treatment of the corresponding parent. Nevertheless, the zone of the chromatogram in which this peak appears corresponds to that of more polar compounds, chlorophyll dephytolate derivatives, and therefore the compound could be an Mg- and phytol-free derivative of the MeO-lactone chl *a* called MeO-lactone-pho *a* (**2.4a**). In series *b* this band was shown by HPLC to consist of two isomers (Fig. 3b, peaks 3 and 3′) and the form of the UV–Vis absorption spectrum resembles that of pheophytin *b* (**1.2b**) [12], although the maximum shows a small hypsochromic displacement of 4–6 nm. The Mg-free derivative of MeO-lactone-chl *b* (**2.2b**) shows the same spectroscopic characteristics as this compound but a considerably smaller capacity factor; this indicates that it has a more polar character and may possibly be MeO-lactone-pho *b* (**2.4b**), that is, the corresponding phytol-free derivative. The absence of red fluorescence under 366-nm UV irradiation for band D together with the remaining chromatographic and spectroscopic properties of the single peak that comprises this band in series *a* (peak 5, Fig. 2b) and series *b* (Fig. 3b, peak 4) lead to their identification as purpurin *a* (**3.4a**) and purpurin *b* (**3.4b**) respectively. The definitive identification was confirmed by co-chromatography with the corresponding pure standard.

4. Conclusions

The alkaline treatment of chlorophylls under mild conditions and in the presence of atmospheric oxygen fosters the formation of compounds with a wide range of polarity. The study of the components of the apolar fraction indicated the presence of chlorophyllic derivatives obtained from both the solvolysis of the isocyclic ring generating Mg-phytyl-chlorin e_6 (**4.1a**) [or Mg-phytyl-rhodin g_7 (**4.1b**)] and the oxidative opening of the same forming Mg-phytyl-purpurin

(**3.1**) and MeO-lactone-chl (**2.1**). In the more polar fraction we have characterized degradation products in which a de-esterification at C-17^3 has occurred simultaneously with the oxidation of C-13^2 producing the corresponding phytol-free derivatives: Mg-chlorin e_6 (**4.2a**) [or Mg-rhodin g_7 (**4.2b**)], Mg-purpurin (**3.2**) and MeO-lactone-chld (**2.3**). The absence of chlorophyllides (**1.3**) and pheophorbides (**1.4**) in this group is important because this demonstrates that the specific formation of these dephytilate derivatives in a product is an evident sign that de-esterification has occurred by action of the chlorophyllase enzyme. This result reveals that in presence of atmospheric oxygen it is not possible to induce the specific de-esterification of the phytol by chemical treatment of the chlorophyll without promoting another series of oxidative reactions at C-13^2. The change in colour observed in green table olives processed according to modifications introduced in the industrial processing system are probably directly related to the oxidation of chlorophylls caused by recirculation of the brines that introduces atmospheric oxygen under alkaline conditions. This situation would be in accord with the detection of oxidation products of chlorophylls in the final product of an industrial process that would have not been detected previously in fruits processed in the traditional manner in a pilot study in our laboratory [24]. The investigation carried out in this work has allowed the definitive preliminary identification of these derivatives (peaks 16 and 17 in Fig. 2 in Ref. [12]). The chromatographic and spectroscopic properties of these derivatives correspond to phytyl-rhodin g_7 and phytyl-chlorin e_6, respectively. This identification was confirmed by co-chromatography with the respective pattern.

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JOURNAL OF
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Prediction of specific retention volumes in gas chromatography by using Kováts and molecular structural coefficients

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Abstract

The Kováts coefficients, $K_{c,Z}$, of a stationary phase and the solute's molecular structural coefficients, $S_{c,i}$, depend both on the specific retention volume V_g, of a solute or homologous series and on the "log-plot" slope, b, of a chromatographic column. In view of this dependence, the feasibility of predicting V_g in three instances was investigated: (a) V_g prediction of any n-alkane from $K_{c,Z}$ and retention data of n-decane; (b) V_g prediction of any solute from the temperature dependence of the above parameters and (c) V_g prediction of any term of a homologous series from the correlations of the S_c increments, ΔS_c, with the organic structural function. The possibilities of the method are evaluated in the light of the analysis of the deviations of the predicted V_g values from the measured values.

1. Introduction

The determination of specific retention volume, V_g, is very difficult as the pressure and flow of the carrier gas, column temperature, mass of the stationary phase and other parameters must be controlled. Therefore, it would be very convenient to predict V_g values from other, more easily obtained retention data. In a previous study [1], the dependence of the molecular structural coefficient of Takacs, $S_{c,i}$, of 100 solutes on temperature, polarity of the stationary phase (SP) and chemical nature of the solute was investigated. A good correlation with proposed equations was found in all cases. $K_{c,Z}$ and $S_{c,i}$ are terms proposed by Takacs [2], the sum of which equals the retention index, I_i, of a solute at a given temperature and carrier gas; together with b these data are of crucial importance in the prediction of the specific retention volumes, and can be easily determined from an n-alkane mixture (provided that these probes are used as standards) when SP of low, medium or moderately high polarity are considered.

At a given temperature, the "log-plot" slope, b, is a characteristic of a given SP. Its value depends on the polarity of the SP and on the column temperature; the effect of the latter on b is described by an Antoine-type curve, according to Hawkes [3] and Tarjan et al. [4]:

$$b(T) = A/T + B \quad (1)$$

Therefore, b also must serve for predicting V_g at variable temperatures (between 80 and 180°C) and SP polarity [retention polarity (RP) up to 72.0] [5] and, in fact, should be included in retention data libraries [4], in the same way as temperature limits or polarity data. Not much

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work has been done on the effect of polarity on b. Apparently no equation for this dependence has been reported.

$K_{c,Z}$ and $S_{c,i}$ [4] are a function of the V_g values of n-alkanes and non-n-alkanes, respectively, as seen in their defining equations:

$$K_{c,Z} = 100[Z - \log V_{g,Z}/b] \quad (2)$$

$$S_{c,i} = (100/b) \log V_{g,i} \quad (3a)$$

Replacing i by Z in Eq. 3a, we obtain

$$S_{c,Z} = (100/b) \log V_{g,Z} \quad (3b)$$

the Z n-alkane's molecular structural coefficient; $K_{c,Z}$ has a constant value and does not depend on the Z value provided that $Z \geqslant 7$.

2. Mathematical expressions used in the predictive studies of V_g

The following three cases have been considered:

(I) Calculation of V_g of an n-alkane of carbon number Z from the V_g of decane at 120°C.

From Eq. 3b, we have

$$\log V_{g,Z} = 0.01bS_{c,Z} \quad (4a)$$

and

$$\log V_{g,10} = 0.01bS_{c,10} \quad (4b)$$

for a Z n-alkane and decane, respectively. Subtracting Eq. 4b from Eq. 4a and substituting b for its value, we obtain

$$S_{c,Z} = S_{c,10} + 100(Z - 10) \quad (5)$$

Dividing Eq. 3a by Eq. 3b and bearing in mind Eq. 5, we have

$$\log V_{g,Z} = F \log V_{g,10} \quad (6)$$

where

$$F = 1 + 100(Z - 10)/(1000 - K_{c,Z}) \quad (7)$$

i.e., the logarithm of $V_{g,Z}$ for the Z n-alkane is equal to the logarithm of the specific retention volume of decane multiplied by a factor F related to the methylene group effect.

(II) Relationship between $S_{c,i}$, $K_{c,Z}$ and b and the column temperature. Application to the prediction of V_g.

Eq. 1 allows the calculation of b at any temperature. In addition there is one equation

$$K_{c,Z}(T) = a_1T + a_2 \quad (8)$$

for each stationary phase, and one equation

$$S_{c,i}(T) = \alpha T + \beta \quad (9)$$

for each solute, where a_1, a_2, α and β are LSR parameters. Therefore, V_g can be calculated from the equation

$$\log V_{g,i}(T) = 0.01b(T)S_{c,i}(T) \quad (10a)$$

for non-n-alkane solutes, and from the equation

$$\log V_{g,Z}(T) = 0.01b(T)K_{c,Z}(T) \quad (10b)$$

for n-alkanes.

(III) ΔS_c and solute chemical functions.

The dependence of ΔS_c of a series of solutes [1] with a given functional group on the stationary phase RP, calculated according to Szentirmai et al. [5], is linear:

$$\Delta S_c(\text{function}) = mRP + n \quad (11)$$

where m and n are obtained by a least-squares fit. As $\Delta S_c(\text{function}) = S_{c,i} - S_{c,Z}$, we have

$$S_{c,i} = S_{c,Z} + \Delta S_c(\text{function}) = (mRP + n) + (100Z - K_{c,Z}) \quad (12)$$

(bearing in mind Eqs. 2 and 3b). $S_{c,i}$ is the S_c of the term of the homologous series with functional group to be determined. Hence the V_g of a solute i will be given by

$$\log V_{g,i} = 0.01b[(100Z - K_{c,Z}) + (mRP + n)] \quad (13)$$

Eq. 13 gives $V_{g,i}$ as a sum of two terms: a first term that decreases with increasing $K_{c,Z}$, which in turn increases with increasing SP polarity, and a second term that increases with the polarity of both the stationary phase and the solute. $V_{g,i}$ also depends on the b value, which renders a good prediction difficult, since it depends on too many parameters, each with its own uncertainty.

Indirect determinations of V_g were tried by Fernández Sanchez et al. [6], who calculated V_g values for 24 silicone-type SPs from relative retention data for n-C_{10}, n-C_{12} and the ten McReynolds probes, with an error of 1.2%. In a second calculation [7], the retention indices of the ten McReynolds probes had a mean error of 2.2%.

Abraham et al. [8] reported an equation for calculating $\log V_g$ of a series of solutes in a given SP as a sum of a constant and five terms, each consisting of a coefficient multiplied by a parameter. Hence they could account for each solute–stationary phase interaction: a molar refractive term, a solute dipolarity moment term, two H-bond terms (acidity and basicity) and a structural term containing the solvation model on which they assume interactions occur. The coefficients were calculated by an MLRA fit. The results were very good, as was to be expected from a five-parameter equation.

3. Experimental

The apparatus, solutes, stationary phases, carrier gas, etc., have been described elsewhere [1]. Retention data used in the calculations were taken from Refs. [9–13].

4. Results and discussion

Some V_g predictions for the above three cases are discussed. The agreement between the calculated or predicted and the measured V_g values is evaluated from both absolute deviations and percentage relative errors. Eqs. 6, 10a, 10b and 13 were used in calculations.

4.1. *Prediction of V_g of any n-alkane from decane at 120°C*

Eq. 6 was used for this prediction. $S_{c,10}$ is the S_c of decane. Table 1 gives the parameters RP, $K_{c,Z}$, $S_{c,10}$ and F necessary for the calculation of V_g and the V_g absolute deviations, δ, for dodecane for 21 stationary phases of different polarities. It is seen that F tends to increase with increasing polarity. The calculated and measured V_gs are in good agreement, the V_g mean absolute deviation being 0.2 unit. The V_g δ values for other n-alkanes (averaged for all the stationary phases) were found to be as follows: hexane, −0.07; heptane, −0.08; octane, −0.07; nonane, +0.09; and undecane, +0.18 unit.

Table 2 presents calculated V_g values for n-alkanes from $Z = 6$ to 12 on OV-22. δ was −0.12 unit.

Fig. 1 shows the plots of the V_g mean absolute deviations of the n-alkanes, excluding the decane reference, for each column versus the column retention polarity. A straight line can be drawn at zero δ, showing that most stationary phases have negligible V_g mean absolute deviations, with the exception of the polyethylene glycols (Carbowax, Superox, etc.), which are the most polar of the SPs and, therefore, show the worst reproducibility. In Fig. 1 the V_g mean deviations for heptane are also included.

From these results it seems that the method works well, especially for low-polarity SPs; in

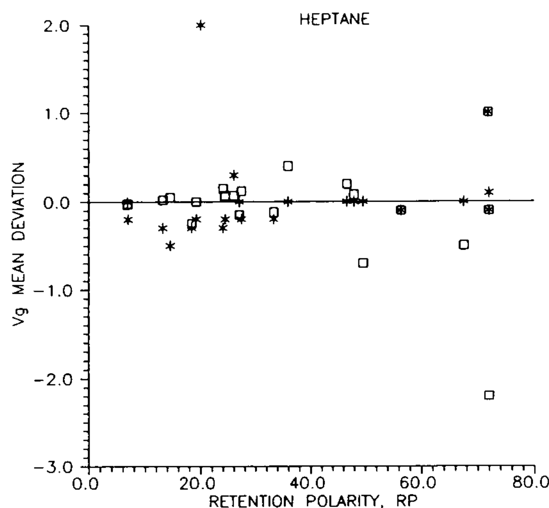

Fig. 1. V_g prediction for n-alkanes. Plots of the absolute deviations V_g(exp.) − V_g(calc.) vs. retention polarity of the 21 stationary phases listed on Table 1. V_g calculated from Eq. 6. * = δ values for heptane and □ = averaged δ value for the n-alkanes $Z = 6,7,8,9,11$ and 12 averaged for each stationary phase.

Table 1
Retention polarities (RP) and Kováts coefficients ($K_{c,Z}$) for 21 stationary phases, molecular structural coefficients, $S_{c,10}$, of n-decane, F factors and V_g absolute deviations for dodecane

Stationary phase[a]	RP	$K_{c,Z}$	$S_{c,10}$	F	δ (dodecane)
OV-101	6.8	170	830	1.241	0.0
PS-255	6.9	139	861	1.232	0.9
OV-3	13.2	173	827	1.242	0.7
OV-105	14.5	149	851	1.235	1.9
OV-7	18.4	193	807	1.248	−0.4
DC-550	19.2	193	807	1.248	0.7
OV-61	24.0	229	771	1.259	1.7
OV-11	24.4	223	777	1.257	1.1
TFPS26%	26.0	212	788	1.254	−0.6
DIOPH	26.0	219	781	1.256	−2.4
OV-17	27.4	244	756	1.264	0.9
OV-22	33.3	278	722	1.277	−0.2
OV-25	35.9	302	698	1.286	1.8
QF-1	46.6	303	697	1.287	0.5
OV-215	47.9	267	733	1.273	0.0
Ucon 50HB-2000	49.6	329	671	1.298	−1.1
OV-225	56.4	284	716	1.279	−0.3
Igepal Co-990	67.5	527	472	1.424	0.0
CW 6000	72.0	416	584	1.342	−0.6
CW 20M	72.0	430	570	1.351	−0.4
Superox 20M	71.9	354	646	1.309	0.0

RP = Retention polarity of Szentirmai et al. [5]; $K_{c,Z}$, calculated from Eq. 2; S_c of decane, calculated from $S_{c,10} = 1000 - K_{c,Z}$; $F = 1 + 100(Z-10)/S_{c,10}$.
[a] DIOPH = Diisooctyl phthalate; TFPS26% = 26% trifluoroporpylsiloxane.

Table 2
Prediction of V_g of the n-alkanes $Z = 6$–12 on OV-22 from decane data

Z	F	V_g(exp.)	V_g(calc.)	Deviation, δ
6	0.4464	6.2	6.3	−0.1
7	0.5848	11.0	11.2	−0.2
8	0.7232	19.6	19.7	−0.1
9	0.8616	34.9	34.9	0.0
10	1.0	61.9		
11	1.1384	109.4	109.5	−0.15
12	1.2768	193.6	193.8	−0.2
				Mean: −0.12

Data: $K_{c,Z} = 277.5$; $S_{c,10} = 722.5$; $\log V_{g,10} = 1.7915$; $F = 1 + 100(Z-10)/722.5$.

Table 3
Linear regression of the "log-plot" slope, b, vs. the reciprocal of the absolute temperature, $b(T) = A/T + B$

Stationary phase	A[a]	B[a]	r_1
PS-255	208.3 ± 2.4	−0.2785 ± 0.006	0.9993
OV-105	207.7 ± 0.84	−0.2795 ± 0.002	0.99998
Didecyl phthalate	217.2 ± 4.0	−0.2689 ± 0.01	0.99959
QF-1	189.1 ± 3.0	−0.2749 ± 0.007	0.99985
OV-215	182.0 ± 6.1	−0.2572 ± 0.015	0.99888
Superox 20M	189.9 ± 5.4	−0.2575 ± 0.014	0.99920

[a] Values ± standard deviations ($n = 5$).

Table 4
α and β LSR parameters of $S_{c,i}$ vs. the absolute temperature and A' and B' LSR parameters of log $V_{g,i}$ vs. reciprocal of absolute temperature for five solutes in six stationary phases

Solute	Stationary phase	α	β	r_2	A'	B'	r_3
Dodecane	OV-105	−1.085	1468	0.992	2547	−3.9	0.9999
Benzene		−0.812	850	0.991	1390	−2.2	0.99995
1-Butanol		−1.204	1007	0.993	1520	−2.5	0.9996
2-Pentanone		−1.033	965	0.995	1479	−2.4	0.9978
Pyridine		−0.752	926	0.998	1578	−2.4	0.99999
Dodecane	PS-255	−1.118	1494	1.000	2750	−4.3	0.99887
Benezene		−0.781	831	0.997	1492	−2.5	0.99927
1-Butanol		−1.142	955	0.99	1496	−2.5	0.9979
2-Pentanone		−1.343	1051	0.99	1581	−2.7	0.9994
Pyridine		−0.835	935	0.971	1652	−2.7	0.99866
Dodecane	Didecyl phthalate	−1.14	1442	0.998	2665	−3.96	0.9993
Benzene		−0.84	861	0.995	1522	−2.4	0.9994
1-Butanol		−1.46	1126	0.997	1846	−3.1	0.9985
2-Pentanone		−1.19	1021	0.993	1727	−2.8	0.998
Pyridine		−0.96	1035	0.993	1866	−2.9	0.994
Dodecane	QF-1	−2.06	1710	0.999	2333	−4.1	0.9996
Benzene		−1.903	1248	0.971	1476	−2.7	0.9993
1-Butanol		−1.763	1229	0.997	1527	−2.8	0.9979
2-Pentanone		−1.69	1360	0.987	1809	−3.2	0.9995
Pyridine		−1.593	1351	0.992	1843	−3.2	0.9992
Dodecane	OV-215	−2.13	1764	0.993	2327	−4.0	0.99999
Benzene		−1.310	1053	0.95	1470	−2.6	0.99986
1-Butanol		−1.567	1181	0.999	1508	−2.7	0.99987
2-Pentanone		−1.64	1378	0.987	1780	−3.0	0.99972
Pyridine		−1.21	1228	0.992	1702	−2.8	0.99973
Dodecane	Superox 20M	−1.235	1385	0.991	2163	−3.5	0.99993
Benzene		−0.83	1001	0.990	1592	−2.5	0.99993
1-Butanol		−1.4	1378	0.990	2077	−3.4	0.99995
2-Pentanone		−1.585	1303	0.926	1859	−3.2	0.99693
Pyridine		Unavailable					

contrast, the method cannot be recommended for polar SPs owing to the known deficiencies concerning the adequacy of n-alkanes as standard probes in polar SPs, and further, the measured retention times of the smaller n-alkanes in these SPs are erratic [14,19]. Obviously, the easiest way to obtain the specific retention volume of any n-alkane is by means of the plot of log $V_{g,Z}$ vs. Z, which has to be available, but the method suggested in this paper calculates V_g values using the V_g of decane and the Kováts coefficient, $K_{c,Z}$, b not being needed. According to Eq. 2, however, b would be needed to calculate an experimental value of $K_{c,Z}$ but the method with Eq. 6 uses an empirical value of $K_{c,Z}$ obtained by its correlation with the RP of the SP (Table 1 shows that $K_{c,Z}$ increase with increasing RP of 21 SPs). The author has worked on this correlation [15], finding a correspondence between these two magnitudes, and subsequently some empirical equations allowing $K_{c,Z}$ to be calculated were found; hence in this case it is sufficient to chromatograph decane to be able to predict the V_g of any n-alkane for any stationary phase of the known first five McReynolds constants at 120°C.

Table 5
V_g prediction for ten solutes on OV-215 and for 1-butanol on Superox 20M at four temperatures

Ten solutes on OV-215

Solute	Temperature (°C)	V_g(exp)	V_g(calc.)	Rel. error (%)
Nonane	100	35.2	34.9	0.8
Benzene	100	20.5	20.1	1.9
Pyridine	100	63.5	61.9	2.5
N,N-Dimethylaniline	155	52.4	51.2	2.2
1-Pentanol	160	10.0	9.7	3.2
1-Octanol	100	217.1	217.8	0.3
2-Octanone	145	57.7	55.9	3.1
Butyronitrile	130	36.9	35.2	4.6
Ethyl acetate	100	27.1	25.1	7.3
Pentylbenzene	115	142.7	145.1	−1.7
				Mean: 2.4

1-Butanol on Superox 20M

Temperature (°C)	b[a]	$S_{c,i}$[b]	V_g(exp)	V_g(calc.)	Rel. error (%)
90	0.2656	869.5	204.5	203.9	0.3
110	0.2383	841.5	102.8	101.2	1.5
130	0.2137	813.5	55.3	54.7	1.1
150	0.1914	785.5	31.6	31.9	0.9
					Mean: 0.9

[a] $b = 189.9/T - 0.2575$.
[b] $S_{c,i}(T) = -1.4T + 1378$.

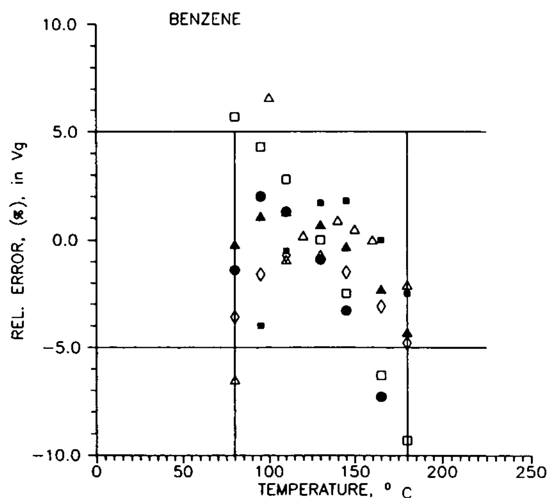

Fig. 2. Relative error (%) in V_g (calculated from Eq. 10a) for benzene vs. column temperature on six stationary phases: PS-255, OV-105, didecyl phthalate, QF-1, OV-215 and Superox 20M. Temperature range, 80–180°C.

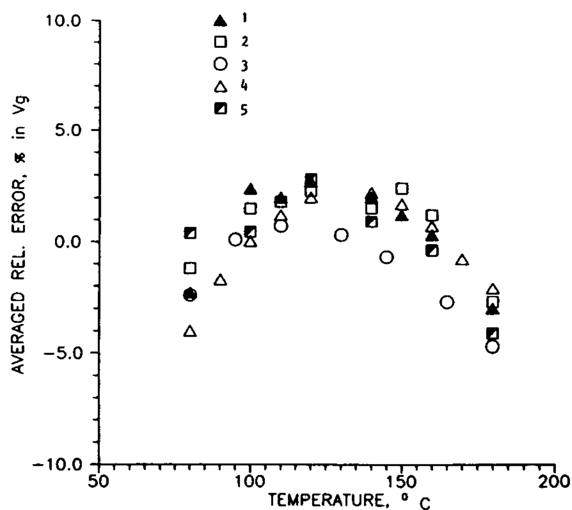

Fig. 3. Relative error (%) in V_g (calculated from Eqs. 10a and 10b) averaged for six stationary phases (PS-255, OV-105, didecyl phthalate, QF-1, OV-215 and Superox 20M) for the solutes octane (5), benzene (3), 1-butanol (1), 2-pentanone (4) and pyridine (2).

4.2. Prediction of V_g of any solute at any temperature

Retention data at several temperatures for six stationary phases are available, from which the dependences of b, $K_{c,Z}$ and $S_{c,i}$ on temperature can be obtained. $V_{g,i}$ values at any temperature can be calculated from Eqs. 10a and 10b for non-n-alkanes and n-alkanes, respectively. The equations for the dependence of $K_{c,Z}$ on temperature are given elsewhere [1,9]. Other data necessary for carrying out the calculations are given in Tables 3 and 4.

Table 3 presents the b temperature-dependence equations for six SPs [9–13]. Table 4 shows the α and β LSR parameters of the $S_{c,i}$ temperature-dependence equations and the A' and B' LSR parameters of the log V_g vs. $1/T$ dependence equations for the solutes dodecane, benzene, 1-butanol, 2-pentanone and pyridine on the same six stationary phases [9–13].

The V_g values in Table 4 are the experimental values against which the V_g values calculated from Eqs. 10a and 10b are compared.

Table 5 shows (a) calculated V_g values for ten solutes on OV-215 stationary phase, yielding a mean error of 2.4%, and (b) the predicted V_g values of 1-butanol at four temperatures in Superox 20M. Using as data the equations in Table 3 and the equation $\log V_g = 2077.5/T - 3.41$, taken from Ref. [12], a mean relative error of 0.9% is obtained. Calculated V_g values for benzene and dodecane on six SPs at 120°C [9–12] are shown in Table 6. The mean relative errors are −0.7 and −1.4%, respectively.

Hence, at 120°C and at moderate temperatures between 70–80°C and 180–200°C, the V_g prediction of the tested solutes on the studied SPs is acceptable.

To find the temperature limits for this prediction, the V_g relative errors of n-alkanes and solutes in Table 4 on the six SPs were plotted versus temperature over the range 80–180°C. Fig. 2 shows the plot for benzene. A band of permissible relative errors was obtained by drawing two horizontal lines at relative errors of 5% and −5%. If two vertical lines are drawn at the temperatures 80 and 180°C, most points fall inside the rectangle of the 5% permissible error. The solute is described by six curves, one for each stationary phase. Similar results were found when the other solutes were studied.

Fig. 3 shows the plot of the sum of the relative errors in V_g for octane and the four non-n-

Table 6
V_g prediction for benzene and dodecane on six stationary phases at 120°C

Solute	Stationary phase	V_g(exp.)	V_g(calc.)	Rel. error (%)
Benzene	PS-255[a]	32.1	32.3	−0.6
	OV-105	21.0	20.9	0.3
	DNDPH[b]	31.6	32.0	1.3
	QF-1	10.5	10.7	−1.9
	OV-215	12.6	13.0	−3.2
	Superox 20M	33.2	33.2	0.0
				Mean: −0.7
Dodecane	PS-255[a]	997.5	1034.9	−3.7
	OV-105	392.2	388.8	0.9
	DNDPH[b]	639.3	658.7	−3.0
	QF-1	70.5	71.7	−1.7
	OV-215	81.0	80.6	−0.5
	Superox 20M	106.1	106.2	−0.01
				Mean: −1.4

[a] Temperature 100°C.
[b] DNDPH = Didecyl phthalate.

alkanes, averaged over the six SPs in the same temperature range. The curves show a maximum at about 130°C, and most errors are under 5%. Hence, the method seems to be applicable from 80 to 180°C.

4.3. Prediction of V_g in a homologous series at 120°C

Theoretically, this type of prediction looks feasible, although with some reservations. Experimental data [9–13] and ΔS_c values for PS-255, QF-1, OV-215 and Superox 20M are available [1]. Therefore, V_g values of different members of some homologous series were tried. Prediction of V_g for the members $Z = 7$, 8 and 9 of the 1-alkanols on Superox 20M yields a mean relative error of 5.7%; prediction of V_g of the $Z = 3$, 6, 7 and 9 members of 1-alkanols on OV-215 yields a mean error of 3.4%, and unbranched nitriles $Z = 6$, 7 and 8 on QF-1 yield a mean error of 3.9%.

Better results are obtained for the TFPS26% stationary phase [13], as Table 7 shows for the functional groups methyl ketones, 1-alkanols, *n*-alhylamines and unbranched nitriles, with mean errors of 1.8, 3.3, 4.8 and 0.3%, respectively. However, in other cases in which predictions were made testing Eq. 13 on McReynolds data [16] the predicted V_g values differed widely from the experimental values, irrespective of whether the correlations of ΔS_c with RP were fair [1]; the failure may be due to the fact of V_g may not depend only on a single variable when chemical functions are involved because the magnitudes taking part in the V_g approximate calculations, i.e., $K_{c,Z}$, b and $S_{c,i}$, may also depend on some additional factors to the SP polarity itself, e.g., the solute polarity; obviously, a multiparametric equation similar to that suggested by Abraham et al. [8] would yield much better results. The work of Takacs [17] and Peng et al. [18] shows the way in which these investigations must be carried out.

Hence the equation proposed in this paper only works when experimental data on the functional group investigated are available, and

Table 7
V_g relative errors of members of four homologous series on the TFPS26% stationary phase at 120°C

Homologous series	Compound	V_g(exp)[a]	V_g(calc.)	Rel. error (%)
Ketones	2-Pentanone	28.4	28.6	−0.7
	2-Hexanone	48.0	48.6	−1.3
	2-Nonanone	230.7	238.9	−3.5
				Mean: −1.8
1-Alkanols	Ethanol	5.3	5.5	−3.7
	1-Hexanol	46.7	46.0	−1.5
	1-Heptanol	80.5	78.2	−2.8
	1-Nonanol	238.7	225.7	−5.4
				Mean: −3.3
Amines	Ethylamine	4.6	4.8	−4.2
	n-Heptylamine	70.8	67.7	−4.3
	n-Octylamine	122.4	113.1	−5.9
				Mean: −4.8
Nitriles	*n*-Hexanitrile	93.7	93.9	−0.2
	n-Octanitrile	270.0	271.1	−0.4
				Mean: −0.3

$\Delta S_c = 344$ for ketones, 334 for 1-alkanols, 307 for amines and 468.5 for nitriles [1]. $RP = 27$; $K_{c,Z} = 212$; $b = 0.2303$.
[a] Measured V_g values taken from Ref. [13].

therefore its use in predictive studies of V_g in all instances cannot be recommended.

5. Conclusions

The $S_{c,i}$ values of solutes and the $K_{c,Z}$, *RP* and *b* parameters of stationary phases can be used to calculate in an approximate way the specific retention volumes of a series of solutes in two cases: (a) at 120°C, for low-polarity stationary phases, the V_g of any *n*-alkane if the $K_{c,Z}$ of the stationary phase (or $S_{c,10}$) and the V_g of *n*-decane are known; (b) at any temperature between 80 and 180°C, it is possible to predict with acceptable errors $V_{g,i}$ values for a series of solutes provided that the relevant $K_{c,Z}$, $S_{c,i}$ and *b* temperature dependences are known.

Extension of ΔS_c to predictive V_g determinations is not suitable for functional groups unless data on the relevant chemical function are available. It is emphasized that the model proposed in this paper is valid only if the assumption is made that the retention mechanism occurs exclusively by gas–liquid partitioning.

Symbols

SP	stationary phase
RP	retention polarity
$K_{c,Z}$	Kováts coefficient for a given SP
$S_{c,Z}$	molecular structural coefficient of a *Z* *n*-alkane
$S_{c,i}$	molecular structural coefficient of a solute *i*
$S_{c,10}$	S_c value for decane
I_i	retention index of a solute *i*
100*Z*	retention index of a *Z* *n*-alkane
$V_{g,Z}$	specific retention volume of a *Z* *n*-alkane
$V_{g,i}$	specific retention volume of a solute *i*
ΔS_c(function)	molecular structural coefficient increment of a functional group
b	slope of the plot of log $V_{g,Z}$ vs. the number of carbon atoms, *Z*
F	methylene group factor
LSR	least-squares regression
MLRA	multiple linear regression analysis
a_1, a_2	LSR parameters for the temperature dependence of $K_{c,Z}$
α, β	LSR parameters for the $S_{c,i}$ temperature dependence
A, *B*	LSR parameters of the *b* temperature dependence
A', *B'*	LSR parameters of the log $V_{g,i}$ temperature dependence
m, *n*	LSR parameters of the dependence of ΔS_c(function) on *RP* of a stationary phase
r_1, r_2, r_3	correlation coefficients
δ	V_g absolute deviation: $V_{g,i}$(exp) − $V_{g,i}$(cal)
$V_{g,i}$(exp)	measured $V_{g,i}$
$V_{g,i}$(cal)	specific retention volume of a solute *i* calculated by means of Eqs. 6, 10a, 10b and 13
Rel. error (%)	Relative error of V_g: $100\{[V_{g,i}(\text{exp}) - V_{g,i}(\text{cal})]/V_{g,i}(\text{exp})\}$

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Gas chromatographic determination of volatile alkenes with on-column bromination and electron-capture detection

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Abstract

A method is described for the gas chromatographic–electron-capture detection determination of alkenes via on-column bromination reactions. Pyridinium bromide perbromide (PBPB) was used as the Br_2 source, and a cholesterol–glass beads mixture, treated with methanol, was used to remove excess Br_2. The optimum ratio of cholesterol to glass beads was found to be 1:10, at which 93% of the bromine released from PBPB can be removed, without removal of the derivitized analytes. The conversion efficiency of alkene to the brominated derivative is extremely low (less than 2%) for ethene, whereas for propene and 1-butene it is 41 and 79%, respectively. For C_3–C_5 alkenes, this method is 200–300 times more sensitive than analysis of the underivitized analytes by using conventional flame ionization detection.

1. Introduction

Few hydrocarbons (or volatile organic compounds, VOCs) are toxic in their own right at the concentrations found in the ambient atmosphere, and their main contribution to air pollution stems from their role in the atmospheric formation of ozone, peroxyacetyl nitrate (PAN), hydrogen peroxide and other secondary air pollutants [1]. Because of their extremely low ambient concentrations [volume mixing ratios of $<10^{-12}$ ppt (v/v) to 10^{-9} ppb (v/v)], detection of hydrocarbons in the atmosphere requires sensitive analytical methods. As the more reactive hydrocarbons (e.g., alkenes) have a greater reactivity towards OH radicals (and thus have higher potential for ozone formation) than the non-reactive hydrocarbons (e.g., alkanes), the priority in atmospheric monitoring programmes which focus on photochemical ozone production is the determination of the reactive VOCs, rather than of the non-reactive species. Under some circumstances it may therefore be advantageous to utilize a detection system that has enhanced sensitivity towards alkenes but is relatively insensitive to alkanes and other less reactive VOC species.

Attempts have been made to develop analytical methodologies that have enhanced sensitivity towards alkenes relative to the behaviour of conventional flame ionization detection (FID). For example, recently, reduction gas detection (RGD), originally developed for detecting the reducing gases CO and H_2 [2], has been used for the detection of acetaldehyde and acetone [3] and isoprene [4]. The response of RGD to a variety of reactive hydrocarbons has been investigated using gas chromatography

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(GC) with packed columns [5]. It was shown that it is considerably more sensitive to alkenes than is FID, and has much greater sensitivity to alkenes than alkanes. RGD has also been developed successfully for the capillary GC determination of hydrocarbons up to C_6 with high resolution [6,7].

In this work, electron-capture detection (ECD) was applied to the detection of alkenes following a derivitization step. ECD is a very sensitive and selective GC detection method for organic compounds containing highly electronegative elements such as Cl, Br and N. Its sensitivity towards organic molecules that do not contain O, N or halogen atoms is very low. However, if an element of high electronegativity could be added to the alkene via an on-column reaction, then it might be expected that alkenes could be detected by ECD with much higher sensitivity than by FID.

The GC–ECD detection of alkene compounds via bromination reactions was investigated. This required the attachment of bromine atoms to the alkenes, to render them amenable to ECD, and removal of excess bromine prior to detection.

2. Experimental

2.1. Principle

To use bromination reactions successfully for the ECD of alkenes requires that the derivitization reactions be very rapid and proceed to completion within a few seconds, otherwise the eluting peaks will be small and broad in the chromatogram, and the sensitivity will be reduced. The principle of this work is based on the reactions of bromine with alkenes to form the *o*-dibromoalkane derivatives:

$$RCH{=}CHR' + Br_2 \rightarrow RCHBrCHBrR' \quad (1)$$

This reaction is an electrophilic addition reaction, and can be affected significantly by the presence of polar substances. In most cases it can proceed very quickly and quantitatively at room temperature in the polar liquid phase, without light or catalysis.

Although the possible role of bromine compounds in the chemistry of the atmosphere has promoted the study of the kinetics of the reactions of bromine atoms with hydrocarbons [8–10], little information is available on the addition reactions of bromine molecules with alkenes in the gas or vapour phase. However, it is expected that, so long as polar conditions can be realized, these reactions may also proceed very quickly in the gas phase and thus may be used for this purpose.

Although the hydrogen atoms in alkanes can be easily displaced by bromine atoms under conditions of light, heat or catalysis, alkanes do not react with bromine in darkness and at room temperature. It should therefore be possible to control the conditions of bromination to prevent the bromination of alkanes while still derivatizing the more reactive alkenes.

2.2. GC system

GC measurements were made using a Hewlett-Packard 5890 Series II gas chromatograph fitted with an electron-capture detector. The carrier and the auxiliary gas was helium. The configuration of the whole GC–ECD–bromination system is shown in Fig. 1. The GC column used was an HP-1 (10 m × 0.53 mm I.D., film thickness 2.65 μm). Tubes 1 and 2 are glass tubes (10 cm × 3 mm I.D.). Tube 1 was packed with either glass beads (80–100 mesh, 0.254–0.284 cm) or the brominating reagent, and tube 2 was packed with either glass beads or the substance for removing the excess bromine, depending on the purpose of the experiment.

2.3. Materials

Pyridinium bromide perbromide (PBPB) and cholesterol were obtained from Aldrich. A 15 ppm (v/v) standard vapour mixture of C_2–C_6 1-alkenes (Scotty) was used. This contains equal concentrations of ethene, propene, 1-butene, 1-pentene and 1-hexene. Their reaction products with bromine will thus be 1,2-dibromoethane, 1,2-dibromopropane, 1,2-dibromobutane, 1,2-dibromopentane and 1,2-dibromohexane, respectively. 1,2-Dibromoethane, 1,2-dibromo-

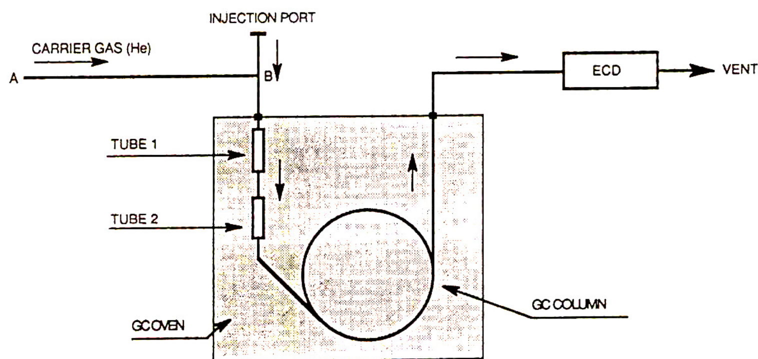


Fig. 1. Schematic diagram for the GC–bromination–ECD system.

propane and 1,2-dibromobutane were obtained from Aldrich for peak identification by comparison of retention times. Their standard vapour mixtures were prepared by the static dilution bottle methods [11,12]. Known amounts of liquid dibromoalkanes were injected into a 1-l glass flask which had been purged with nitrogen for 3 min. Further serial dilutions were made by withdrawing known amounts of vapour from the flask and injecting them into other similar flasks. Flasks and gas-tight syringes were kept at 55 ± 5°C in order to prevent vapour condensation of adsorption.

3. Results and discussion

3.1. Release of Br_2 from pyridinium bromide perbromide (PBPB)

PBPB is a red, crystalline salt ($C_5H_5NHBr \cdot Br_2$, M_r 319.84, m.p. 135–136°C). From its structural formula it is expected that it can release free bromine molecules, so when substituted for bromine in any standard bromination procedure the reaction should proceed in the normal manner. Because this reagent can be handled and stored more easily than liquid bromine, it has been used as a brominating reagent since the 1940s [13–15]. However, all such applications of PBPB have been in the liquid phase in which the free bromine was released from solutions of PBPB in glacial acetic acid or methanol. Its use as a source of vapour-phase Br_2 molecules for the on-line derivitization of alkenes was first proposed by Simmonds [16].

In order to check if the perbromide can release bromine vapour from its solid phase or not, tube 1 in Fig. 1 was packed with 0.27 g of PBPB and tube 2 with 0.72 g of glass beads (80–100 mesh). It was observed that the rate of release of Br_2 by the PBPB, as detected by ECD, increased with increasing carrier gas flow-rate passing through it, and especially with increasing GC oven temperature. The ECD signals responding to the bromine released from the PBPB varied from a few thousand mV to the maximum (i.e., saturation of the ECD). Even at a constant carrier gas flow-rate (e.g., 20 ml/min) and GC oven temperature (e.g., 30°C), the ECD signal due to the bromine released from the PBPB still varied significantly, from 2000 to 5000 mV.

3.2. Bromine filter

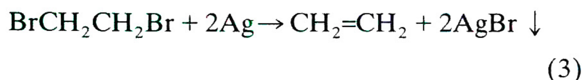
Following derivatization of the alkenes in an air sample, excess bromine from the PBPB will still be present in the gas flow and must be removed prior to entry into the detector. This is necessary for at least three reasons: (1) to allow detection of trace amounts of the brominated analytes the concentration of free Br_2 must be reduced to an absolute minimum; (2) the ECD sensitivity will be progressively reduced by formation of $NiBr_2$ when it is continuously exposed

to bromine vapour; (3) to avoid damage of the GC capillary column by bromine due to its highly oxidizing properties. The bromine filter should ideally be in the solid phase under normal conditions, and it should be able to remove bromine (by adsorption or absorption) efficiently, but it should not have any effect on the brominated products.

Silver powder was investigated for this purpose, and it was found that it can remove bromine efficiently at room temperature by forming AgBr via the following reaction:

$$2Ag + Br_2 \rightarrow 2AgBr \downarrow \quad (2)$$

However, the brominated alkenes were also removed very efficiently by reaction with silver via the following reaction (taking 1,2-dibromoethane as an example):



$$BrCH_2CH_2Br + 2Ag \rightarrow CH_2{=}CH_2 + 2AgBr \downarrow \quad (3)$$

Hence silver and other similar metals cannot be used for this purpose.

Large organic molecules with double bonds may also be able to remove bromine via bromine addition reactions, but may have no effect on the brominated alkenes. Cholesterol ($C_{27}H_{46}O$, M_r 386.67, m.p. 146–149°C) is a white, crystalline solid with a double bond and its use for this purpose was suggested by Breuer [17]. Its molecular structure is shown in Fig. 2.

In order to check if cholesterol can remove bromine or not, tube 1 in Fig. 1 was packed with 0.27 g of PBPB and tube 2 with 0.20 g of cholesterol. A carrier gas flow-rate of 20 ml/min, optimized on the basis of peak width and Br_2 production rate, and an oven temperature of 30°C, kept as low as possible to minimize the release rate of Br_2 from the PBPB and to minimize the volatilization of cholesterol, which contains an OH group to which the ECD is sensitive, were used. Under these conditions, it was observed that the ECD signal from the PBPB was not reduced by the cholesterol. This suggests that cholesterol itself does not react sufficiently rapidly with bromine. This may be due to the lack of polar conditions, since the addition reactions of bromine with alkenes are electrophilic.

In order to create the optimum polar conditions for the cholesterol–bromine addition reactions, a series of different mass ratios of cholesterol–glass bead mixtures, treated with methanol, were prepared: 1:1, 1:2, 1:4, 1:5, 1:7, 1:9, 1:10, 1:11, 1:12 and 1:15. These mixtures were prepared by dissolving known amounts of cholesterol in methanol (20 ml) with gentle heating. Known amounts of glass beads were added to the solution and the methanol was removed by evaporation to dryness. At ratios of 1:1 and 1:2, no reduction in the ECD signal due to the PBPB was observed. At ratios of 1:4 and 1:5, the ECD signal was reduced to 700–800 mV for about 10 min, and then increased rapidly to above 1000 mV again. At a ratio of 1:7, the ECD signal from the PBPB was reduced to and stabilized at about 600 mV, and at ratios of 1:9, 1:10, 1:11 and 1:12 the signal stabilized at about 300 mV. Considering the average ECD signal due to PBPB alone (3000 mV), the ECD baseline signal (about 30 mV) and the ECD signal from cholesterol alone (about 50 mV) under the conditions used, the bromine removal efficiency of the cholesterol–glass bead mixture at ratios of 1:9, 1:10, 1:11 and 1:12 is about 93%. At a ratio of 1:15, the ECD signal from the PBPB was reduced to about 400 mV, but increased rapidly again to above 1000 mV, suggesting that insufficient cholesterol was available for complete continuous reaction with the bromine.

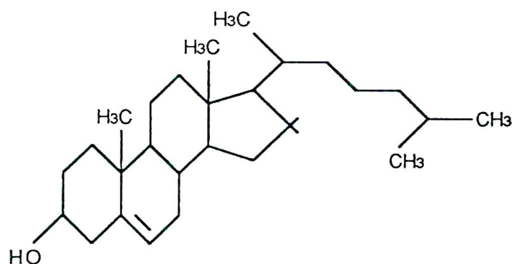


Fig. 2. Molecular structure of cholesterol.

3.3. Effect of different cholesterol–glass beads ratios on the bromination of alkenes

It is expected that the methanol-treated cholesterol–glass bead mixture can provide suitable polar conditions, not only for bromine removal by cholesterol, but also for bromine addition reactions with the alkenes. As in the case of bromine removal by cholesterol, the mass ratios of the cholesterol–glass bead mixture may also be important for the bromine addition reactions with alkenes. In order to investigate this effect, the same amounts of C_2–C_6 1-alkenes were injected into the system at cholesterol-to-glass bead ratios of 1:7, 1:9, 1:10, 1:11 and 1:12. A typical chromatogram is shown in Fig. 3. On the basis of retention times relative to those of the standard dibromoalkanes, it is concluded that the alkenes were successfully derivitized under the conditions used and that sufficient excess Br_2 has been removed by the bromine filter to allow their detection by ECD.

The response factors for the brominated compounds (assuming complete derivitization of the alkenes) were calculated at different cholesterol-to-glass bead ratios and the results are shown in Fig. 4. Because the elution time of 1,2-dibromohexene is very long (70 min, see Fig. 3),

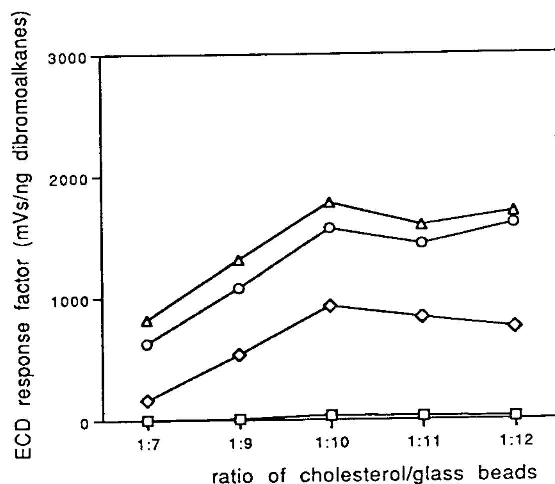


Fig. 4. Variation of ECD response factors for brominated compounds with mass ratio of cholesterol to glass beads. □ = 1,2-Dibromoethane; ◇ = 1,2-dibromopropane; □ = 1,2-dibromobutane; △ = 1,2-dibromopentane.

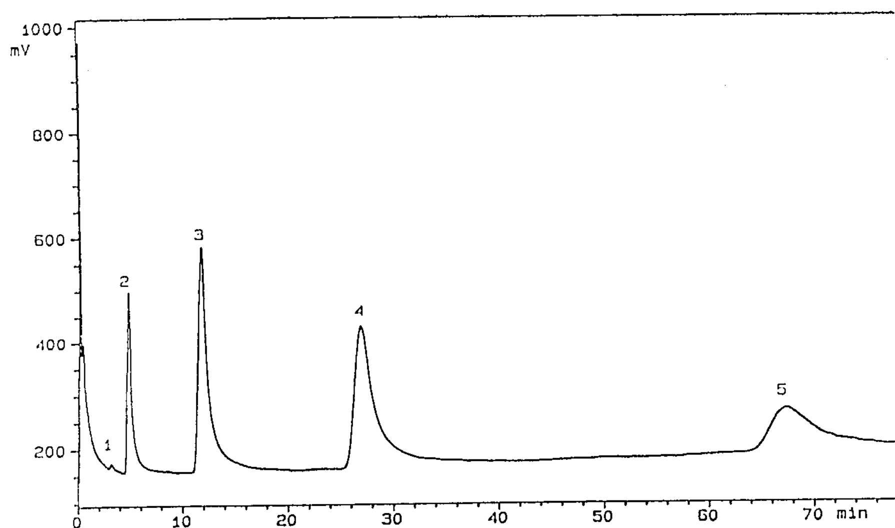


Fig. 3. Chromatograms of brominated compounds: 0.2 ml 15 ppm alkenes (C_2–C_6). GC oven temperature, 30°C; carrier gas flow-rate, 20 ml/min; column, 10 × 0.53 μm HP-1; tube 1, PBPB; tube 2, cholesterol–GB (1:10). Peaks: 1 = 1,2-dibromoethane; 2 = 1,2-dibromopropane; 3 = 1,2-dibromobutane; 4 = 1,2-dibromopentane; 5 = 1,2-dibromohexane.

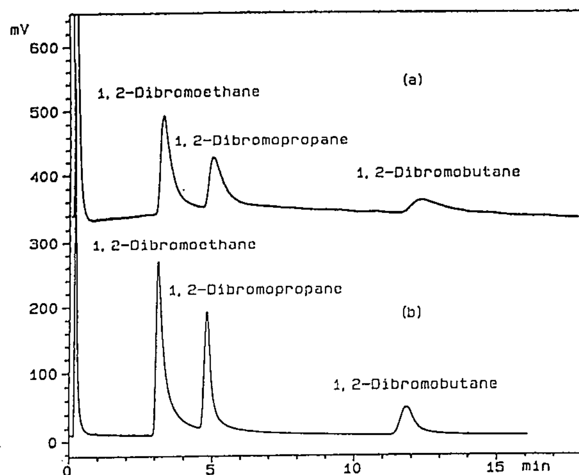


Fig. 5. Chromatograms of dibromoalkanes. GC oven temperature, 30°C; carrier gas flow-rate, 20 ml/min. (a) Tube 1, PBPB; tube 2, cholesterol–glass beads. (b) Tubes 1 and 2, glass beads.

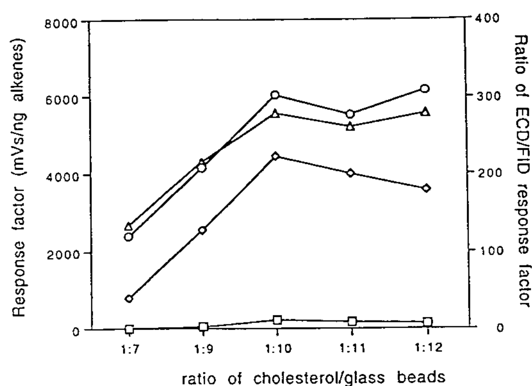


Fig. 6. Variation of the ECD response factors and the mass ratios of ECD and FID response factors for alkenes with the mass ratio of cholesterol to glass beads. □ = Ethene; ◇ = propene; ○ = 1-butene; △ = 1-pentene.

alone. However, even by considering the adsorption effect of the PBPB and the cholesterol–glass bead mixture (see the results in parentheses in Table 1), the conversion efficiency of ethene is still extremely low (less than 2%). This suggests that the bromine addition reaction with ethene is so inefficient that the method may not be suitable for the determination of ethene.

3.5. *Comparison of the sensitivity of FID with the bromination–ECD method for alkenes*

Fig. 6 shows the ECD response factors for the derivitized alkenes as a function of the mass of alkene introduced, and relative to the response factors of FID to the alkenes (about 20 mV s/ng), at different ratios. It can be seen that with cholesterol–glass bead mixtures of 1:10–1:12, ECD is about 200–300 times more sensitive to the C_3–C_5 alkenes via the bromination reactions than is FID to the underivitized alkenes. Little increase in sensitivity is achieved by bromination–ECD relative to FID for ethene.

The minimum detectable amounts of alkenes using the present GC–bromination–ECD system, based on a signal-to-noise ratio of 2, were 0.08 ng for propene, 0.11 for 1-butene and 0.14 ng for 1-pentene. Although they are higher than the typical detection limits currently obtainable with high-resolution capillary GC–FID (about 0.01 ng), it will be possible to lower them substantially by improving the chromatography and hence the peak shapes of the derivitized compounds.

3.6. *System linearity*

In order to check if the bromination–ECD system is linear with respect to the amount of alkene injected, different amounts of alkenes were injected on to the GC column, with tube 1 packed with PBPB and tube 2 packed with a 1:10 cholesterol–glass bead mixture. The results are shown in Fig. 7. It can be seen that the method is linear up to masses of ca. 2, 3 and 8 ng for propene, 1-butene and 1-pentene, respectively. That is, the range of linearity becomes broader with increasing carbon number. This is consistent with the fact that the conversion efficiency of the alkenes to the brominated derivatives increases with increasing carbon number. Hence, the range of linearity depends on the conversion efficiency of the bromination reactions. As the conversion efficiency for ethene is extremely low, its range of linearity is not relevant in this context.

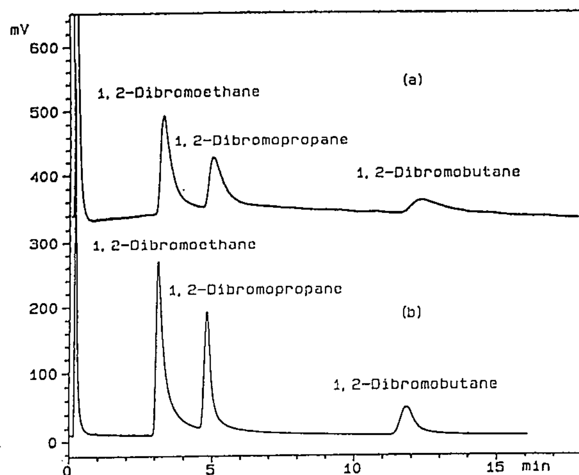
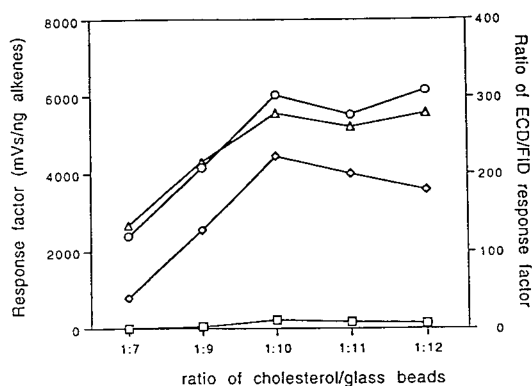


Fig. 5. Chromatograms of dibromoalkanes. GC oven temperature, 30°C; carrier gas flow-rate, 20 ml/min. (a) Tube 1, PBPB; tube 2, cholesterol–glass beads. (b) Tubes 1 and 2, glass beads.



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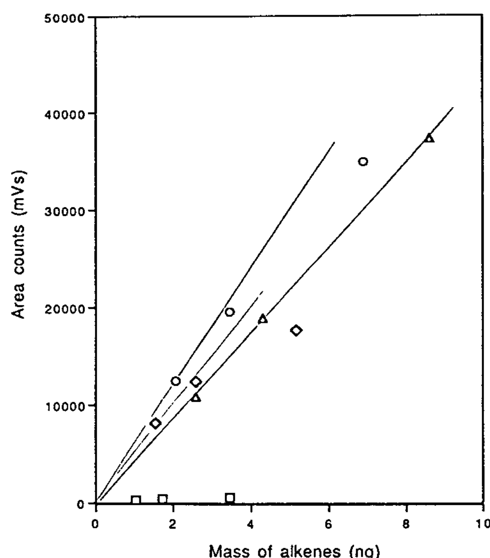

Fig. 7. Linearity of the ECD responses to the brominated alkenes. □ = Ethene; ◇ = propene; ○ = 1-butene; △ = 1-pentene.

3.7. Further discussion on the bromination of alkenes

The above results suggest that the rates of the addition reactions of bromine with alkenes are much lower for the lighter molecules, especially for ethene. This may be due to the low temperature (30°C) at which the alkene bromination reactions were carried out throughout this work. The reaction rates may be improved by using higher reaction temperatures, but this would be inappropriate in this application as the bromine-releasing rate of PBPB is extremely sensitive to temperature change, as is the volatilization rate of cholesterol. Experiments were carried out at 40°C, but the brominated products were not observable against an enhanced background ECD signal of ca. 1000 mV.

An attempt was made to use a higher GC oven temperature without enhancing the Br_2 production rate by removing the tube containing the PBPB (tube 1) from the oven and placing it between points A and B in the carrier gas line shown in Fig. 1. With this configuration, the same amounts of C_2–C_6 alkenes as those used above were injected on to the GC column but, surprisingly, no brominated products appeared. This suggests that the alkene vapour must pass through the PBPB tube to allow sufficient reaction with bromine molecules. It therefore appears that the bromination reactions have to be carried out isothermally at or around 30°C.

Without further development the method may not be suitable for the determination of heavier alkene molecules ($\geq C_6$). In addition, the insertion of the two packed tubes, negates some of the chromatographic advantages given by capillary columns and no additional benefit will result from using very high-resolution columns.

4. Conclusions

ECD has been successfully used for the GC determination of alkenes via on-column bromination reactions. The most important aspects of the method can be summarized as follows:

(i) PBPB can release bromine vapour at room temperature, and its bromine-releasing rate increases with increasing carrier gas flow-rate and, especially, with increasing temperature.

(ii) A methanol-treated cholesterol–glass bead mixture may be used to remove the excess bromine produced by the PBPB. The electrophilic nature of the addition reactions of Br_2 with alkenes requires the use of a polar medium: reaction with cholesterol alone is extremely slow. At the optimum ratio of the cholesterol–glass bead mixture of 1:10, 93% of the bromine released from PBPB can be removed.

(iii) The conversion efficiencies of the individual alkenes to their brominated products is very low for ethene (less than 2%), but increases with carbon number, reaching 74% for 1-butene.

(iv) The sensitivity of ECD to brominated C_3–C_5 alkenes is about 200–300 times higher than that of conventional FID without derivitization. Current detection limits with the GC–bromination–ECD system are higher than those with high-resolution GC–FID. It is expected that by using a two-oven system, that is, the reaction oven (for tubes 1 and 2) and the oven for the separation (for the capillary column), significant improvements in the chromatography, and hence

the detection limits, of the derivitized compounds will be obtained since the temperatures of the two ovens can be controlled separately.

(v) The amount of brominated compound produced from the alkene derivitization procedure increases linearly with increasing mass of analyte, but the range of linearity depends on the conversion efficiency of the bromination reactions.

The method described here allows the extremely sensitive detection of C_3–C_5 alkenes. Further development work (using a two-oven system) will be carried out in the future to allow application of the method to the detection of heavier alkenes ($\geq C_6$) at low concentrations in ambient air; the work to date provides a framework for this purpose.

Acknowledgements

This work arose from initial discussions with Dr. P.G. Simmonds and we gratefully acknowledge his assistance. We thank Dr. S. Breuer for suggesting the use of cholesterol as a bromine filter and for stimulating discussions. We thank the Government of the People's Republic of China, the British Council and the Natural Environment Research Council for funding.

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Gas chromatographic determination of volatile alkenes by on-column bromination and electron-capture detection

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Abstract

A method is described for the GC–electron-capture detection determination of ultra trace quantities of alkenes via on-column bromination reactions. Copper bromide coated onto a non-polar solid support, Gas Chrom Q (100–120 mesh) acted as the bromine source. At a temperature of 90–110°C, steel wool may be used to remove selectively up to 90% of the bromine bleed from the reactor. The conversion efficiency of an alkene to the dibrominated derivative is extremely high, up to 90% for ethene, propene, butene and pentene. The bromination of acetylene is also possible, but is not as efficient.

1. Introduction

Alkenes are a series of hydrocarbons that are of both natural and anthropogenic origin distributed throughout the global environment. They are comparatively unstable compounds in the atmosphere due to their high photochemical reactivity. The low-molecular-mass alkenes are believed to be precursors for peroxyacylnitrates (PANs) [1]. Alkenes are also an urban health hazard owing to their metabolic conversion to genotoxic epoxides [2,3].

The approximate atmospheric concentrations and lifetimes of the more important alkenes are shown in Table 1.

The bulk of the alkenes enter the environment through their widespread industrial use and from the incomplete combustion of fossil fuels. Ethene is the most abundant naturally produced alkene, a product of the ripening of fruit, hence the atmospheric concentration from this source is seasonal.

The accurate determination of alkenes in the atmosphere is hindered by both their extremely low concentrations (<1 ppb) and the detection limit of the current analytical instruments, which are based on gas chromatographic separation coupled to flame ionization detection (FID).

The limit of sensitivity of FID is approximately 50 pg per component, and increases in proportion to the number of hydrogen and carbon atoms present in the analyte. This sensitivity is insufficient to determine the concentrations of alkenes present in ambient air without the use of extensive preconcentration systems.

Electron-capture detection (ECD) is particularly responsive towards electrophores and hence has a high sensitivity towards halogen containing

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Table 1
Approximate atmospheric concentration and lifetimes of alkenes

Alkene	Approximate atmospheric concentration (ppt)	Approximate lifetime
Ethene	150–1000	36 h
Propene	150–1000	10 h
Acetylene	150–1000	Days
1-Butene	50–150	10 h
Isobutene	50–150	5 h
Isopropene	50–150	3 h
1-Pentene	1–50	9 h
cis-2-Butene	1–50	5 h
trans-2-Butene	1–50	4 h
Propadiene	1–50	20 h
1,3-Butadiene	1–50	4 h

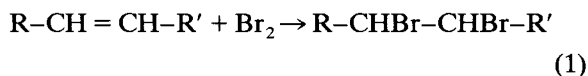
compounds, in the order of 0.05 to 1 pg, with a linear range from 10 ppt to 1 ppb. The device has been used extensively for the monitoring of halogen-containing compounds, e.g. chlorofluorocarbons in the ambient environment [4,5].

However, the ECD response towards the low-molecular-mass alkenes is poor because of the lack of an electrophoric group. If alkenes could be derivatised, through the addition of an electronegative atom (bromine), by an in situ procedure immediately prior to the chromatographic column, this may then permit the near real-time detection of atmospheric alkenes.

2. Experimental

2.1. Principle

The successful use of on-column bromination reactions for the ECD of alkenes requires the derivatization to be very rapid and proceed to completion within a few seconds, otherwise the eluting peaks will be broad and diffuse, so reducing resolution and detection limit. The principle of the present work is based on the reactions of bromine with alkenes to form the dibromoalkane derivatives.

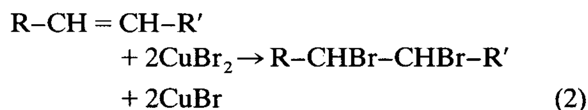


$$R\text{–}CH = CH\text{–}R' + Br_2 \rightarrow R\text{–}CHBr\text{–}CHBr\text{–}R' \quad (1)$$

This is an electrophilic addition reaction.

In most cases it can proceed rapidly and quantitatively at room temperature in a polar liquid phase, without light or catalysis, but this is not compatible with a routine method of gas chromatographic analysis.

For the vapour phase bromination of volatile alkenes, the gas is passed through a heated tube containing a metal halide catalyst coated on an inert support, such as $Cu(II)Br_2$ on activated alumina [6].

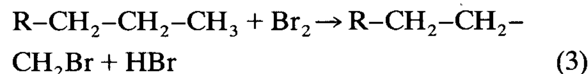


$$R\text{–}CH = CH\text{–}R' + 2CuBr_2 \rightarrow R\text{–}CHBr\text{–}CHBr\text{–}R' + 2CuBr \quad (2)$$

The temperature required for reaction 2 is dependent on the ease with which the respective olefins can be polarised. Hence, hexene undergoes bromine addition at lower temperatures than ethene. Acetylene is particularly difficult to brominate completely due to the extremely high bond energy of the σ and two π bonds. The bromo derivative of acetylene most readily formed, is 1,2-dibromoethene.

The possible role of bromine compounds in the chemistry of the atmosphere has promoted study of the kinetics of the reaction of bromine molecules with hydrocarbons [7]. Work has been carried out on the halogenation of olefins in the liquid phase [8,9], but little information is available on the bromination of very volatile alkenes in the gas or vapour phase [10–12].

Conditions were sought that would maximize the bromine addition reaction with alkenes while minimizing substitution (3) reactions with alkanes naturally present in the complex matrix of an ambient atmospheric air sample.



$$R\text{–}CH_2\text{–}CH_2\text{–}CH_3 + Br_2 \rightarrow R\text{–}CH_2\text{–}CH_2\text{–}CH_2Br + HBr \quad (3)$$

2.2. Instrumentation

System 1

A Pye 104 fitted with an FID system allowed both the efficiency of the bromination phase and

identification of the derivative peaks to be determined. Replacing FID with ECD allowed determination of the detection limit.

Two packed, wide-bore glass columns were used (2 m × 7 mm I.D.), containing a non-polar separation phase [5% (w/w) silicone gum OV-101–Gas Chrom Q (100–120 mesh)]. One column contained 400 mg of the bromination phase packed into the inlet, the second column containing separation phase only, to be used as a reference.

System 2

To enhance the detection limit of each of the brominated alkenes, the GC configuration was altered to incorporate a capillary system. The equipment involved the use of two Pye 104 gas chromatographs coupled via a heated fused-silica capillary tube acting as a gas transfer line. One oven housed a column containing the brominating phase (38 cm × 0.762 mm I.D.), preceding the separation column (25 m × 0.5 mm I.D. support-coated open tubular OV-101), in the second oven. The object of this was to allow separate temperature control of both the brominating reaction phase and chromatographic separation column. The detector used was a ^{63}Ni ECD system.

2.3. Reagents and materials

For preparation of the brominating phase, iron(III) bromide, chromium(III) bromide and pyridine hydrobromide perbromide polymer bound, were purchased from Aldrich (Gillingham, UK). Copper(II) bromide only and copper(II) bromide coated onto alumina 30% (w/w), were obtained from Fluka (Gillingham, UK). Inert chromatographic support phases used were silica, 80–120 mesh, obtained from BDH (Poole, UK) and Gas Chrom Q and Chromosorb W, 100–120 mesh, from Alltech (Deerfield, IL, USA). Aluminium oxide to be used as a support phase for copper(II) bromide was also purchased from BDH.

Materials used for the removal of bromine bleed were steel wool (fine grade), from a commercial source and iron powder (pure) from BAS (Middlesborough, UK). Copper turnings and silver wool were purchased from Alltech. Squalene, copper(II) nitrate and thallium(I) nitrate were obtained from BDH.

2.4. Preparation of alkene standards

For the preparation of low concentration gaseous mixtures, a 1000 ppm (v/v) alkenes standard (Scotty, Alltech) was used. The mixture contained equal concentrations of ethene, propene, but-1-ene, pent-1-ene and hex-1-ene. For the preparation of 10 and 1 ppm (v/v) standards, two 40-ml glass bottles were filled with nitrogen while submerged in water and stoppered with gas-tight seals. Using a gas syringe, 400- and 40-μl samples of the Scotty standard were injected into the bottles, preparing 10 and 1 ppm (v/v) standards, respectively.

Standard solutions (1 ppm and 1 ppb) of the respective dibromoalkane derivatives were prepared by serial dilution, employing doubly distilled hexane from an all-glass system.

2.5. Preparation of bromination phases

Thermogravimetric analysis (TGA) of the metal bromides, iron(III) bromide, chromium(III) bromide and copper(II) bromide, was carried out using a Stanton Thermobalance TR-1, capable of reaching 900°C. The results indicated the copper halide to be most suitable for the purpose of a brominating phase. Copper(II) bromide is a black crystalline salt but reduction at 150°C to copper(I) bromide is accompanied by a colour change from black to cream.

A series of brominating phases were prepared by coating copper(II) bromide onto: 100–120 mesh Chromosorb W (50:50, w/w); 100-120 mesh Gas Chrom Q (50:50, w/w); aluminium oxide (50:50, w/w); 80–120 mesh silica (20:80, w/w).

The same method of preparation was used for each phase.

Copper(II) bromide was dissolved in hot methanol and added to the support phase, preheated to 100°C. Most of the excess methanol boiled off immediately, while the remainder was

removed under vacuum. The phase was then conditioned under nitrogen for 24 h at 60°C.

Pyridine hydrobromide perbromide polymer bound, and copper(II) bromide coated on alumina (30:70, w/w), were purchased in a state ready for direct use as brominating phases.

2.6. Preparation of a phase to remove bromine bleed

Three metals, copper turnings, silver wool, iron powder and iron wool, were examined as scrubbing phases to adsorb the bromine bleed from the brominating phase. Other possible scrubbing phases were also investigated but required preparation using simple manipulative procedures.

(i) Copper coated onto Chromosorb W (25:75, w/w).

(ii) Copper coated onto Gas Chrom Q (25:75, w/w).

(iii) Thallium(I) nitrate coated onto Chromosorb W (25:75, w/w).

(iv) Thallium(I) nitrate coated onto Gas Chrom Q (25:75, w/w).

(v) Silver coated onto Chromosorb W (25:75, w/w).

(vi) Silver coated onto Gas Chrom Q (25:75, w/w).

(vii) Copper(I) bromide coated onto Gas Chrom Q (50:50, w/w).

(viii) Squalene coated onto Gas Chrom Q (20:80, w/w).

Preparation of phases i and ii

Copper(II) nitrate was dissolved in water and a slurry prepared with the support phase. The slurry was placed in a muffle oven at 450°C to decompose the copper(II) salt and form the oxide. This phase was then cooled and packed into a glass column which was placed in a GC oven at 150°C, with hydrogen passing through it. Coupling the column to a FID system and introducing air at the detector allowed the hydrogen to be burnt as the copper oxide was reduced.

Preparation of phases iii and iv

Thallium(I) nitrate was dissolved in water and a slurry prepared with the support phase. This mixture was then dried in an oven at 110°C.

Preparation of phases v and vi

These phases were prepared using silver(I) nitrate, preparing a slurry with the support phase and heating it to 450°C, producing the desired silver phase.

Preparation of phase vii

The phase was prepared by heating the phase copper(II) bromide/Gas Chrom Q, at 150°C for approximately 24 h until the colour change of the phase from black to cream indicated the formation of copper(I) bromide.

Preparation of phase viii

Squalene was dissolved in acetone and a slurry prepared with the support phase. The solvent was removed under a vacuum while heated at 60°C. Further conditioning was carried out at 85°C for 4 h.

3. Results and discussion

3.1. The bromination phases; general observations

The thermal gravimetric analyses of the bromination phases mentioned indicated the temperatures at which these metal bromides were reduced. This provided an estimate of the decomposition temperature and the quantity of bromine bleed caused by an increase in temperature. Such information provided an assessment of the suitability of each metal halide for the purpose of on-column bromination. The results are summarized in Fig. 1.

Chromium(III) bromide undergoes steady reduction at temperatures beginning below 100°C up to 310°C and was thus expected to cause problems with continuous bromine bleed.

Iron(III) bromide appears to be reduced at 250°C, while below this temperature little bromine appeared to be released. The metal

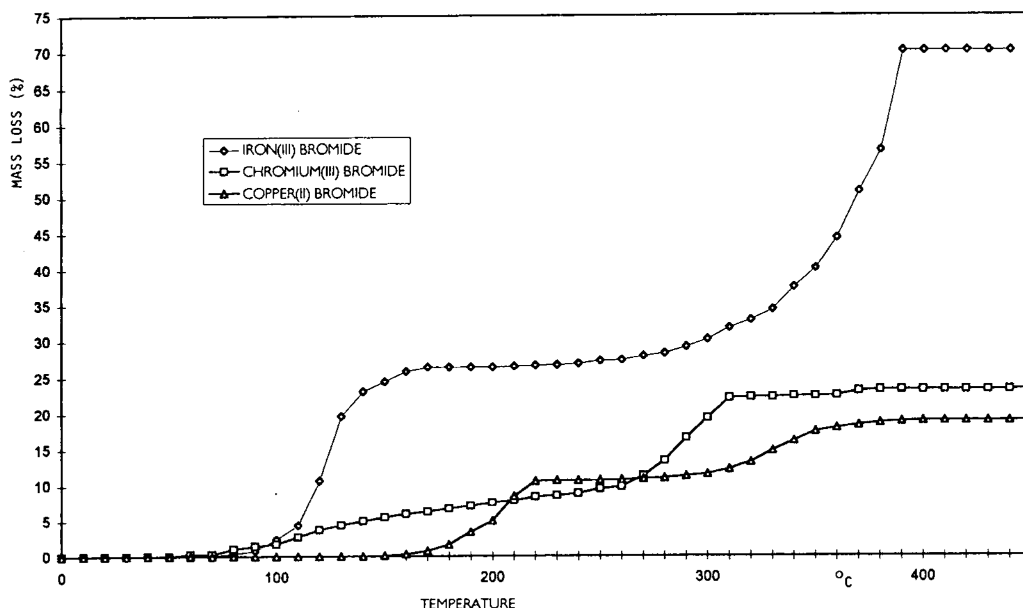


Fig. 1. TGA of (□) chromium(III) bromide, (◇) iron(III) bromide and (△) copper(II) bromide. Graph of temperature against % mass loss of metal halides.

halide, when supported on Gas Chrom Q or Chromosorb W, 100–120 mesh (50:50, w/w), did not afford bromine addition of the alkenes. It was apparent that a series of substitution reactions were occurring as shown by the elution of compounds with very long retention times, when compared to those of the dibrominated haloalkane standards used for peak identification purposes.

Copper(II) bromide decomposes and is reduced at 150°C with some bromine bleed below this temperature. The metal halide, when supported on Chromosorb W, was seen to bring about bromine addition. The efficiency of bromination can be increased by using a less polar support phase, Gas Chrom Q 100–120 mesh. However, when aluminium oxide was used as a support phase for the metal bromide, no evidence of bromination was observed. Although the dibromo derivative was probably formed on passing through the reactor, dehydrobromination also occurred, resulting in reformation of bromine and the respective alkene [13]. This was confirmed by passing a dibromoethane vapour sample through the brominating phase, which resulted in two peaks with the retention times of bromine and ethene.

Silica, 40–100 mesh, coated with the copper(II) bromide (80:20, w/w), was an effective bromination reagent. However, considerable adsorption of the brominated derivatives was evident due to a substantial increase in their peak widths, thought to be caused by the incomplete coating of silica.

Pyridine hydrobromide perbromide, polymer bound, was also tested as a bromination reagent [14,15], but resulted in continuous bromine bleed at temperatures above ambient. Derivatization of the alkenes using system 1 proved possible at 30°C, although such a low temperature resulted in generally poor chromatography, with inadequate detection limits. Increasing the temperature of this phase above 35°C improved the yield of the dibromoalkanes, but resulted in a dramatic rise in the level of bromine bleed. The bleed made the use of this particular brominating material difficult when using system 1 fitted with ECD. None of the scrubbing phases tested had adequate capacity to remove selectively the level of excess bromine, and yet at the

same time not adversely effect the elution of the dibromoalkane derivatives.

From observations made using the various bromination phases, copper(II) bromide coated onto Gas Chrom Q 100–120 mesh appeared to be the most promising phase for vapour phase bromination.

3.2. Using copper(II) bromide coated onto Gas Chrom Q (50:50, w/w) as the brominating phase

In order to check that bromine was being released from the copper(II) bromide phase, 100 mg were packed into a stainless-steel column, 51 cm × 0.762 mm I.D., and placed in the first GC oven (system 2) prior to the separation phase in the second oven. The temperature of the GC oven housing the bromination phase was increased incrementally from 55 to 96°C. The ECD standing current decreased linearly as the temperature of the copper(II) bromide phase was increased, indicating that the release of bromine was temperature dependent.

When using 100 mg or less of the copper(II) bromide phase, 80% of the ECD standing current (1.00 nA) was maintained at temperatures up to 96°C. Thus, if bromination of the alkene vapours was possible at or below this temperature, the problem of excess bromine bleed would be ameliorated. When using amounts of bromination phase in excess of 150 mg, a scrubbing phase is essential to remove the bromine bleed, and thus facilitate a good ECD standing current.

The release of bromine bleed was generally independent of the nitrogen carrier gas flow-rate. A decrease in the ECD standing current was observed during the initial moments when increasing the flow-rate, however the original value was soon restored.

3.3. Identification of the haloalkane derivatives

Using FID (system 1) allowed the identification of the derivatives to be made without regard to the bromine bleed. Nitrogen carrier gas flow-rates were adjusted to give identical retention times for the elution of a reference gas, methane, through each column. The alkenes were injected onto the column containing the bromination phase, also a standard solution of the respective haloalkanes (1 ppm) was injected onto the reference column for identification purposes.

3.4. Determination of the efficiency of bromine addition

Again using FID (system 1), integrated peak areas of both the unreacted alkene and brominated derivative allowed an indication of the efficiency of the brominating phase to be determined at different temperatures. Due to the lack of sensitivity of FID, the procedure meant that relatively high concentrations of alkene vapours had to be used to enable detection. Hence, a true measure of the efficiency of a very small amount of the brominating phase could not easily be determined using this instrumental system.

The brominating efficiency of the copper(II) bromide/Gas Chrom Q was found to be dependent upon two parameters: (i) temperature of the copper(II) bromide phase and (ii) contact time between the olefin and the brominating phase.

(i) There appears to be a minimal temperature, above which bromine addition to an individual olefin would occur, and a maximum temperature that the brominating phase may be employed before bromine substitution also occurred. From the preliminary investigations with the system 1 configuration, using 400 mg of bromination phase, temperatures in excess of 50°C but below 125°C were suitable for conversion of the C_2–C_5 olefins to dibromoalkanes. However, a more detailed investigation was performed using a system 2 experimental arrangement, employing 70 mg of bromination phase with no scrubbing phase present. The results (Fig. 2) show maximum bromination of propene requires temperatures in excess of 85°C. The optimum temperature for the bromination of butene appears to be in the region of 80°C, with any increase resulting in bromine substitu-

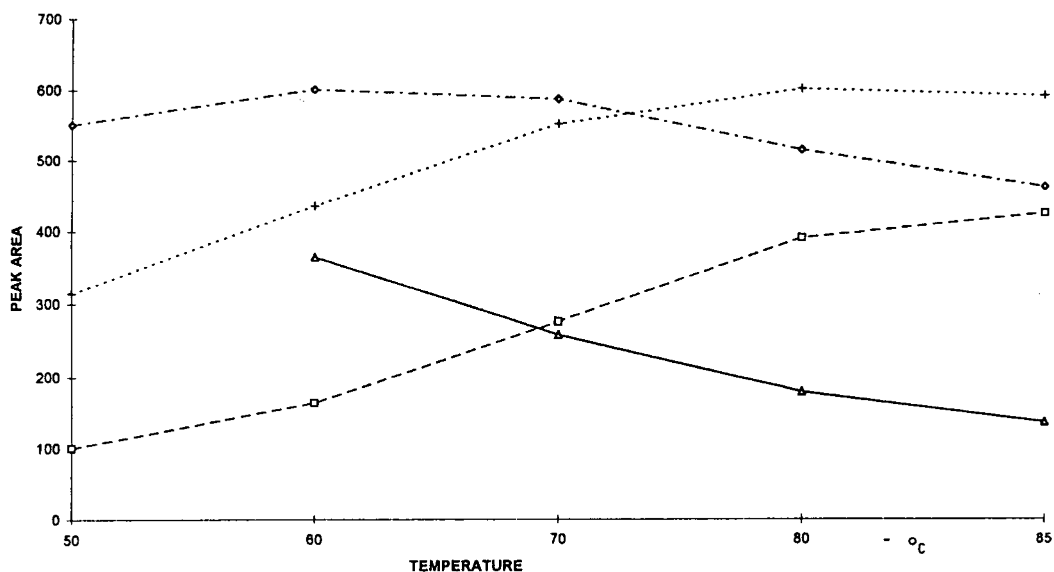


Fig. 2. Using system 2. Graph showing the effect of the temperature of copper(II) bromide phase on the addition of bromine to olefins. From this, the optimum temperature of the phase for bromine addition is indicated. □ = Dibromopropane; + = dibromobutane; ◇ = dibromopentane; △ = dibromohexane.

tion reactions, causing a loss in peak area of the dibromo derivative. However, for pentene a significantly lower temperature, around 60°C, allowed maximum conversion to dibromopentane with substitution occurring above 70°C. With hexene, temperatures below 60°C could not be employed since this did not allow ready elution (<1 h), of the corresponding brominated compound from either the bromination phase or the capillary column. Clearly substitution reactions became significant for hexene derivatization, shown by the successive decrease in peak area with an increase in temperature of the bromination phase.

(ii) Increasing the contact time between the bromination phase and the olefin also improved the reaction efficiency. As with the temperature of the bromination phase, an optimum contact time was sought that allowed maximum bromine addition to the lowest-molecular-mass olefins without interference of substitution reactions for the heavier ones.

Using system 1 with 400 mg of the bromination phase at 90°C, the percentage bromination of ethene as a function of carrier gas flow-rate was examined.

The percentage of ethene dibrominated decreased linearly as the carrier gas flow-rate was increased. The optimum flow-rate for 80% bromination of ethene was 6.5 ml/min, equivalent to a contact time between the ethene and bromination phase of 15 s.

Using system 2 the maximum amount of copper(II) bromide phase used to maintain a good ECD standing current, without the need of a bromine scrubbing phase, was 100 mg. Incremental changes in the carrier gas flow-rate (2.5–14.0 ml/min) allowed the effect on the bromination of olefins to be studied. Fig. 3 portrays these results. The conversion of ethene to 1,2-dibromoethane was too low to be successfully measured, particularly at the higher flow-rates, thus only data for propene and higher olefins is given. For propene the flow-rate must be 2.5 ml/min or less to allow maximum conversion, this being equivalent to a contact time between the propene and bromination phase of 5.6 s. At shorter contact times, i.e. higher flow-

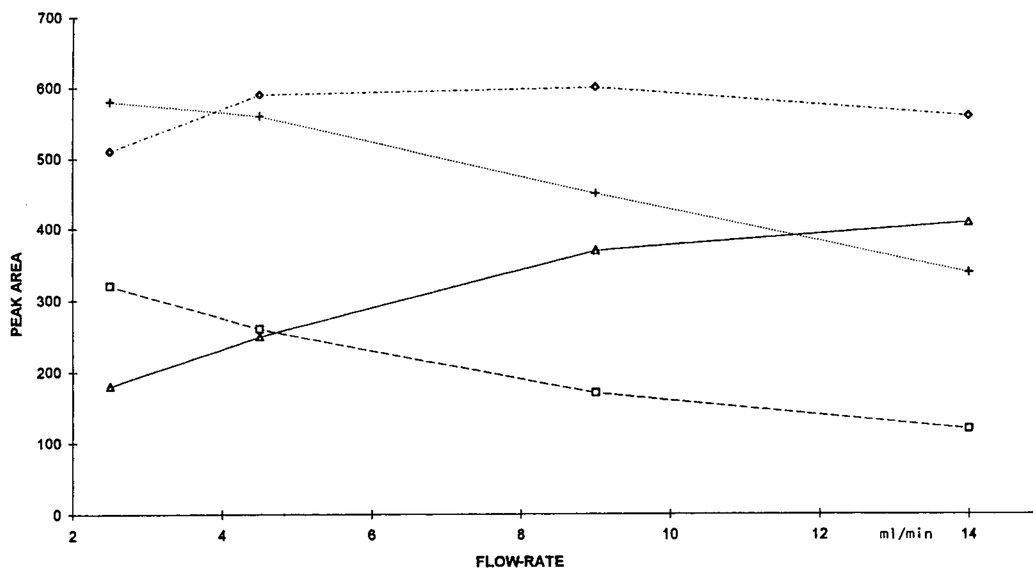


Fig. 3. Using system 2. Graph showing the effect of the carrier gas flow-rate, i.e. contact time between olefins and bromination phase, on the efficiency of bromine addition to olefins. From this the optimum carrier flow-rate is indicated. Symbols as in Fig. 2.

rates, the conversion of propene was less efficient. A similar result is seen for the conversion of butene. However, there is little change in the conversion of pentene which is essentially complete with a contact time of 3.2 s. Longer contact times for hexene allow substitution reactions to occur, thus causing a decrease in the amount converted to dibromohexane, producing a fall in the area of the sought for peak. Thus as contact time is reduced, opportunities for substitution reactions to occur are minimized.

Therefore, temperatures in excess of 70°C, with a contact time of approximately 5 s, provides a reasonable compromise for optimum conversion of all the olefins. While these conditions will not allow complete bromination of ethene, they are optimum for the conversion of propene and olefins of higher molecular mass, allowing minimal substitution. Such a performance is satisfactory for clean ambient air samples since, the level of ethene is very much higher than the levels of the higher-molecular-mass olefins, which are principally derived from vehicular emissions. Where urban air samples are to be investigated, the lowering of the bromination phase temperature together with an increase in carrier gas flow-rate, could be beneficial.

3.5. Determination of the detection limit of olefins

For the determination of detection limits of brominated alkenes, system 1 was used, but fitted with ECD in place of FID. Detection of the derivatives was restricted due to the reduced ECD standing current caused by the continuous bromine bleed from the brominating phase. This problem was alleviated by the use of phases prepared to remove the bromine selectively. The bromine scrubbing phase was packed in the glass column after the brominating phase, but prior to the separation phase and the detector.

Due to the relatively low flow-rate of carrier gas required and the presence of the scrubbing phase, the chromatography because of band spreading, did not allow good detection limits to be achieved. The values are ethene 2 $\mu l \times 10$ ppm (v/v) (25 pg), propene 1 $\mu l \times 10$ ppm (v/v) (19 pg), butene 1 $\mu l \times 10$ ppm (v/v) (25 pg).

Using system 2 with 70–100 mg of the cop-

per(II) bromide phase at temperatures between 55 and 85°C, allowed detection of very low concentrations of C_2–C_5 olefin vapours.

Thus with 70 mg of copper(II) bromide phase packed into a 38 cm × 0.762 mm I.D. stainless-steel tube, heated to 68°C and a nitrogen carrier gas flow of 2.5 ml/min (contact time 4.2 s) the detection limits for olefin vapour samples derivatized were (see Fig. 4): ethene 1 μl × 1 ppm (v/v) (1.3 pg), propene 1 μl × 1 ppm (v/v) (1.9 pg), butene 1 μl × 1 ppm (v/v) (2.5 pg), pentene 1 μl × 1 ppm (v/v) (3.1 pg). The detection limit of acetylene was approximately <5 μl × 10 ppm (v/v) (60 pg).

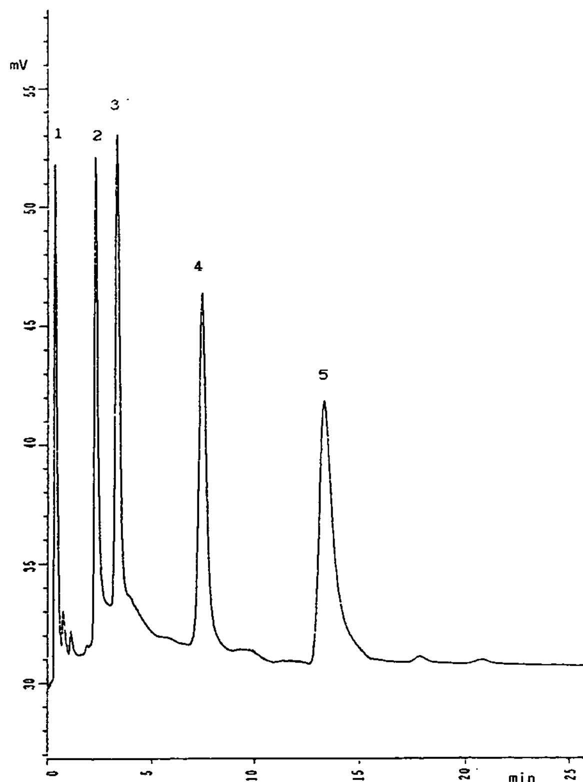


Fig. 4. Using system 2. Chromatogram showing the separation of the brominated olefins from an injection of 5 μl × 1 ppm (v/v) Scotty mix (ethene, propene, butene and pentene). Bromination phase, 70 mg copper(II) bromide phase heated at 68°C and the separation column oven at 100°C. Peaks: 1 = oxygen; 2 = dibromoethane; 3 = dibromopropane; 4 = dibromobutane; 5 = dibromopentane.

Efficiency of the bromination of ethene is limited by the percentage derivatization.

Using 100 mg of copper(II) bromide phase packed into a 51 cm × 0.762 mm I.D. stainless-steel tube, heated to 75°C and a nitrogen carrier gas flow of 5 ml/min, it was evident that the percentage of ethene derivatised increased. An injection of 5 μl × 1 ppm (v/v) of ethene and propene in nitrogen gave two well separated peaks of approximately equal area, indicating efficient bromination of ethene. However, under these conditions, an injection of 5 μl × 1 ppm (v/v) of the Scotty olefin mixture resulted in the elution of only two derivatives, dibromoethane and dibromopropane, the remaining olefins undergoing extensive bromine substitution.

3.6. Reproducibility of detection limits

The relative standard deviation values calculated from the derivatization of 10 μl × 1 ppm alkene standards were: ethene 0.61%, butene 0.71%, propene 0.69%, pentene 0.72%.

3.7. Bromine bleed scrubbing phase

All of the scrubbing phases listed in the Experimental section were found to adsorb the bromine bleed with varying degrees of efficiency. Many of them were also found to adsorb both the olefin derivatives or standard mixtures of the respective halocarbons. Only steel wool, iron powder and squalene were found to discriminate between the bromine bleed and the derivatives produced.

Steel wool was found to selectively remove the bromine bleed, but allowed the elution of the dibromoalkane derivatives at temperatures between 90 and 110°C without causing significant degradation or loss in signal compared to dibromoalkane standards. Injection of dilute dibromoalkane standard solutions allowed the detection of 2 pg of dibromoethane and dibromopropane. The use of this scrubbing phase had the disadvantage of increasing peak widths of the eluting compounds, which in turn reduced detection limits.

The use of pure iron powder as a scrubbing

phase proved possible, but the work was not easily reproduced. The reason for this was thought to be due to the changing degree of oxidation the iron powder underwent while in use or storage. When the iron powder was used initially, it proved too efficient, in that both the bromine bleed and the haloalkane derivatives were adsorbed. After 1 h of operation, only bromine vapour was removed, with good recoveries of the dibromocompounds. After approximately 12 h the bromine vapour broke through as seen by a reduction of the ECD standing current, with consequent loss in detector performance. Repetition of this process using a fresh portion of the iron powder was possible but as the iron powder in the bottle aged (>1 month), rapid breakthrough of the bromine vapour became evident when it was used. Thus, as the degree of oxidation of the iron powder is critical, no further investigation was carried out.

Squalene coated onto Gas Chrom Q 100–120 mesh (20:80, w/w) was able to remove the bromine bleed, while allowing the elution of the derivatives. The main disadvantage was the resulting increase in peak width of the derivatives. The squalene was obviously acting as a further stationary phase towards the dibromoalkanes, both retaining and diffusing the peaks. Increasing the temperature of this phase above 100°C caused severe bleeding of the squalene, hence limiting its use.

4. Conclusions

The use of a copper(II) bromide reagent for the addition of bromine to alkenes in the vapour phase has been demonstrated. The process is efficient with conversion rates normally greater than 80% for the C_2–C_5 olefins. Coupling this bromination reagent with an electron-capture detector permits the direct determination of atmospheric olefins at the 2 pg level.

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Solid-phase extraction and gas chromatographic–mass spectrometric identification of degradation products from enhanced environmentally degradable polyethylene

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Abstract

A solid-phase extraction (SPE) method using unbonded silica (Si) and silica bonded with octadecyl (C_{18}) or aminopropyl (NH_2) groups was developed to separate into five fractions the highly complex mixture of low-molecular-mass degradation products formed from degradable polymers. Application of the method to polyethylene modified with starch and/or a pro-oxidant system, degraded for 30 weeks in water at 95°C, enabled the identification by GC–MS of over three times as many products as when the sample was prepared by liquid–liquid extraction. Over 60 degradation products were identified in each sample; mainly dicarboxylic acids, monocarboxylic acids and *n*-alkanes. In addition, several lactones, aldehydes and alcohols were detected.

1. Introduction

Environmental concerns have promoted the development of degradable plastics. To understand fully the environmental impact of such polymers, their degradation products need to be established. Environmental degradation involves not only biotic factors but also abiotic factors such as sunlight, heat, moisture and oxygen which give rise to products varying in composition, molecular mass and volatility.

The degradability of relatively inert synthetic polymers such as polyethylene (PE) can be significantly improved by incorporating additives that possess a sensitivity towards environmental degradation factors. We have studied PE containing native granular corn starch and a pro-oxidant formulation consisting of a styrene–butadiene copolymer and manganese stearate [1,2]. The first degradation step in such materials is an oxidation which can be triggered by heat, UV radiation, water etc. This oxidation may be enhanced by biodegradation of the starch granules, which produces voids leading to a greater surface/volume ratio and a greater oxygen permeability. In a secondary process, micro-organisms may utilize the abiotic degradation products and low-molecular-mass polymer in anabolic and catabolic cycles [3]. The determination of the abiotic degradation products is thus an important first step towards establishing the products resulting from environmental degradation.

Degradation products from PE have been the focus of several studies but attention has mostly been directed towards determining the volatile

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products formed during short-term thermo-oxidation or pyrolysis [4–7]. Products resulting from thermo-oxidation at lower temperatures, i.e. in the solid state, have also been reported [8–12].In summary, PE yields a complex mixture of products such as water, CO, CO_2, hydrocarbons, carboxylic acids, ketones, aldehydes and cyclic ethers. If other components such as starch and pro-oxidants are incorporated into the formulation, their intermediate breakdown products and possible interactions further increase the complexity.

We have previously attempted to identify the degradation products of PE–starch–pro-oxidant materials resulting from aging at 95°C in aqueous environments [13].The aqueous phase was liquid–liquid extracted and subsequently subjected to GC–MS analysis. Highly complex chromatograms were obtained where many small peaks were obscured by larger ones which consequently limited the number of identifiable compounds. It was thus clear that it is desirable to develop some means of separating the degradation products prior to analysis by GC–MS.

Solid-phase extraction (SPE) has in recent years gained in popularity as a sample preparation technique due to its ability to selectively extract and isolate compounds of interest from various samples. The technique has found particular use in the selective extraction of steroids, lipids, peptides, drugs and pharmaceuticals from matrices such as plasma, blood, urine and culture media [14]. Bonded phase sorbents of silica gel with a wide variety of chemical functionalities are commercially available. They exhibit unusual dimensional stability, i.e. they do not swell or shrink, in virtually all organic solvents and may be used for extractions over a pH range of 1 to 14 [15]. These features make them attractive for the purpose of pre-separating complex mixtures of degradation products in aqueous matrices such as water or basal salt media.

In the present work, an SPE scheme employing bonded phase silica columns of octadecyl, aminopropyl and unbonded silica was developed for the class fractionation of degradation products formed during prolonged (30 weeks) thermal ageing in water at 95°C. The samples were PE containing pro-oxidants and/or corn starch. The extracted products were further separated and identified by GC and/or GC–MS. Products still remaining in the samples were monitored by Fourier transform infrared spectroscopy (FT-IR) at intervals throughout the degradation period. Changes in molecular mass were followed by high temperature size-exclusion chromatography (HTSEC).

2. Experimental

2.1. Materials and degradation procedure

Low-density polyethylene (LDPE) films (30 μm) were made by a conventional blown film process using a Betol extruder with a 25 mm screw of length/diameter ratio 20:1, a blow-up ratio of about 2.5:1 and a die temperature of 185°C. The polymer was a conventional LDPE grade of MFI 2 acquired from ATO (France) which incorporated a conventional thermal stabilizer of undisclosed composition. Pro-degradant additives were incorporated into the LDPE matrix in the form of a master batch in the amount of 20% consisting mostly of corn starch, styrene–butadiene copolymer (SBS), manganese stearate and linear low-density polyethylene (LLDPE) [16,17]. In some samples, the starch was omitted. The samples were all prepared in collaboration with Epron Industries. The degradation took place in water at 95°C for a period of 30 weeks. Samples (2 g) were placed in glass flasks filled with 100 ml distilled water, pH 7.0, equipped with condensors and immersed in a temperature-controlled hot water bath.

2.2. SPE procedure

After the chosen degradation time 25 ml water phase were withdrawn from each flask and extracted by an SPE scheme in portions of 5×5 ml. The sorbents used for extraction were three different chemically functionalized silica gels: silica bonded to octadecyl chains (C_{18}), silica bonded to aminopropyl chains (NH_2) and unbonded silica (Si), each packed in portions of

100 mg in disposable syringe carts. The C_{18} and Si columns were of the Isolute type from International Sorbent Technology (IST) while the NH_2 columns were of the Bond Elut type from Varian. The SPE method was developed using relevant standard compounds that had been chosen on the basis of earlier degradation studies on the same materials [13]. The standards used were fatty acids (C_4, C_6, C_9, C_{12} and C_{18}), benzoic acid, *n*-alkanes (C_9, C_{14}, C_{19} and C_{30}), 1-alcohols (C_6, C_8 and C_{14}), aldehydes (C_7, C_9 and C_{14}), 3-heptanone, 5-methyl-3-heptanone, 2-nonanone, 2-heptadecanone and γ-butyrolactone. Fig. 1 presents the SPE scheme for separation of these products. The procedure was as follows. The pH of the sample was adjusted to about 2. The C_{18} column was activated with methanol (2 ml) followed by water (2 ml, pH 2). The sample was then allowed to penetrate the reversed-phase bed merely by gravitation to ensure a sufficiently low flow-rate to allow interactions by the analyte and the sorbent to be established (this was also done with all the subsequent solvent passages through the sorbent). After very light drying by vacuum analytes were displaced by passing hexane (2 ml) through the column followed by methanol (2 ml). The hexane fraction was then passed through an NH_2 column that had been activated by hexane (2 ml). The column was washed with additional hexane (1 ml), then chloroform (1 ml) followed by final elution with 2% acetic acid in diethyl ether (3 ml), the last step according to Kaluzny et al. [18]. The combined hexane fractions (hexane fraction 2 + hexane wash) were passed through an Si column activated with hexane (2 ml). The column was washed with an additional ml of hexane and analytes eluted with dichloromethane (2 ml). The fractions of chloroform, diethyl ether, hexane 3 and dichloromethane were concentrated to 80 μl and the methanol fraction to 0.4 ml. The fractions were then subjected to GC–MS analysis.

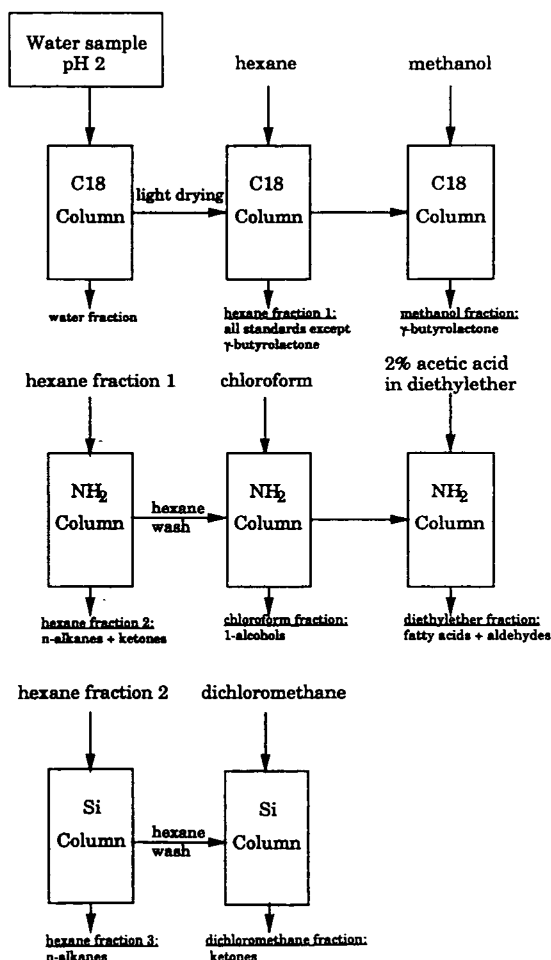

Fig. 1. Solid-phase extraction scheme for the extraction and fractionation of degradation products in aqueous matrices. Standard compounds used for the development of the scheme were fatty acids (C_4, C_6, C_9, C_{12} and C_{18}), benzoic acid, *n*-alkanes (C_9, C_{14}, C_{19} and C_{30}), 1-alcohols (C_6, C_8 and C_{14}), aldehydes (C_7, C_9 and C_{14}), 3-hepatanone, 5-methyl-3-heptanone, 2-nonanone, 2-heptadecanone and γ-butyrolactone.

2.3. GC–MS

Degradation products were separated and identified by means of a Hewlett-Packard 5890 gas chromatograph, equipped with a 60 m × 0.32 mm DB-5 column, film thickness 0.25 μm, coupled to a Model VG-70-250SE mass spectrometer. The oven temperature was held at 50°C for 1 min, raised by 5°C/min to 310°C and held there for 10 min. The carrier gas was helium. The injector temperature was 250°C and the interface was maintained at 300°C. Electron impact spectra were obtained with an electron

energy of 70 eV and an ion source temperature of 200°C. The resolution and scan rate were 1000 and 0.5 s/scan respectively. Compounds were identified by comparison with the national institute of standards and technology (NIST) data base.

2.4. GC

Chromatograms of degradation products and standard compounds were measured with a Varian 3400 gas chromatograph equipped with a J & W 30 m × 0.32 mm DB-5 column, film thickness 0.25 μm, and with a flame ionization detector. The oven temperature was held at 50°C for 1 min, raised by 5°C/min to 310°C and then held there for 10 min. Nitrogen was used as carrier gas. Identifications of compounds were made, complimentary to those by MS, by comparison of retention indices (relative diisooctyl phthalate or eicosane) with those of standard compounds that were analyzed by the same temperature program as the samples. For compounds identified solely by GC, relevant standard compounds were chosen on the basis of information obtained from the mass spectra.

2.5. Size-exclusion chromatography (SEC)

A Waters 150C HTSEC apparatus equipped with two PLgel 10 μm mixed-B columns was used to measure changes in molecular masses and distributions. The mobile phase was 1,2,4-trichlorobenzene (TBC) at 135°C and the flowrate was 1 ml/min. Calibration was performed according to polystyrene standards.

2.6. FT-IR

FT-IR analyses were performed on a Perkin-Elmer 1725x. In the IR spectra special interest was focused on the carbonyl region. Carbonyl absorbance at 1718 cm^{-1} was measured relative to the CH_2 scissoring peak at 1463 cm^{-1}.

3. Results and discussion

Both the samples, with and without starch, demonstrated a considerable loss of molecular mass during degradation as measured by SEC (Fig. 2). The samples without starch showed a slightly larger degradation. The reductions in molecular mass were accompanied by the formation of low-molecular-mass degradation products, some of diffused from the samples to the surrounding aqueous environment. These were separated by means of SPE and further characterized by GC–MS.

The aim of the SPE procedure was to achieve a class fractionation of the different products in order to facilitate the identification of the individual compounds. The five different fractions obtained by the SPE are those soluble in diethyl ether, hexane, methanol, chloroform and dichloromethane. Since the dominating products had previously been found to be fatty acids and hydrocarbons, emphasis was put on separately isolating these and they were found in the diethyl ether fraction and hexane fractions, respectively. Other products such as ketones and aldehydes were not as neatly class separated due to the

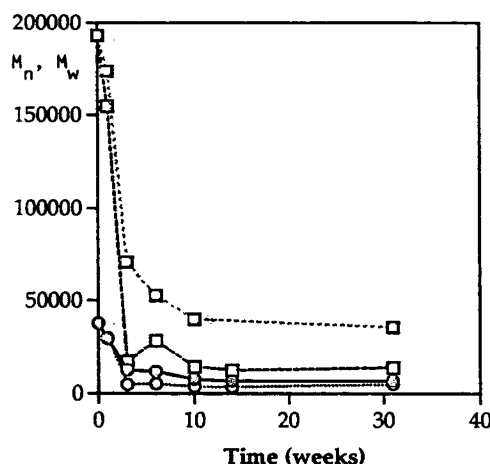

Fig. 2. Molecular mass (number average, M_n and weight average, M_w) as a function of time of degradation in water at 95°C. **O** = LDPE + pro-oxidant + starch, M_n; ○ = LDPE + pro-oxidant, M_n; **□** = LDPE + pro-oxidant + starch, M_w; □ = LDPE + pro-oxidant, M_w.

wide range of differing polarities and some compounds were distributed in two fractions rather than one. The main part, however, was in one of the fractions as shown in Fig. 1. Fig. 3 shows typical gas chromatograms of a liquid–liquid extracted sample and of the five different fractions from the SPE of the same sample. This separation of the products enables each individual fraction to be adequately concentrated and reveals compounds which in the total product mixture are obscured by other compounds. A knowledge of the kinds of products to expect (in terms of polarity) also simplified the identification process. Earlier [13], when employing liquid–liquid extraction (LLE), we were typically able to identify about 20 degradation products in each sample, regardless of the actual number and amounts of products present. In fact, we have found that increasing the degradation time and thereby the amount of products forme often leads to a decrease in the number of identifiable compounds due to the additional complexity. The SPE method allowed us to identify about three times as many products in each sample as was possible in our earlier work employing LLE as the isolation step. The number of identifiable compounds in the LLE fraction was in this study very low. The chromatogram was similar to that of the methanol fraction from the SPE method, but only a few shorter fatty acids and dicarboxylic acids, benzoic acid and a series of furanones were identified. The SPE method thus proved to be a powerful separation method.

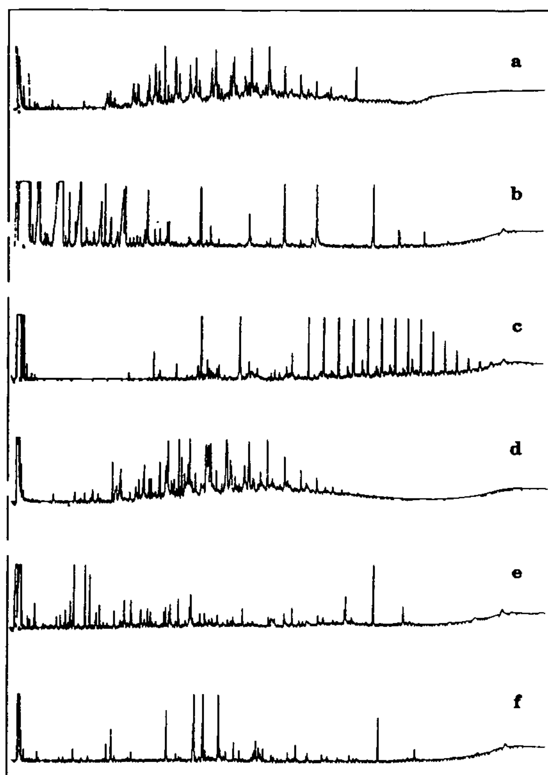

Fig. 3. Examples of GC chromatograms after liquid–liquid extraction (LLE) or solid-phase extraction (SPE), according to the scheme in Fig. 1, of degradation products present in water. The sample was LDPE + pro-oxidant degraded for 30 weeks in water at 95°C. (a) LLE with dichloromethane, (b) SPE, diethyl ether fraction, (c) SPE, hexane fraction, (d) SPE, methanol fraction, (e) SPE, chloroform fraction, (f) SPE, dichloromethane fraction.

The products identified in the different fractions are presented in Table 1. In the diethyl ether fraction mostly fatty acids ranging from C_6 to C_{22} were detected. Emulsions were often formed when using LLE, which is ascribed to the presence of fatty acids or other partly water-soluble compounds, and this reduced the extractability. Fatty acids were found in about the same yields from samples with starch and/or pro-oxidant. These compounds are one of the main types of degradation products formed, as established by earlier studies. A few branched acids were also identified. The formation of carboxylic acid is proposed as the result of various reactions involving alkoxy or peroxy radicals as precursors [8–11,16]. These radicals are formed in the initial autoxidation steps of the material. A possible route of carboxylic acid formation is a radical decomposition of hydroperoxide groups via aldehyde groups (Fig. 4) [9]. This is supported by the fact that only trace amounts of octanal and nonanal were found since the aldehydes serve only as intermediate products and are quickly further oxidized. Benzoic acid and benzaldehyde were identified from both sample types. These are known thermal degradation products from polystyrene [17,18], hence, we

Table 1
Compounds formed and identified after degradation of LDPE with starch and/or pro-oxidant in water at 95°C for 30 weeks

Compound	LDPE + pro-oxidant + starch	LDPE + pro-oxidant
Diethyl ether fraction		
2,2-Dimethylpropanoic acid[a]	−	+
Hexanoic acid[a,b]	+	+
Heptanoic acid[a,b]	+	+
2-Ethylhexanoic acid[a]	+	+
Benzoic acid[a,b]	+	+
Octanoic acid[a,b]	+	+
Nonanoic acid[a,b]	+	+
Decanoic acid[a,b]	+	+
Undecanoic acid[a,b]	+	−
Dodecanoic acid[a,b]	+	+
Tetradecanoic acid[a,b]	+	+
Pentadecanoic acid[a,b]	−	+
Hexadecanoic acid[a,b]	+	+
Octadecanoic acid[a,b]	+	+
Eicosanoic acid[a,b]	+	−
Docosanoic acid[a,b]	+	−
Phthalic acid[a,b]	−	+
Benzaldehyde[a,b]	−	+
Octanal[a,b]	−	+
Nonanal[a,b]	+	+
2,6-Di-*tert.*-butyl-4-methylphenol[a,b]	+	+
Butyl-8-methylnonyl phthalate[a]	+	−
Hexane fraction		
Dodecane[a,b]	−	+
Tridecane[a,b]	−	+
Tetradecane[a,b]	−	+
2-Methyltetradecane[a]	+	−
Pentadecane[a,b]	+	+
Hexadecane[a,b]	+	+
3-Methylhexadecane[a]	+	−
Heptadecane[a,b]	+	+
Octadecane[a,b]	+	+
Nonadecane[a,b]	+	+
Eicosane[a,b]	+	+
Heneicosane[b]	+	+
Docosane[a,b]	+	+
Tricosane[a,b]	+	+
Tetracosane[a,b]	+	+
Pentacosane[b]	+	+
Hexacosane[a,b]	−	+
Heptacosane[a,b]	+	+
Octacosane[a,b]	+	+
Nonacosane[b]	−	+
Triacontane[b]	−	+
Hentriacontane[b]	−	+
Dotriacontane[b]	−	+
Tritriacontane	−	+
2,6-Di-*tert.*-butyl-4-methylphenol[a,b]	+	+
Butyl-8-methylnonyl phthalate[a]	+	−
Diisooctyl phthalate[a,b]	+	−

Table 1. *(continued)*

Compound	LDPE + pro-oxidant + starch	LDPE + pro-oxidant
Methanol fraction		
5-Methyldihydro-2(3H)-furanone[a]	+	–
5-Ethyldihydro-2(3H)-furanone[a]	+	+
5-Pentyldihydro-2(3H)-furanone[a]	–	+
5-Hexyldihydro-2(3H)-furanone[a]	–	+
4-Oxopentanoic acid[a]	–	+
Butanedioic acid[a,b]	+	+
Pentanedioic acid[a,b]	+	–
Benzoic acid[a,b]	+	+
2-Oxopentanedioic acid[a]	+	–
Hexanedioic acid[a,b]	+	–
7-Oxooctanoic acid[a]	+	+
Heptanedioic acid[a,b]	+	–
Octanedioic acid[a,b]	+	–
9-Oxodecanoic acid[a]	+	–
Nonanedioic acid[a,b]	+	–
Decanedioic acid[a,b]	+	–
Undecanedioic acid[a,b]	+	–
Dodecanedioic acid[a,b]	+	–
Chloroform fraction		
Hexadecanol[a,b]	+	–
Octadecanol[a,b]	+	–
2,4-Dimethyltetrahydrofuran[a]	–	+
2,5-Dipropyltetrahydrofuran[a]	–	+
5-Ethyldihydro-2(3H)-furanone[a]	–	+
5-Ethyldihydro-5-methyl-2(3H)-furanone[a]		+
5-Pentyldihydro2(3H)-furanone[a]	–	+
1,1-Diethoxyheptane[a]	–	+
Acetic acid, 1-methylethyl ester[a]	–	+
2,6-Di-*tert.*-butyl-4-methylphenol[a,b]	+	+
Benzyl butyl phthalate[a]	+	–
4-Nonylphenol[a]	+	–
Butyl-8-methyinonyl phthalate[a]	–	+
Diisooctyl phthalate[a,b]	–	+
Diisononyl phthalate[a,b]	+	+
Dichloromethane fraction		
1-Hexadecene[a,b]	–	+
1-Octadecene[a,b]	+	–
1-Eicosene[a,b]	+	–
2,6-Di-*tert.*-butyl-4-methylphenol[a,b]	+	+
Butyl-8-methylnonyl phthalate[a,b]	–	+
Diisooctyl phthalate[a,b]	+	–
Benzyl butyl phthalate[a]	+	–
Diisononyl phthalate[a]	+	–

Prior to analysis by GC and/or GC–MS the degradation products in the aqueous phase were extracted and fractionated by the SPE scheme shown in Fig. 1.

[a] Identification by MS by comparison with the National Institute of Standards and Technology (NIST).

[b] Identification by GC by comparison with standard retention indices.

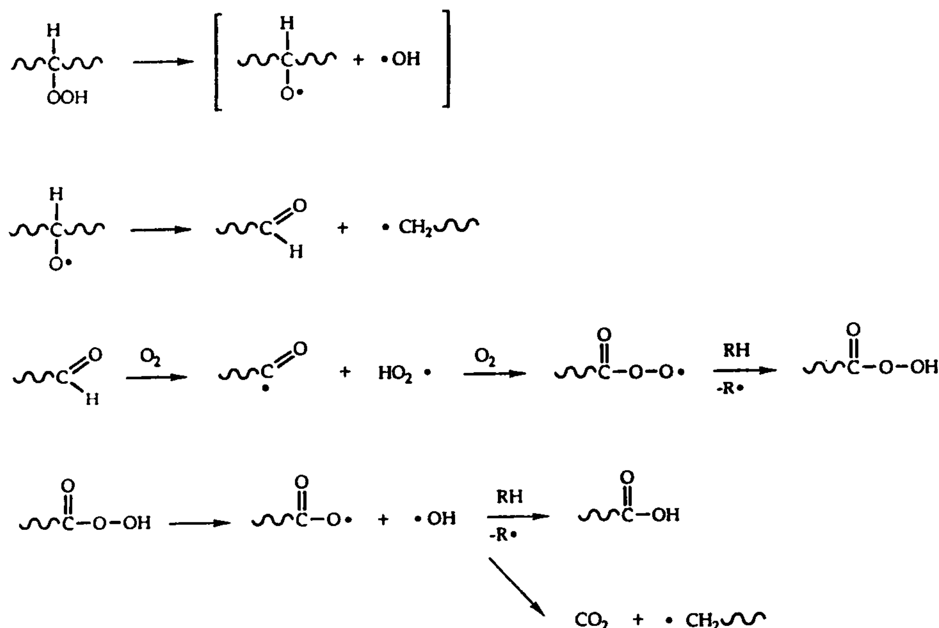

Fig. 4. Examples of carboxylic acid formation during degradation of LDPE.

attribute them to the breakdown of the styrene part of the SBS pro-oxidant.

Aliphatic hydrocarbons constitute another major class of breakdown products formed upon chain scission. A homologous series of *n*-alkanes, C_{12}–C_{33} as well as a few branched alkanes were the main components identified in the hexane fraction. Unsaturated hydrocarbons may be further oxidized and consequently only a few unsaturated hydrocarbons were found in any of the fractions. Only 1-hexadecene, 1-octadecene and 1-eicosene were detected in the dichloromethane fraction.

In both types of sample, with and without starch, the methanol fraction was about five times larger by mass than the other fractions and had an intense yellow colour. A number of species seemed to be responsible for this discoloration since no clear-cut absorption maximum was found in the visible range as observed by UV–Vis spectroscopy (data not shown). We were not able by GC–MS to identify specific compounds that could account for the yellow colour. Dicarboxylic acids dominated among the identified compounds. These can be formed according to the mechanisms for monocarboxylic acid formation involving a dual β-scission of alkoxy radicals. In previous degradation studies [13] we have not encountered these compounds, but they may be formed under the severe oxidation conditions applied in this study. Dicarboxylic acids have been reported as degradation products when polyethylene is oxidized in boiling nitric acid [19]. We detected the dicarboxylic acids as dimethyl esters since reaction had occurred with the methanol from the SPE extraction step. This derivatization reaction took place due to the prolonged contact of the analytes with the methanol and is an advantage since it facilitates the identification of the acids. The dicarboxylic acids were mainly determined from the samples containing starch, and this may be due to the fact that this material more easily allows the products formed to diffuse out to the surrounding environment. Other products in this fraction were a series of alkyl-substituted dihydro-2(3H)-furanones, some of which were also found in the chloroform fraction. In an earlier work [13] we suggested that the starch component contributed to the formation of these products. In this work,

however, these products were detected from both types of sample, with and without starch. The formation of γ-lactones can occur whenever carboxylic acid and hydroxyl groups are generated in the 1,4-positions of the polymer backbone [11]. The decomposition of 1,4-dihydroperoxides [23] according to Fig. 5, reaction 1, and/or through homolysis of a percarboxyl group, reaction 2 [19] are other possible mechanisms.

With the exception of hexadecanol and octadecanol, few alcohols were detected in the chloroform fraction and no ketones in the dichloromethane fraction. Owing to the extent of the degradation, these structures had most probably been further oxidized. We have earlier in less severe oxidation studies detected these types of compounds. Several compounds were detected in each fraction, which we assign to external contaminants introduced during the analytical handling procedure and impurities in the solvents used in the extraction. These include 2,6-di-*tert.*-butyl-4-methylphenol and several types of phthalate esters. The facts that these compounds were found in all fractions and that they are not normally additives in LDPE or the other components support this view.

With the exception of the dicarboxylic acids, we could establish no major differences in degradation behaviour between samples with and without starch by virtue of the degradation products. The SBS pro-oxidant is the primary component initiating the oxidation. As shown earlier [24], the transition metal salt, which is the other part of the pro-oxidant formulation, catalyses a decomposition of any hydroperoxides formed which is a crucial step in the onset of autoxidation [25]. If starch degradation occurs, this can promote the primary oxidative reactions due to the increased oxygen permeability and the increased surface/volume ratio. It also facilitates the release of degradation products from the samples. This can be achieved under biotic conditions when the starch granules are consumed, as by the action of hot water when the starch granules are disrupted.

Degradation products, either of the backbone type or trapped inside the films were monitored by FT-IR throughout the test period. Special interest was focused on the carbonyl region

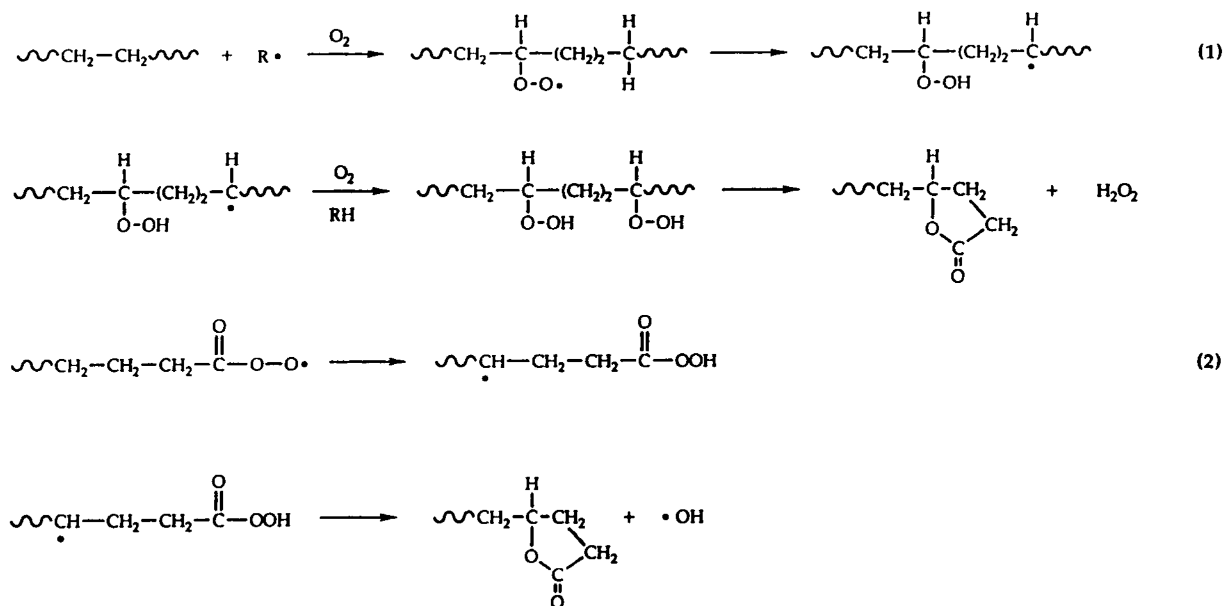

Fig. 5. Examples of γ-lactone formation during degradation of LDPE.

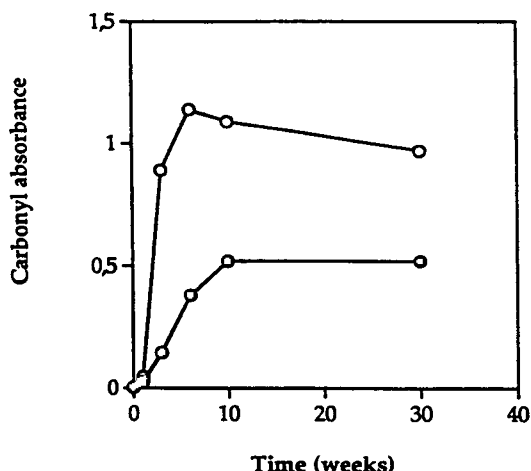

Fig. 6. Carbonyl absorbance as a function of time of degradation in water at 95°C. ○ = LDPE + pro-oxidant + starch; ○ = LDPE + pro-oxidant.

which encompasses several types of compounds with a maximum absorbance at 1718 cm^{-1}. The carbonyl absorbance index is presented in Fig. 6 versus degradation time. The samples not containing starch display a sharp increase in carbonyl content, reaching a maximum after about 6 weeks, after which the absorbance decreases slightly while the samples containing starch reach a maximum which is less than half that value after about 10 weeks. Since the starch-containing samples were expected to allow more of the degradation products to diffuse out to the surrounding environment, these results are reasonable.

4. Conclusions

An SPE method based on solid sorbents of bonded silica of the octadecyl and aminopropyl type and unbonded silica type, has been developed for the separation of highly complex product mixtures. The method is applicable to the class fractionation of products obtained upon degradation of polymers. The products are separated into five different fractions which facilitates the qualitative identification by e.g. GC–MS due to the less complex chromatograms obtained, the possibility of adequately concentrating each fraction and the increased knowledge of what kind of products to expect. Application of this method to LDPE with starch and/or pro-oxidant degraded for 30 weeks in water at 95°C proved to be powerful and enabled the identification of over three times as many products as when liquid–liquid extraction was used followed by GC–MS. Over 60 degradation products were identified in each sample; mainly dicarboxylic and monocarboxylic acids, and *n*-alkanes. The rate of the oxidation processes is altered by the incorporation of additives but we have not, as yet, found that these additives alter the degradation products so as to render these materials less environmentally acceptable than in our earlier degradation studies of pure LDPE.

5. Acknowledgements

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JOURNAL OF
CHROMATOGRAPHY A

Short communication

Improved chiral stationary phase for β-blocker enantioseparations

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Abstract

The previously described α-Burke 1 chiral stationary phase (CSP) was designed for the chromatographic separation of the enantiomers of β-blockers. Difficulties with the reproducibility of the free radical addition reaction, used in the attachment of the chiral selector to the chromatographic support, have required the development of an alternative silane immobilization process (α-Burke 2 CSP). While the enantioselectivity afforded by this new CSP is generally equivalent to that of the original CSP, the α-Burke 2 CSP demonstrates longer analyte retention, necessitating the use of mobile phases of greater eluotropic strength. The increased retention of the new CSP presumably results from a greater surface density of functional selectors, an interpretation which is supported by the observation that the preparative capacity of the α-Burke 2 CSP is greater than that of the original. Some of the factors influencing the retention and separation of a group of 23 β-blockers on the α-Burke 2 CSP are discussed.

1. Introduction

Two fundamentally different immobilization strategies have been employed in the production of brush-type chiral stationary phases (CSPs). The most straightforward approach involves the preparation of a silica-reactive chiral selector (usually an alkoxysilane) followed by the "one-step" immobilization of this selector onto a silica surface (Fig. 1a). A more roundabout "two-step" bonding approach is also frequently employed. With this technique the silica surface is first functionalized with a heterobifunctional silane reagent to afford a reactive stationary phase which is then allowed to react with the chiral selector to afford the CSP (Fig. 1b). Many early CSPs, including the original DNB-phenylglycine "Pirkle column" [1] were made using a "two-step" bonding approach. It has long been realized that the two-step bonding approach, while often convenient, has a number of attendant problems [2].

Perhaps the best recognized of these problems is that CSPs produced via a two-step bonding approach almost always contain residual reactive groups which can function as sites for non-enantioselective retention [3] and lead to a decrease in the enantioselectivity of the column [4]. In addition, these residual reactive groups can be the sites for irreversible reaction with analytes, matrix components, or other entities injected onto the column, resulting in a column which changes properties over time.

Several years ago Pirkle and Burke [5,6]

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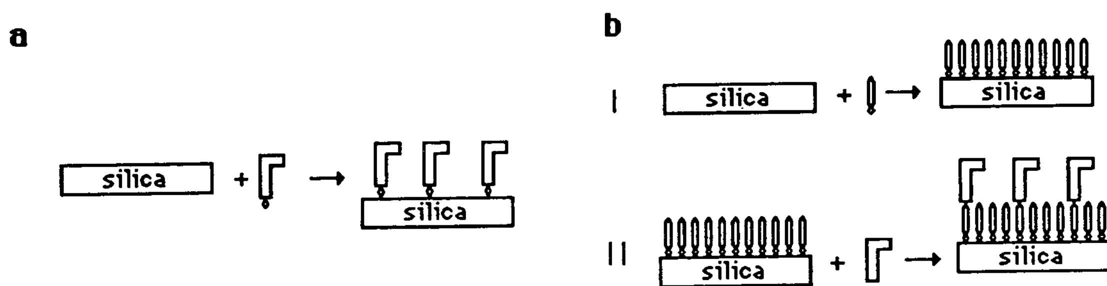

Fig. 1. Comparison of "one-step" and "two-step" approaches to CSP synthesis. (a) In the one-step approach a silica surface is allowed to react with a silica-reactive chiral selector to afford the CSP. (b) In the two-step approach, the silica surface is first allowed to react with a heterobifunctional reagent to afford a reactive stationary phase (step I). In step II, the reactive stationary phase is allowed to react with the chiral selector to afford the CSP.

reported the preparation of a new chiral stationary phase designed specifically for the chromatographic separation of the enantiomers of β-blockers. This column, subsequently commercialized by Regis as the α-Burke 1 [7], has proven to be very useful for the separation of the enantiomers of β-blockers and other analytes [8]. The two-step synthetic protocol used in the production of the α-Burke 1 CSP is illustrated in Fig. 2. Following preparation of mercaptopropyl silica, the enantiopure olefin is allowed to react with thiopropyl silica in the presence of the free radical initiator azobisisobutyronitrile (AIBN) to afford the thioether-linked CSP. This two-step free radical immobilization approach is widely used, being originally applied to the immobilization of alkaloids of the quinine family by Rosini et al. [9], and subsequently routinely used in the immobilization of 3,5-dinitrobenzoyl amino acid derivatives and other compounds by Tambuté and co-workers [10,11]. Pirkle and Burke opted to use this two-step bonding approach following failure of conventional hydrosilyation of the olefin with trichlorosilane [12].

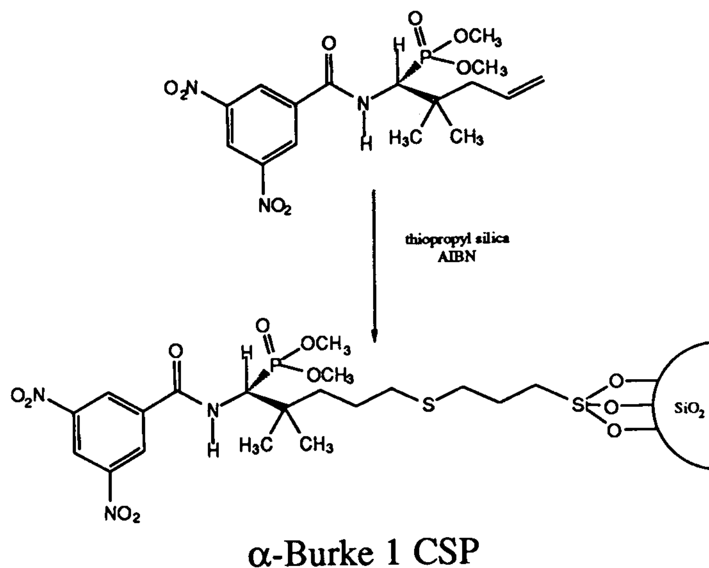

Fig. 2. Preparation of α-Burke 1 CSP. Reaction of enantiopure olefin with thiopropyl silica in the presence of the free radical initiator, AIBN, affords the thioether-linked α-Burke 1 CSP.

Apart from the general problems associated with two-step CSP production, the free radical thioether immobilization method has several additional disadvantages. First, the thiopropyl surface itself is redox active, can be oxidized to form disulfide linkages, and is potentially reactive in redox reactions with analytes, matrix components or metal ions. Second, the reaction of a thiol radical with an unactivated olefin is generally a poor reaction. For example, in the reaction of the olefin precursor with thiopropyltriethoxysilane, a complex mixture of products is obtained with a very low yield of the desired silane reagent [12].

Considering all of these disadvantages, it is perhaps not too surprising that we have experienced some difficulties in producing this CSP in a reproducible manner. After considerable expenditure of time and resources to develop conditions for reproducible preparation of the α-Burke 1 CSP, we chose to reinvestigate the hydrosilylation of the olefin precursor. Like Pirkle and Burke, we were also unable to effect hydrosilyation using trichlorosilane. However, hydrosilylation with dimethylchlorosilane gave no such problem, and the corresponding CSP (α-Burke 2) was produced using conventional one-step methods (Fig. 3).

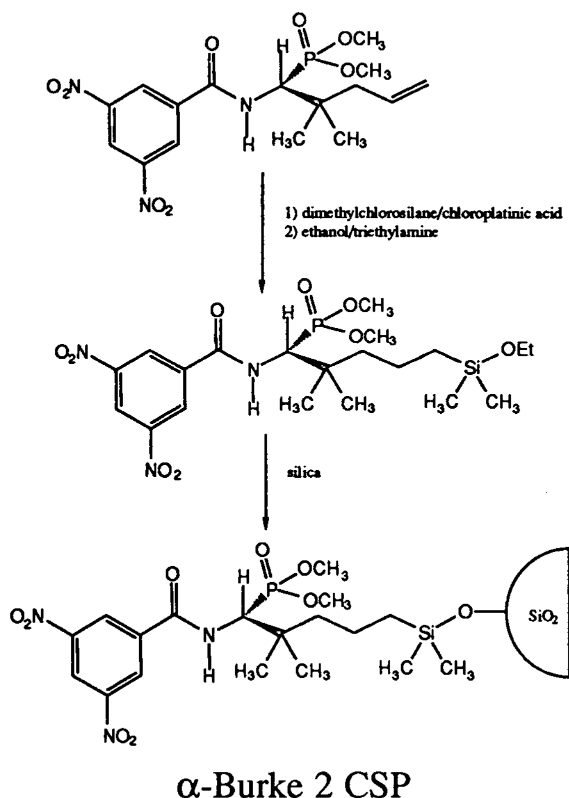

Fig. 3. Hydrosilyation of the enantiopure olefin with dimethylchlorosilane and chloroplatinic acid, followed by conventional immobilization of the resulting silane on silica, affords the silane-linked α-Burke 2 CSP.

A comparison of the separation of the enantiomers of the β-blockers metoprolol and bufuralol was undertaken on the two CSPs. In addition, factors influencing the separation of the enantiomers of a group of 23 β-blockers (Fig. 4) on the α-Burke 2 CSP was studied.

2. Experimental

2.1. Apparatus

Chromatographic analysis was performed using a Kratos Spectroflow 400 pump, a Rheodyne Model 7125 injector fitted with either a 2-ml or a 20-μl sample loop, a Kratos Spectroflow 757 variable-wavelength absorbance monitor, and a Hewlett-Packard HP 3394 integrating recorder.

2.2. Materials

The (R)-α-Burke 1 CSP was obtained from Regis Technologies, Morton Grove, IL, USA. The α-Burke 2 CSPs were prepared by conventional methods following the procedure outlined in Fig. 3. Ammonium acetate and ammonium formate were obtained from Aldrich, Milwaukee, WI, USA. HPLC solvents were HPLC grade. Variable-temperature column jacket was obtained from Aura Industries, Staten Island, NY, USA.

β-Blocker samples were available from previous studies, with many samples kindly donated by Dr. Joe Gal, University of Colorado School of Medicine, Denver, CO, USA. Column void time was measured by injection of 1,3,5-tri-*tert.*-butylbenzene.

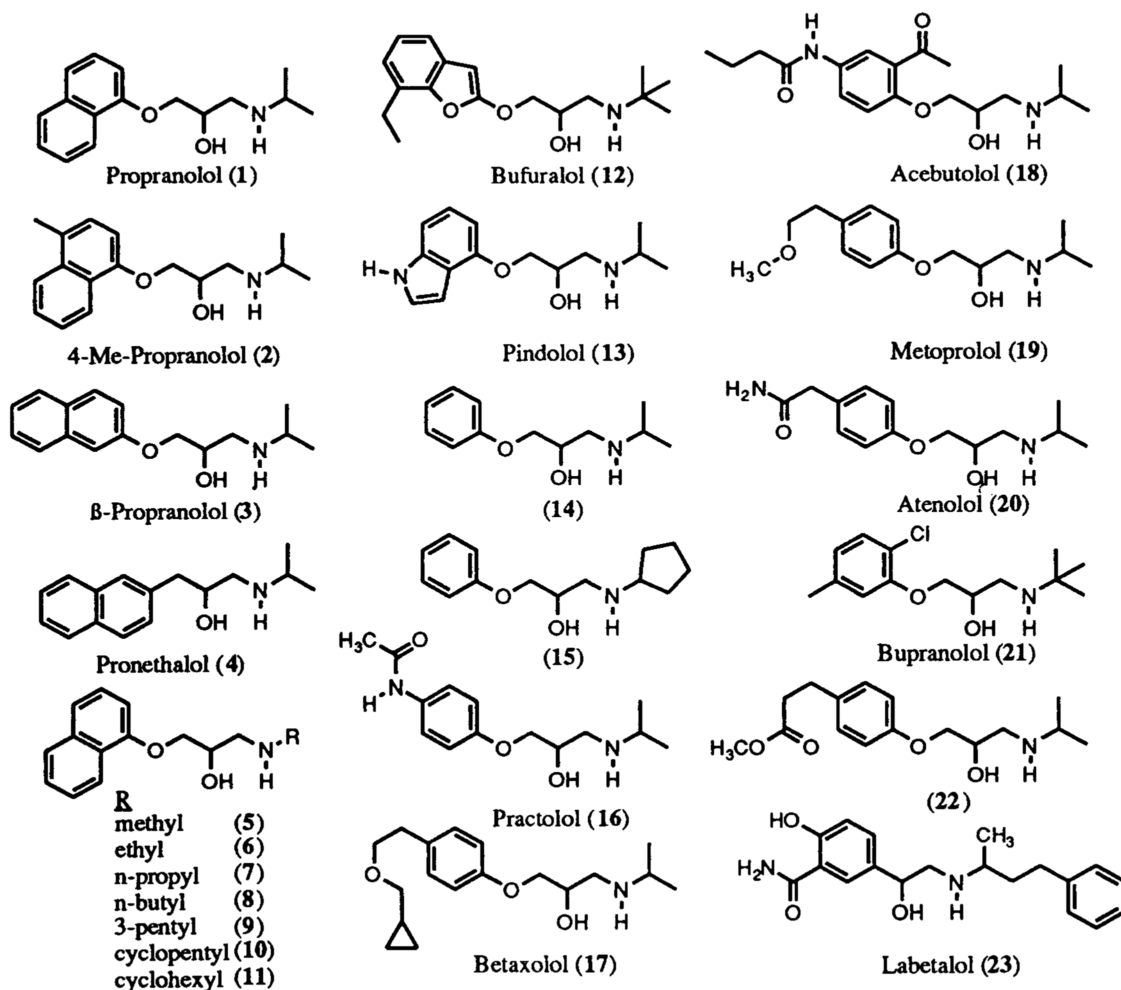

Fig. 4. β-Blocker analytes used in the study.

3. Results and discussion

A comparison of the separation of the enantiomers of the representative β-blockers, metoprolol and bufuralol, on the two CSPs reveals that the silane-linked α-Burke 2 CSP affords enantioselectivities which are more or less equivalent with those obtained with the original α-Burke 1 CSP (Table 1). The retention factors are appreciably greater with the α-Burke 2 CSP, an outcome which is initially somewhat surprising for a CSP in which sites for non-specific adsorption have presumably been eliminated. However, it has often been shown that changes in the length or chemical makeup of CSP tethers can influence both enantioselectivity and retention [2]. In addition, a simpler explanation may obtain in the present case: the greater retention of the α-Burke 2 CSP could also result from a denser population of functional chiral selectors (a higher phase ratio).

A comparison of the preparative capacity of the two CSPs in the separation of propranolol enantiomers (Fig. 5) shows that the (R)-α-Burke 2 column can indeed tolerate a significantly higher sample load than the thioether-linked CSP. This result is consistent with the (R)-α-Burke 2 having a denser coverage of functional chiral selectors. It must be noted that the preparative capacity of a stationary phase also

Table 1
Comparison of the separation of some β-blocker enantiomers on (R)-α-Burke 1 and (R)-α-Burke 2

Compound	(R)-α-Burke 1				(R)-α-Burke 2			
	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s
Metoprolol	2.81	3.42	1.22	1.93	7.35	9.02	1.23	2.45
Bufuralol	3.21	6.59	2.05	7.37	7.36	14.86	2.02	8.32

Mobile phase, ethanol–dichloromethane (10:90) with 10 mM ammonium acetate; flow-rate, 2 ml/min; detection, UV at 280 nm. k'_1 = Retention factor of first-eluted enantiomer; k'_2 = retention factor of second-eluted enantiomer.

depends upon the retention. Nevertheless, changing to a mobile phase which affords greater retention on the (R)-α-Burke 1 CSP has only a marginal effect on improving the loading capacity.

The increased retention of the (R)-α-Burke 2 CSP requires the use of mobile phases of greater eluotropic strength to afford more convenient retention times. Therefore, a survey of the influence of mobile phase composition on the retention and enantioselectivity of the representative β-blockers, metoprolol and bufuralol, was undertaken. The results presented in Table 2 show a rather dramatic decrease in enantioselectivity with increasing concentrations of ethanol. Interestingly, the retention factor exhibits a striking non-linear dependence on ethanol concentration (Fig. 6). Substitution of methanol for ethanol results in only a marginal decrease in retention, which is accompanied by a marked

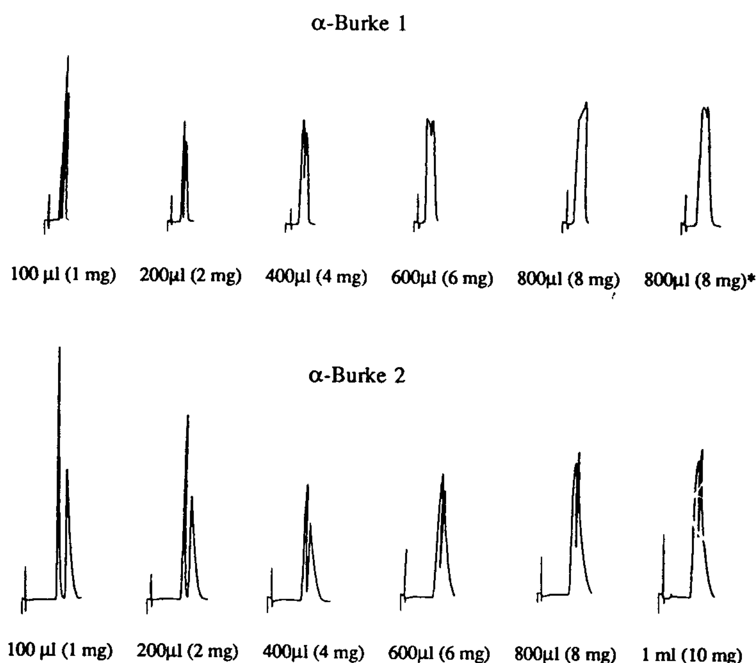

Fig. 5. Preparative separation of propranolol enantiomers on analytical (25 cm × 4.6 mm) (R)-α-Burke 1 and (R)-α-Burke 2 CSPs. Conditions: mobile phase, ethanol–dichloromethane (10:90) with 10 mM ammonium acetate; flow-rate, 2.00 ml/min; detection, UV at 325 nm; sample, propranolol, 10 mg/ml in ethanol. * Mobile phase, ethanol–dichloromethane (5:95) with 10 mM ammonium acetate.

Table 2
Effect of mobile phase composition on the separation of metoprolol and bufuralol enantiomers on (*S*)-α-Burke 2 CSP (2 ml/min; UV 280 nm)

Mobile phase[a]	Metoprolol				Bufuralol			
	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s
Ethanol–dichloromethane (5:95)	7.14	8.95	1.25	2.91	6.84	15.89	2.32	9.35
Ethanol–dichloromethane (10:90)	4.98	6.15	1.23	2.45	5.12	11.01	2.15	8.63
Ethanol–dichloromethane (20:80)	4.66	5.61	1.20	2.08	4.98	9.56	1.94	7.92
Ethanol–dichloromethane (40:60)	5.25	6.18	1.18	2.02	5.63	10.14	1.80	7.05
Methanol	9.85	10.30	1.05	0.94	–	–	–	–
Methanol–dichloromethane (1:1)	4.30	4.67	1.09	1.36	4.82	6.23	1.29	4.23
Isopropanol–dichloromethane (1:1)	7.19	8.53	1.19	1.34	6.67	12.57	1.88	5.84
Ethanol–acetonitrile (1:1)	7.24	8.04	1.11	1.55	6.92	9.32	1.35	4.07

[a] All mobile phases contain 10 m*M* ammonium acetate.

decrease in enantioselectivity. Consistent with previous studies [7], the enantioselectivity afforded by isopropanol-based mobile phase is marginally greater than that afforded by ethanol-based mobile phases. However, the much greater efficiency provided by the ethanol-based mobile phase results in significantly greater resolution.

Increasing environmental concerns about the use of halogenated chromatographic solvents prompted the investigation of some non-halogenated co-solvents. Although the acetonitrile-based mobile phase affords significantly less enantioselectivity and resolution than the dichloromethane-based mobile phase, the resolution provided is sufficient for baseline resolution of many β-blockers. Methyl *tert.*-butyl ether (MTBE) was also evaluated as a co-solvent, but

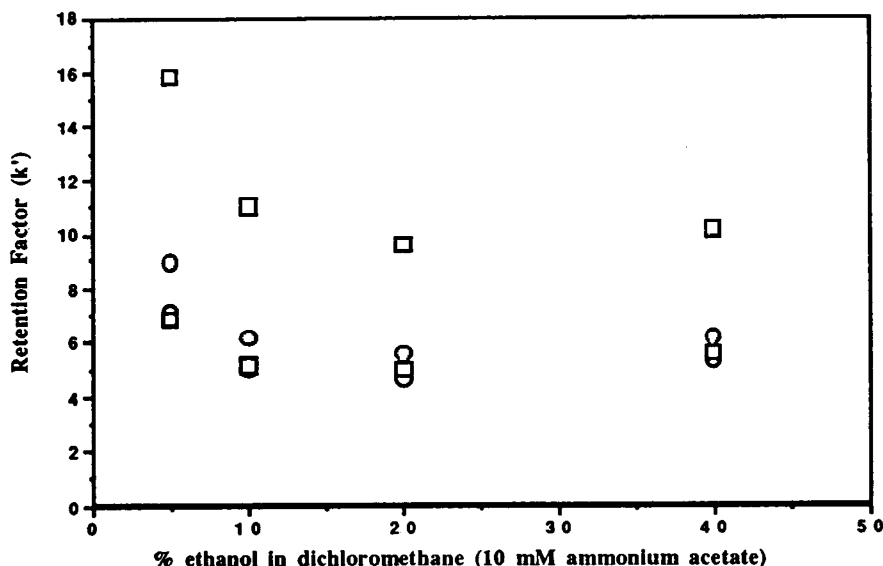

Fig. 6. Influence of ethanol content on the retention of metoprolol (○, **O**) and bufuralol (□, **□**) enantiomers on (*S*) α-Burke 2 CSP. ○, □ = k' of first-eluted enantiomer; **O**, **□** = k' of second-eluted enantiomer.

found to afford an even greater decrease in enantioselectivity and resolution. In addition, MTBE has a poor ability to solubilize the required ammonium acetate modifier.

As noted by Pirkle and Burke, an effective way to control retention is to adjust the concentration of the polar modifier [6]. They found that increasing concentrations of ammonium acetate caused a decrease in retention factor, with little effect on separation factor. We also found that increasing salt concentration decreases retention. However, there is an accompanying decrease in separation factor, which is more evident for the analytes which are well resolved (Table 3). The use of ammonium acetate in these studies should not be taken as an indication that this is the optimum polar modifier. For example, Perrin found that the use of ammonium formate has a greater influence on decreasing retention, on a per mole basis, than does ammonium acetate [7]. In addition, a range of other salts have been used with good effect in a related system, sometimes affording significantly enhanced separations [13]. The separation of β-blocker enantiomers on the α-Burke 2 CSP appears to be dramatically influenced by both the nature and concentration of salt additives, and may be of importance to researchers trying to optimize individual separations.

The separation of the enantiomers of a group of 23 β-blockers (Fig. 4) was studied on (*R*)-α-Burke 2 CSP using both a dichloromethane-based and an acetonitrile-based mobile phase (Table 4). As with the work of Pirkle and Burke, enantioselectivity appears to be best for those analytes bearing the most π-basic aryl groups (compounds 1–13). Analytes bearing sites for strong non-selective interactions with the CSP (compounds 16, 18, 20, 23) are resolved most poorly, and are strongly retained, presumably owing to non-specific adsorption.

Most of the analytes shown in Table 4 are baseline resolved under the specified conditions. For those which are not, a change in mobile phase composition or column temperature may be helpful. The unusual influence of temperature on β-blocker enantioseparations found by Pirkle and Burke using the α-Burke 1 CSP is also observed with α-Burke 2 CSP. For β-blockers, a reduction in column temperature generally affords decreased retention and increased enantioselectivity on this column. For example, decreasing column temperature from room temperature to 0°C decreases the retention of analyte 22 by about one half, while the resolution improves from 1.35 to 1.60. Further decreases in column temperature oftentimes provide even greater improvements.

4. Conclusions

While the enantioselectivity afforded by the α-Burke 2 CSP is generally equivalent with that of the original α-Burke 1 CSP, the new CSP affords greater retention, necessitating the use of mobile phases of greater eluotropic strength. The increased retention of α-Burke 2 CSP is believed to result from a greater surface density of functional selectors, an interpretation supported by the observation that the preparative capacity of the new CSP is greater than that of

Table 3
Effect of ammonium acetate concentration on the separation of some β-blocker enantiomers on (*R*)-α-Burke 2

Compound	10 m*M* Ammonium acetate				20 m*M* Ammonium acetate			
	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s
Metoprolol	7.35	9.02	1.23	2.45	2.40	2.94	1.23	2.02
Bufuralol	7.36	14.86	2.02	8.32	2.77	4.71	1.70	5.97

Mobile phase, ethanol–dichloromethane (10:90) with 10 or 20 m*M* ammonium acetate; flow-rate, 2 ml/min; detection, UV at 280 nm.

Table 4
Separation of some β-blocker enantiomers on (R)-α-Burke 2 CSP using two different mobile phases (2 ml/min; UV 280 nm)

Compound[a]	Ethanol–dichloromethane (10:90) with 20 mM ammonium acetate				Ethanol–acetonitrile (20:80) with 12 mM ammonium formate			
	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s
1	4.09	5.59	1.37	3.27	8.60	10.45	1.22	3.31
2	4.36	6.26	1.44	3.73	10.74	13.48	1.26	3.93
3	3.63	4.22	1.16	1.60	7.28	7.94	1.09	1.46
4	4.30	4.98	1.16	1.59	8.24	8.50	1.03	0.58
5	7.77	9.94	1.28	1.95	11.83	13.97	1.18	1.91
6	5.48	7.40	1.35	3.03	9.91	11.99	1.21	2.94
7	3.68	4.97	1.35	2.95	8.42	10.08	1.20	2.92
8	3.01	4.05	1.35	2.40	7.55	9.06	1.20	2.88
9	11.90	2.29	1.21	1.82	5.63	6.41	1.14	2.13
10	1.89	2.28	1.21	1.82	5.63	6.41	1.14	2.10
11	2.81	3.77	1.34	3.07	8.67	10.45	1.21	2.94
12	2.77	4.71	1.70	5.97	5.52	7.32	1.33	4.34
13	1.66	16.11	1.38	3.30	9.04	11.00	1.22	3.30
14	3.05	3.49	1.14	1.56	5.09	5.52	1.08	1.18
15	2.45	2.96	1.21	2.08	5.43	5.92	1.09	1.37
16	8.02	8.89	1.11	0.91	7.83	8.45	1.08	1.12
17	2.04	2.41	1.18	1.48	4.63	5.10	1.10	1.57
18	5.81	6.34	1.09	0.96	6.50	7.03	1.08	1.07
19	2.40	2.94	1.23	2.02	4.99	5.50	1.10	1.56
20	12.43	13.77	1.11	1.11	8.86	9.54	1.08	1.19
21	2.55	3.16	1.24	2.03	5.42	6.23	1.15	2.28
22	2.48	2.97	1.20	1.35	4.65	5.13	1.10	1.68
23[b]	4.44	5.16	1.16	1.14	7.01	8.06	1.15	0.82

[a] See Fig. 4.
[b] Only two peaks were obtained for this separation (diastereomers were not resolved).

the original. Some factors influencing retention and enantioselectivity have been explored, and conditions for baseline resolution of a number of β-blockers have been provided.

Acknowledgements

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Use of Sep-Pak C_{18} cartridges to clean up free amino acids from coniferous needles ☆

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Abstract

Reversed-phase Sep-Pak C_{18} cartridges were investigated to evaluate their ability to purify free amino acids extracted from jack pine (*Pinus banksiana* Lamb.) and white spruce [*Picea glauca* (Moench) Voss] needle tissue samples for HPLC analysis. Twenty-one amino acids from a standard only and amino acids from conifer needles with added standard were eluted through Sep-Pak C_{18} cartridges. An average recovery of 98% was found for all standard amino acids. Using norleucine as an internal standard, recovery for all amino acids except alanine and methionine averaged 104% for jack pine and 97% for white spruce tissue. Alanine co-chromatographed with an unknown peak and recovery appeared to exceed 130%. Methionine, with less than 33% recovery, was probably degraded during the extraction and purification procedures. Aside from alanine and methionine, Sep-Pak C_{18} cartridges appear to be a faster and more effective method for purifying conifer foliage extracts prior to amino acid analysis than the traditional ion-exchange column purification method.

1. Introduction

Extracts of biological samples for amino acid analysis are commonly purified with a strong cation ion-exchange resin [1]. In our laboratory, Amberlite IR-120 is used in an automated ion-exchange column system to clean extracts of samples collected from coniferous tree tissues [2,3].

However, even automated ion-exchange chromatography sample purification is time consuming. Sep-Pak C_{18} cartridges (Sep-Pak) appeared to be a potentially less time-consuming alternative purification method [4,5]. Hart and White [6] used Sep-Paks to purify amino acid samples hydrolyzed from protein using trifluoroacetic acid (TFA) by Waters' method (Waters, Mississauga, Canada) and found different amino acid yields because of retention differences in the Sep-Pak. These retention differences were corrected by Cohen et al. [7] when they modified the procedure to use hydrochloric acid instead of TFA to elute amino acids from the Sep-Pak.

The purpose of this study was to observe the efficacy of the Waters' modified (using HCl) Sep-Pak method [7] for purification of conifer needle

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☆ Use of brand names does not constitute an endorsement by the Ministry of Natural Resources for any specific purpose.

[1] Former research scientist (deceased) with Ministry of Natural Resources.

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tissue amino acid extracts for free amino acid analysis by the Waters' Pico-Tag method.

2. Experimental

2.1. Sample preparation

Randomly collected 2 + 0 jack pine and 3 + 0 white spruce seedlings from Midhurst Nursery, Ontario, Canada were rinsed with cold tap water, rinsed with reagent-grade water and drained. Current-year needles were separated from stems, frozen in liquid nitrogen and lyophilized for 72 h. Dry samples were ground in a Wiley mill with a 20-mesh sieve and stored at −20°C until extraction.

2.2. Extraction of amino acids

A 200-mg amount of dried sample was placed in a 15-ml centrifuge tube, 10 ml of distilled water were added, and vortexed. Tube was sealed and shaken at 50°C for 30 min in a horizontal position to extract. Tube was centrifuged at 1850 g for 10 min. The supernatant was decanted into a 100-ml evaporating flask. The extraction was repeated twice and the sample was washed with 10 ml of water prior to being decanted for a total of 40 ml of solution after centrifugation. The combined solution was evaporated to dryness in a rotary evaporator at 50°C. To five of ten dried samples, 400 μl of 2.5 μM/ml standard amino acids were added and the samples were redried. The ten samples in the flasks were redissolved with 2 ml of 1.0 mM norleucine (internal standard) in water. The aliquots of samples were then centrifuged in 1.5-ml micro tubes at 8160 g for 10 min.

2.3. Sep-Pak C_{18} cartridge purification of samples

The Sep-Pak C_{18} Plus cartridge was preconditioned with 10 ml of methanol and 10 ml of water. The Sep-Pak cartridge was loaded with 0.5 ml of 1 M HCl and 0.5 ml of sample with internal standard. Amino acids were eluted with 1.5 ml of 1 M HCl, and then 2.5 ml of 30% acetonitrile in 1 M HCl [7]. Because tryptophan is not stable in acid, the amino acids were eluted into a vial containing 0.5 ml of 1.0 M sodium hydrogencarbonate solution.

2.4. Derivatization and separation of amino acids

A 50-μl volume of sample was derivatized using phenylisothiocyanate [7]. A 20-μl volume of the derivatized samples was then injected into the Waters Maxima 820 HPLC system with a 30 × 0.39 cm stainless-steel Pico-Tag column. Calibration was carried out using a Pierce (IL, USA) amino acid standard H with asparagine,

Table 1
Recovery of 250 pmol/injection of each standard amino acid by the Sep-Pak cartridge clean-up method

Amino acid	Recovery (%) ($n = 4$)	R.S.D. (%) ($n = 4$)
Asp	94.2	4.2
Glu	96.0	2.9
Ser	101.1	3.4
Asn	95.8	2.1
Gly	95.6	2.2
Gln	95.8	2.6
His	99.5	8.5
GABA	108.3	4.1
Thr	99.3	1.5
Ala	102.8	3.1
Arg	96.8	2.5
Pro	99.3	2.5
Tyr	95.7	2.8
Val	96.4	3.0
Met	95.4	3.5
Ile	95.9	3.0
Leu	96.7	2.6
Norl	100.5	2.7
Phe	99.2	2.7
Trp	97.0	2.7
Lys	97.3	2.6
Overall average	98.0	3.1

Three-letter abbreviations are standard abbreviations for amino acids. GABA = γ-Aminobutyric acid; Norl = norleucine.

glutamine, γ-aminobutyric acid, tryptophan and norleucine added. The amino acids were separated with solvent 1 [70 m*M* sodium acetate, pH 6.55, 2.5% (v/v) acetonitrile] and solvent 2 (acetonitrile–methanol–water, 45:15:40) [8] using a standard procedure for free amino acid analyses [7]. Blank tests were performed with underivatized samples and with derivatized blanks.

All amino acid concentrations were calculated based on 20 μl of injected samples and the amino acid concentrations in Table 2 were corrected to 100% using norleucine as an internal standard.

3. Results and discussion

Standard amino acid recoveries using Sep-Pak cartridges are shown in Table 1. The average yield of the 21 standard amino acids was 98% with a relative standard deviation (R.S.D.) of 3.1%. The recovery of norleucine, which was used as an internal standard, was 100.5% which confirmed it as a good internal standard.

The recoveries of most amino acids in jack pine needles except alanine and methionine were close to the expected amount with an average of 104% with a R.S.D. of 2.9% (Table 2). The average yield in white spruce was 97% (R.S.D.

Table 2
Sep-Pak purified amino acid concentrations extracted from jack pine and white spruce seedling needles with and without the addition of 250 pmol of standard (std.)/injection

Amino acid	Jack pine		White spruce	
	Concentration without std. (pmol ± S.E.)	Recovery with std. (%)[a]	Concentration without std. (pmol ± S.E.)	Recovery with std. (%)[a]
Asp	24 ± 2	108.5 (3.0)	34 ± 1	77.0 (6.7)
Glu	88 ± 1	104.8 (3.0)	91 ± 2	105.1 (5.0)
Ser	27 ± 1	111.2 (2.6)	24 ± 2	101.1 (4.3)
Asn	5 ± 0	99.8 (1.7)	2 ± 0	99.8 (3.3)
Gly	5 ± 1	93.1 (3.3)	8 ± 1	94.0 (4.0)
Gln	7 ± 1	99.5 (1.9)	21 ± 2	97.2 (5.5)
His	5 ± 1	98.5 (4.4)	8 ± 2	95.4 (5.4)
GABA	61 ± 1	100.9 (3.2)	28 ± 2	97.7 (4.6)
Thr	11 ± 2	103.8 (4.6)	2 ± 0	94.4 (4.6)
Ala	12 ± 2	152.0 (3.2)	7 ± 3	131.8 (8.9)
Arg	129 ± 2	105.9 (3.2)	394 ± 6	99.0 (4.6)
Pro	63 ± 2	115.6 (4.0)	40 ± 2	105.6 (6.2)
Tyr	8 ± 0	109.9 (2.4)	30 ± 3	94.4 (5.6)
Val	8 ± 0	112.4 (3.2)	7 ± 1	95.3 (4.6)
Met	4 ± 2	21.9 (51.4)	5 ± 1	32.8 (55.8)
Ile	4 ± 0	111.4 (2.2)	5 ± 1	102.9 (3.2)
Leu	8 ± 0	108.2 (0.9)	10 ± 1	105.0 (1.0)
Phe	11 ± 0	105.1 (1.1)	12 ± 1	97.8 (2.6)
Trp	38 ± 1	96.1 (1.7)	78 ± 1	97.2 (3.0)
Lys	12 ± 0	83.1 (5.3)	17 ± 1	85.6 (4.0)
Overall average[b]		104 (2.9)		97 (4.3)

S.E. = Standard error.
[a] Relative standard deviations (%) in parentheses; $n = 5$.
[b] Alanine and methionine were excluded from the calculation of the mean.

4.3%) of the expected concentration except for alanine and methionine. White spruce showed a higher R.S.D. than did jack pine. The methionine recovery from jack pine needles was 21.9% and from white spruce needles it was 32.7% while the recovery of the methionine standard was 95.4% (Table 1). R.S.D. in the both species also showed over 50% (Table 2). Although it was not confirmed experimentally, something from the plant extract probably degraded methionine in the acidic eluting solution, a phenomenon that was also observed in extracts purified by the cation ion-exchange resin [9].

Although the Sep-Pak procedure was effective at cleaning up most contaminants, there were some quantification problems usually caused by co-eluting unknown peaks especially in samples from outdoor grown trees. For example, alanine concentration was higher than its actual concentration because of an unknown co-eluting peak found in underivatized samples. In addition, samples from outdoor grown trees showed a higher unknown co-eluting peak near alanine than did greenhouse samples. Some field samples also had unknown co-eluting peaks which, at low concentrations of amino acid (less than 10 pmol per injection), interfered with threonine resolution and quantitation and aspartic acid quantitation. The unknown co-eluting peak interference to resolution could be largely overcome by rerunning samples with a known amount of standard amino acid added to increase the amino acid peak size. After amino acid identity was clearly established the computer was used to reprocess peak data. Lysine, the last peak in the run, showed a reduced yield of 83.1% in jack pine and 85.6% in white spruce. Actual lysine concentration was calculated using the correction factor based on percent recovery of the internal standard norleucine.

The Sep-Pak method requires about 10 min to elute each sample whereas the ion-exchange column method requires 3–4 h when column regeneration time is included. We estimate that in our laboratory for each set of 16 samples, the time savings of the cartridge method over the automated ion-exchange column method (with 10 columns running at one time) is one entire working day. The Sep-Pak method also yields excellent recovery rates for most amino acids and we currently use this method routinely in our laboratory for amino acid analysis of conifer foliage and root tissue and find it yields consistent results. In our evaluation, the Sep-Pak method is superior for cleaning up conifer foliage extracts for amino acid analysis.

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Separation of polymer and on-line determination of several antioxidants and UV stabilizers by coupling size-exclusion and normal-phase high-performance liquid chromatography columns

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Abstract

A procedure consisting of connecting in series two different HPLC columns, one for size-exclusion chromatography (SEC) and the second one a normal-phase (silica) column has been developed. An automatic three-way switching valve was placed between the two columns. Through the valve, the polymer was drained whereas the rest of the compounds, a group of antioxidants and UV stabilizers, were separated and analyzed in the second column. The behaviour of the SEC column in different organic phases is studied. Detection limits about 0.1 $\mu g\ ml^{-1}$ were obtained for BHT, Tinuvin 326 and Tinuvin 327; 0.2 $\mu g\ ml^{-1}$ for Irganox 1076, and 1.1 $\mu g\ ml^{-1}$ for Cyasorb UV 9 and Cyasorb UV 1084. R.S.D. values of the whole process are lower than 4%.

1. Introduction

The determination of additives such as antioxidants and UV stabilizers in plastic packaging materials in contact with food is carried out by means of an extraction step, in which the compounds contained in the polymer are transferred to the organic solvent. The extraction can be carried out by classical extraction, in which the organic solvent is shaken with the polymer, by Soxhlet (continuous extraction) or by an ultrasonic bath. The ultrasonic bath has been shown as one of the most efficient systems in which most of the components present in the packaging material are transferred to the liquid organic phase. As a result of this extraction step, all the additives and small molecules, some oligomers and sometimes the polymer, can be present in the organic solution obtained. Obviously, a second step of clean-up is necessary to achieve the separation of the compounds of interest. Several methods have been proposed for the clean-up of such organic solutions [1–3] but among them, size-exclusion chromatography (SEC) is the most frequently used [4–8] due to its simplicity and short analysis times.

In most cases, the interfering compounds have larger sizes than the additives and consequently, they are eluted from the SEC column before the additives. However, SEC columns elute the compounds grouped in bands, instead of in narrow peaks corresponding to individual com-

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pounds. Consequently, a second separation column is necessary.

Fraction collectors connected to SEC columns are a useful alternative to facilitate the second separation step. Once the desired fraction is concentrated, it can be analyzed either by gas chromatography, reversed-phase [9–16] or normal-phase [14–20] high-performance liquid chromatography, but in all cases this behaviour implies working in batch, which is complex and time consuming.

The possibility of connecting on-line two HPLC columns with different stationary phases has been tried by different authors [21–28]. Nevertheless, this is not an easy task. First, the mobile phase has to be compatible with both columns, and secondly, the major compounds separated in the first column, e.g. SEC, should not be introduced into the second column. Johnson et al. [29] described a coupled column chromatography connecting size-exclusion and a reversed-phase columns each one with a different mobile phase for the analysis of some additives in rubber. Two independent HPLC systems were necessary to achieve the effective coupling, in which the first pump worked with tetrahydrofuran (THF) in the isocratic mode, with the SEC column and a UV variable-wavelength detector; the second system pumped water–acetonitrile through a gradient with a reversed-phase column and another UV detector. Between the columns, a switching valve acts as injector of the second HPLC system. This is a very powerful approach although two HPLC systems and a switching valve are necessary.

The present paper shows another system which allows the separation of polymer and the analysis of antioxidants and UV stabilizers in organic media in only one step. The system consists of two HPLC columns in series, one for SEC and the other one a normal-phase (silica). Between them, an automatic switching valve permits the polymer being drained at controlled time. Once the major compounds are eliminated by one of the valve ways, after the switch the rest of compounds passes through the other way to the second column, where their separation is improved and they can be quantified properly. The behaviour of each column and the analytical features for the determination of several antioxidants and UV stabilizers in polymers are discussed.

2. Experimental

2.1. Reagents

2,6-Di-*tert.*-butyl-4-metylphenol (BHT) was from Fluka (Buchs, Switzerland), pure quality, 2-hydroxy-4-methoxybenzophenone (Cyasorb UV 9), styrene and benzophenone were from Sigma (St. Louis, MO, USA), analytical-reagent quality; octadecyl-3,5-di-*tert.*-butyl-4-hydroxyhydrocinnamate (Irganox 1076), 2,2′-thiobis(4-*tert.*-octylphenolate)-*n*-butylamine nickel (Cyasorb UV 1084), 2(3′-*tert.*-butyl-2′-hydroxy-5′-methylphenyl)-2H-5-chlorobenzotriazole (Tinuvin 326) and 2-(2′-hydroxy-3′,5′-di-*tert.*-butylphenyl)-2H-5-chlorobenzotriazole (Tinuvin 327), were supplied by courtesy of Ciba-Geigy (Basle, Switzerland). All of them were used without further purification. Polystyrene was from Aldrich (Steinheim, Germany), average M_r 280 000. Chloroform, cyclohexane, dichloromethane, *n*-hexane and tetrahydrofuran (without stabilizer) were from Merck (Darmstadt, Germany), HPLC quality. Alugram Nano-SIL G/ UV_{254} thin-layer chromatography (TLC) plates were from Macherey–Nagel (Düren, Germany).

2.2. Apparatus

A Kontron Instruments liquid chromatograph (Milan, Italy) with two pumps 420, autosampler 460, oven controller 480, dual UV–Vis detector 430 and 80286 personal computer with Data System 450, version 1.85 was used.

2.3. Procedures

Two on-line coupled columns were used: a Hewlett-Packard (Palo Alto, CA, USA) PL-Gel 50 Å, 300 × 7.5 mm I.D. with pre-column (same characteristics but 50 × 7 mm I.D.), and a Scharlau (Barcelona, Spain) Nucleosil 100-7 OH, 7

μm, 25 × 4.6 mm I.D. Two UV wavelengths were set, 280 and 254 nm. The mobile phase composition was *n*-hexane–dichloromethane (73:27, v/v). The flow-rate was 0.9 ml min^{-1}. The oven temperature was 35°C in all cases.

To allow the separation of residual polymer from the studied compounds, a Rheodyne (Cotati, CA, USA) Model 7030 ARV three-way valve with electric two-position actuator (Thar Designs, Pittsburgh, PA, USA) was used. The automatic control of this valve was via software.

Solvents were degassed by ultrasonic bath (15 min) and they were filtered through PTFE 0.45-μm filters before use. A 10-μl volume of the THF sample solution was injected into the column. Broadening appeared in peak shapes when injecting larger volumes (more than 20 μl), due to the higher polarity of THF compared with the mobile phase used. Alternatively, standard solutions of the compounds dissolved in mobile phase were injected without problems.

Samples were also filtered through syringe PTFE filters (luer lock type, 0.45 μm) before its injection in the HPLC system.

Spiked samples of polymer and antioxidants were prepared as follows: 0.01 g of polystyrene were added to 10 ml dichloromethane solution which contained 25 μg ml^{-1} of each antioxidant and UV stabilizer. This solution was used to optimize the analytical procedure with the two columns connected in series.

3. Results and discussion

3.1. Behaviour of the SEC column

The stationary phase used in SEC is usually a copolymer of styrene–divinylbenzene which has a neutral behaviour against mobile phases as THF, dichloromethane, chloroform or N,N-dimethylformamide, although these gels can be used in a wide range of polarities from cyclohexane to acetonitrile or methanol. The common mechanism of separation is size-exclusion, and the solvent used as mobile phase has the ability of swelling up the stationary phase. Consequently, the pore size changes and the separation capacity is modified, too. This effect is shown as a variation of the retention time of each compound.

However, when using lower-polarity solvents such as *n*-hexane, cyclohexane or mixtures of them with those previously cited, an adsorption effect appears as was described before in different works with SEC [30–32]. This has been confirmed by studying the behaviour of benzophenone and styrene. So, the plot of retention times differences (directly related with the resolution) vs. the percentage of *n*-hexane in the mixture used as mobile phase showed that when the percentage of *n*-hexane is lower than 70%, the predominant mechanism is size-exclusion, whereas at higher proportions of *n*-hexane the adsorption effect is very clear.

3.2. Determination of antioxidants and UV stabilizers

Once the behaviour of the SEC column was established, the determination of some antioxidants and UV stabilizers commonly used in packaging materials for food contact was carried out. All the compounds studied have a similar chemical structure. In consequence, the SEC column is not enough to achieve their analytical separation. When two identical SEC columns were connected in series, the resolution was slightly improved, but still insufficient. Furthermore, under these conditions, the time of analysis was doubled in comparison to that when using only one column.

As one of the major components in the packaging materials is the polymer, the analytical procedure involves the separation of the polymer from the rest of the studied compounds, and this first separation was successfully achieved by the SEC column.

In order to get the whole process, separation of major components, usually called clean-up, and analytical separation in only one step, two different HPLC columns were connected in series. The first one was the SEC column, and the second one a normal-phase (silica) column. A three-way switching valve was connected between the two columns, as shows Fig. 1. Both

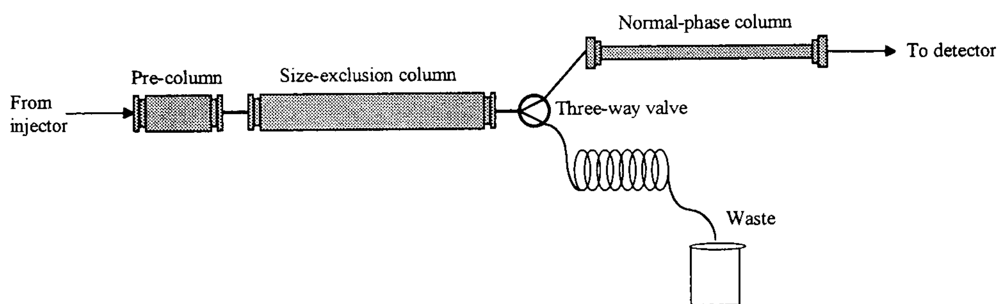


Fig. 1. Diagram of the system with SEC and normal-phase columns and a switching valve between them for on-line clean-up and analysis of antioxidants and UV stabilizers from polymer extracts.

columns are compatible with the organic mixture of *n*-hexane and dichloromethane used as mobile phase. The ratio of *n*-hexane and dichloromethane was previously optimized by TLC using UV light to show the obtained spots. An optimum ratio of dichloromethane–*n*-hexane (27:73) was found.

Fig. 2a shows the chromatogram obtained when a solution containing polystyrene and a mixture of antioxidants and UV stabilizers was analyzed using only the SEC column. Fig. 2b shows the chromatogram obtained using only the silica column (without polymer), and Fig. 2c the chromatogram of the same solution when the whole system of Fig. 1 was applied.

Compared to the work of Johnson et al. [29] our procedure presents some advantages: (1) it is applied satisfactorily (only 30 min/analysis) to the determination of antioxidants and UV stabilizers, even if the resolution is low in some cases, as commented above; (2) no gradient is used, so that no equilibration time is needed, and a second HPLC gradient pump is unnecessary; (3) sensitivity is much higher than in the cited work because around 10 ml —the fraction between 9 and 20 min shown in Fig. 2a— are transferred to the second column instead of 10–50 μl in the previously commented case.

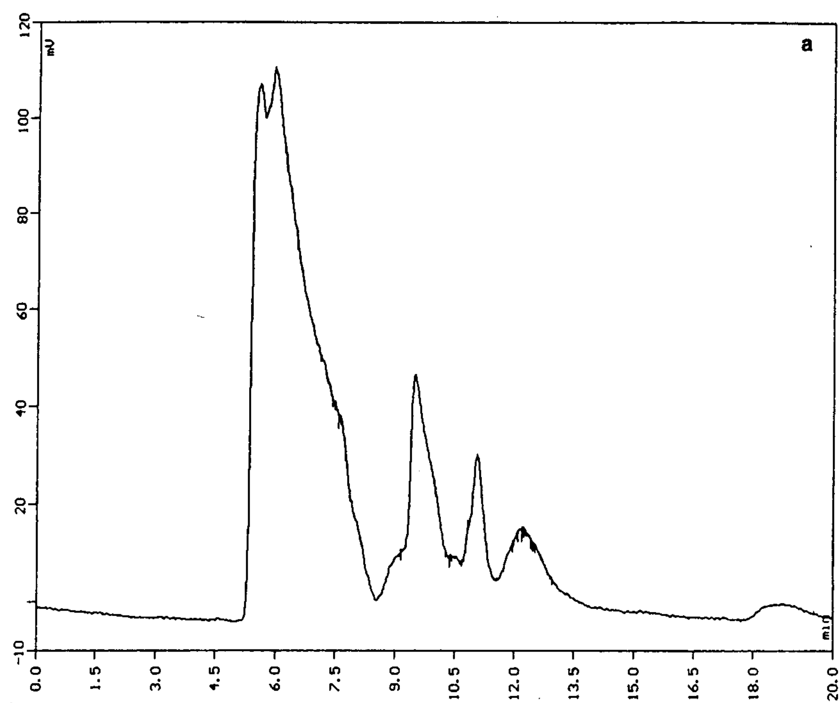
As can be seen, the separation of the compounds has been improved by the two columns in series. The polymer has been drained off through the valve and it is not introduced into the second column, so that the silica column is preserved from any damage and its operative life extended.

When the automatic valve changes from the drainage position to the normal-phase column position, the pressure of the system is slightly increased during a period of 30 s from 27 to 49 bar. This allows the drainage without problems. A 2-ml capillary loop was connected in the outlet of the valve in the drainage mode to simulate pressure of the system during the sudden break. Although the polarity gradient of solvent is supported by this system, the isocratic mode was used throughout to avoid long stabilization periods between different runs.

3.3. Analytical features

The detection limits, for all the compounds studied expressed as the equivalent concentration to three times the area of the background noise, are shown in Table 1. As can be seen, in all cases these values are about 0.1 μg ml^{-1} with the only exception of Cyasorb UV 1084 (because of its low sensitivity at the wavelengths used) and Cyasorb UV 9, because of its chemical nature (benzophenone derivative), has a very strong interaction with the stationary phase and a broad peak appears in the chromatogram.

The analysis of a standard solution containing all the mentioned compounds showed very good precision and accuracy, even in the case of peaks with incomplete separation, and quantitative accurate results were obtained (Table 1). A



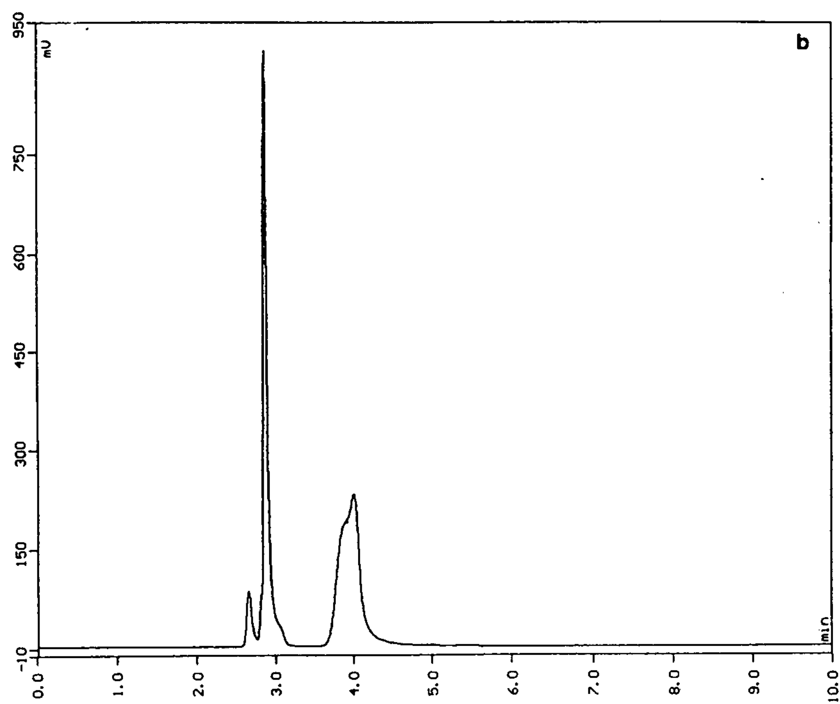


Fig. 2. (*Continued on p. 235*).

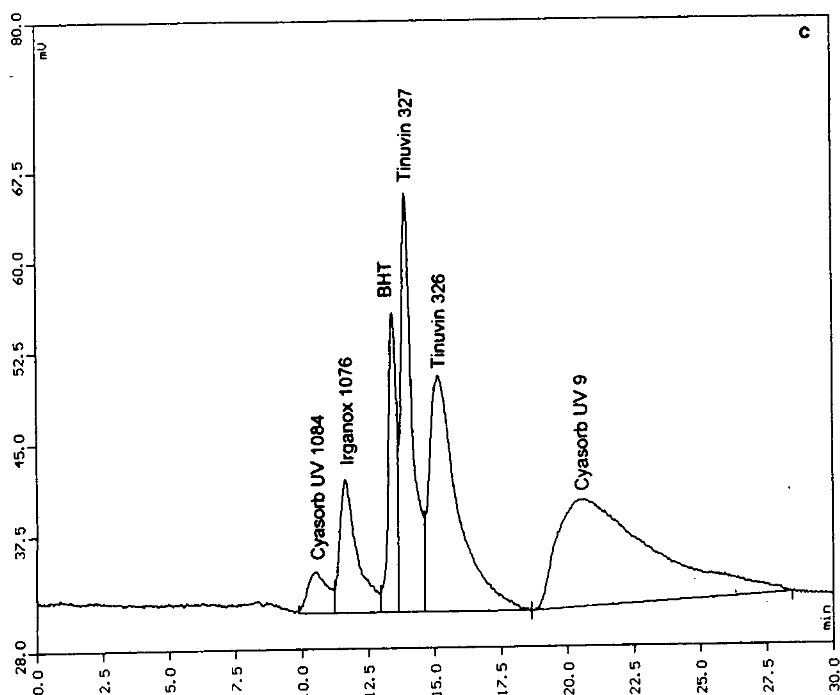


Fig. 2. Chromatograms obtained from (a) polymer (first peak) and additives only with SEC column and (b) additives only with normal-phase column. (c) Final chromatogram of the analysis of additives from polymer sample according to the proposed method. Conditions (for all cases): flow 0.9 ml min^{-1}; mobile phase n-hexane–dichloromethane (73:27, v/v); column temperature 35°C.

Table 1
Analytical characteristics of the determination of antioxidants and UV stabilizers by coupling both HPLC columns (SEC + normal phase)

Compound	Retention time (min)[a]	Detection limit (μg ml^{-1})	Linearity (r)	Precision (R.S.D., %)[b]	Accuracy[a,c]	Linear range (μg ml^{-1})
Cyasorb UV 1084	0.50 ± 0.08	1.1	0.9989	3.2	24.4 ± 0.8 (24.8)	5.0–200
Irganox 1076	11.62 ± 0.07	0.2	0.9992	2.4	25.6 ± 0.6 (25.0)	1.0–200
BHT	13.44 ± 0.05	0.1	0.9994	3.7	25.4 ± 0.9 (24.9)	0.5–200
Tinuvin 327	13.95 ± 0.11	0.1	0.9981	4.0	24.1 ± 1.0 (24.8)	0.2–200
Tinuvin 326	15.12 ± 0.09	0.1	0.9978	2.9	25.8 ± 0.8 (25.3)	0.5–200
Cyasorb UV 9	20.64 ± 0.34	1.1	0.9976	4.2	26.0 ± 1.1 (25.5)	5.0–200

[a] Confidence level 95%, 6 replicates.
[b] Calculated with 6 replicates (ca. 25 μg ml^{-1}).
[c] Found value; real value in parentheses.

slightly higher sensitivity was obtained at 280 nm than at 254 nm. A wide linear range was obtained for all the compounds, which allows the direct determination of the compounds in the common plastics used.

4. Conclusions

The main conclusion is that on-line coupling of two high-resolution columns with different stationary phases —exclusion and adsorption— is an attractive and powerful methodology for the simultaneous analysis of different compounds by HPLC. So, this method permits the automatization of clean-up and analysis in one step, decreasing both sample handling and analysis time.

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Calibration and determination of volatile fatty acids in waste leachates by gas chromatography

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Abstract

Low-level radioactive waste leachates were analyzed for volatile fatty acids by gas chromatography as part of the continuing waste management program at the Chalk River Laboratories. An existing method was optimized whereby carboxylic acids were detected at the mg/l level with a precision of 5% or better for C_2–C_7 acids and an accuracy of 3% or better for acetic acid. Parameters such as sample handling, calibration and accuracy are discussed.

1. Introduction

Low-level radioactive wastes (LLRWs) contain large amounts of cellulose-based materials such as paper towels, used clothing, corrugated board, etc. After waste burial, disposal, or in leachates, microbial degradation of the organic substrate produces high quantities of dissolved organic matter (DOM) [1–4]. Volatile fatty acids (VFAs), defined here as C_2–C_7 monocarboxylic aliphatic acids, were the most abundant class of compounds in the DOM present in landfills or LLRW leachates [1,5–7]. VFAs, particularly acetic acid, may have an impact on Pu migration in soils [8] thus monitoring its contents and production with time in the leachates could be important. An experimental program was carried out at the Chalk River Laboratories (CRL) to monitor the decomposition products of LLRWs. Wastes were collected and compacted into bales, and eight such bales were leached with water in a closed-loop recirculation system [1].

The VFA content in the leachates was determined by liquid–liquid extraction of the aqueous phase with ether, and a 10-μl aliquot of the organic phase injected in a GC system equipped with flame ionization detection (FID) [9–12]. In this method, since the VFAs have to transfer from the aqueous to the organic phase, the reproducibility and calibration could represent a problem, especially if the extraction is not quantitative. The original method [9,11] did not include a calibration procedure, so we had to develop a calibration method for this work. We have used a commercial mixture of C_2–C_7 carboxylic acids, which were liquid–liquid extracted similarly to the leachate samples. However, when the slope of the individual calibration curve of each VFA was plotted as a function of the number of carbon atoms, the signal did not increase linearly as it should [13] for acetic and propionic acids. We suspected that this was due

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to a non-quantitative extraction of acetic acid. We have also used a total carbon analyzer as an independent method to check for dissolved organic carbon content in the standards. Our work aims to verify the accuracy of the calibration procedure for the preparative work and analysis of the samples, and to improve our precision. Potential problems associated with calibration will be briefly discussed.

2. Experimental

2.1. Instruments

A Perkin-Elmer Model 8500 GC-FID system was used with a stainless-steel column (6 ft. × 0.085 in. I.D.; 1.83 m × 0.216 cm I.D.) packed with GP-10% SP-1200/1% H_3PO_4 on 80–100 mesh Chromosorb W AW (Supelco, Bellefonte, CA, USA). The carrier gas was helium (Linde, high purity) at a flow-rate of 30 ml/min. The detector base and injection port temperatures were set at 250°C. The runs were performed using a temperature program consisting of a 3-min isothermal period at 70°C, followed by a 10°C/min temperature ramp to 130°C, a second temperature ramp (5°C/min) to 180°C, and a 1-min isothermal period at 180°C. The stainless-steel column used may not represent an adsorption or ghosting problem as the amounts of VFAs are high [1] and this combination of gas flow-rate and temperature programming gives a good separation (Fig. 1).

The total carbon analyzer used for the calibration was a Dohrmann DC-80 (Rosemount Analytical, Santa Clara, CA, USA). The instrument was calibrated in the 400 mg C/l range with potassium hydrogen phthalate. The VFA content of the standards agreed within 2% with this instrument for up to 500 mg C/l. Above this level, dilution was necessary.

2.2. Sample preparation

The leachate samples were acidified at pH 2 using concentrated nitric acid for dissolved metal analysis for a separate study. An aliquot of this solution was taken for VFA analysis. This change in acid (sulfuric acid was used in the original procedure [9]) did not change the accuracy (results not shown). A 1-ml aliquot of the acidified leachate was shaken along with 1 ml of diethyl ether for approximately 30 s. The supernatant ether phase was quantitatively transferred to a volumetric flask using a Pasteur pipette. The volume of ether extract was adjusted to the 1-ml mark and a small amount (approximately 0.2 g) of anhydrous magnesium sulfate was added to absorb traces of water. The extract was allowed to stand for approximately 10 min in an ice bath and it was then transferred to a 1-ml reaction vial with a Mininert cap. A 10-μl volume of this extract was injected into the GC system using a syringe. The extracts could be stored at room temperature in these vials for up to a week.

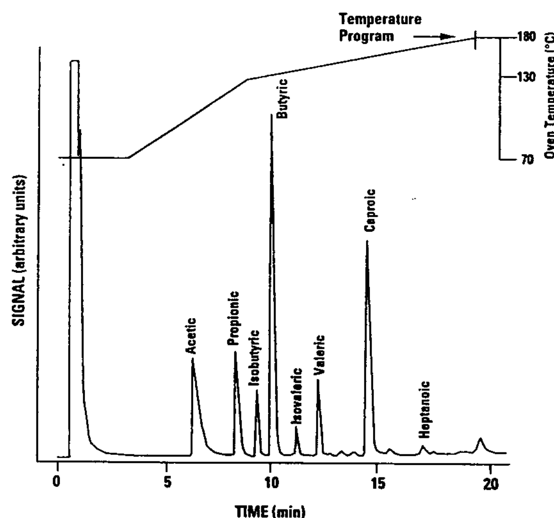

Fig. 1. Chromatogram of VFA extract from a waste leachate sample, also showing the GC temperature program.

2.3. GC calibration and VFA standards

A series of standards for the calibration curves was prepared in the same manner as described above. Individual calibration curves were obtained for each compound using the standard VFA mixture, in concentrations ranging from 0 to 10 m*M* for all VFAs except for acetic acid (0

to 100 m*M*). The standards used for the successive extractions were prepared using dilute acetic acid (calibrated with the carbon analyzer) mixed in equal proportions with the primary VFA standard to obtain a final concentration of 0.057 *M* for acetic acid and 0.005 *M* for the other VFAs.

2.4. Chemicals

The primary C_2–C_7 VFA standard mixture was purchased from Matreya (Pleasant Gap, PA, USA; catalog No. 1075). These other chemicals were used: anhydrous diethyl ether (Fisher; 99 + % purity), anhydrous magnesium sulfate (Fisher, 98.0%) and potassium hydrogen phthalate dried overnight at 60°C (Fisher, 99.95–100.5%, ACS primary standard).

3. Results and discussion

Fig. 1 shows a chromatogram from the analysis of an actual leachate sample. The 3-min isothermal period allowed for the complete elution of ether, so the signal reached the baseline prior to elution of acetic acid. A gradual temperature gradient was used after 9 min to ensure a good separation of the heavier VFAs. Table 1 shows the precision for three separate extractions. In general, our experiments have shown that there was a larger variability between duplicate extractions than between duplicate injections of the same organic aliquot. We have consistently obtained a precision of 5% or better in separate extractions.

The leachate samples and calibration standards (C_2–C_7 VFAs) were liquid–liquid extracted under the same conditions after pH adjustment to <2 to ensure protonation of the carboxylic acid groups. Despite the caution used to perform the extractions, the slopes of the calibration curves did not increase proportionally with the number of carbon atoms for all the VFAs (□, Fig. 2). This lead us to examine the extraction step in detail.

Theoretical considerations (Tables 2 and 3) suggested that quantitative transfer was possible for the more hydrophobic VFAs, but not for acetic and propionic acids. The data in Tables 2 and 3 use the capacity of a substrate to transfer to the organic phase, given by:

$$k' = P \cdot \frac{V_o}{V_a}$$

where k' = capacity factor, P = liquid–liquid partition coefficient, V = volume of solution, and the subscripts o and a stand for organic and aqueous phases, respectively. P is defined as [14]:

Table 1
Results showing the precision of triplicate extracts from a leachate sample

Acid	Peak area for injection No: (arbitrary units)			Average	R.S.D. (%)[a]
	1	2	3		
Acetic	1599	1657	1706	1654	2.7
Propionic	913	930	950	931	1.6
Isobutyric	446	452	455	451	0.9
Butyric	2643	2665	2701	2667	1.0
Isovaleric	167	169	171	169	0.9
Valeric	652	654	664	657	0.8
Caproic	2060	2060	2107	2071	1.2
Heptanoic	56.9	53.3	60.5	56.9	5.2

[a] R.S.D. = Relative standard deviation = 100 · standard deviation/mean.

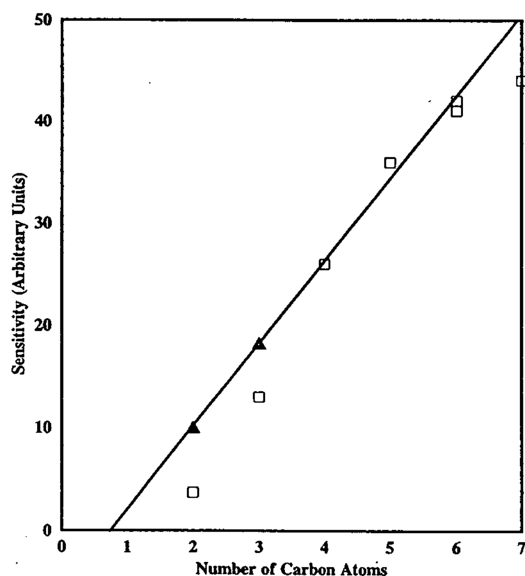

Fig. 2. Sensitivity (or response factor) of the individual VFAs as a function of the number of carbon atoms of the compounds. Note that the line does not go through the origin, since the FID signal is weak for oxygenated carbon compounds such as carboxylates [13]. □ = Experimental; ▲ = corrected sensitivity; line = fitted C_2–C_6.

$$P = \frac{[\mathrm{VFA}]_o}{[\mathrm{VFA}]_a}$$

where the brackets [] denote concentrations. Table 2 shows that there is a linear relationship between the number of carbon atoms of the VFA and log P. The branched VFAs (isobutyric acid, etc.) are included in the relationship for chloroform, thus the C_2–C_6 relationship including the branched VFAs should also be linear for ether.

We consider a liquid–liquid extraction quantitative if 95% or more of the analyte is extracted into the organic phase, which translates to a log $k' \geq 1.25$ or log $P \geq 1.25$ for a 1 ml to 1 ml extraction. Note that in the Supelco method [11], a 1:2 organic-to-aqueous ratio was used, where theoretically only the C_6 or higher VFAs could transfer quantitatively. We have performed three successive extractions of the same aqueous standard, each time with a fresh ether layer to determine if the liquid–liquid extractions were quantitative with the C_2–C_7 VFAs (Table 4). Note that the individual VFA values (determined separately with the GC method) agreed within 3–11% of the label-declared value, and the sum of all the VFAs combined was within 1% of the total DOM value given by the carbon analyzer. In a separate run, the carbon analyzer values for acetic acid agreed within 3% with the GC method.

Table 3
Calculated log P for monocarboxylic aliphatic acids using the relationship of Table 2

Acid	No. of carbon atoms	Log P	
		From [14]	This work, calculated
Acetic	2	−0.34	−0.38
Propionic	3	0.2	0.18
Isobutyric	4		0.75
Butyric	4	0.68	0.75
Isovaleric	5		1.31
Valeric	5	1.24	1.31
Isocaproic	6		1.87
Caproic	6	1.95	1.87
Heptanoic	7		2.43

Table 2
Relationships between log P and the number of carbon atoms for C_2–C_6 monocarboxylic aliphatic acids for chloroform–water and diethyl ether–water (data from [14])

Solvent	Slope a	Intercept b	n	r^2
Chloroform	0.6032	−2.7118	11	0.9885
Diethyl ether	0.5620	−1.5020	5	0.9946

Log $P = ax + b$, where x = number of carbon atoms.

Table 4
Peak areas from successive extractions of the same VFA mixture with fresh ether phase

Acid	Peak area for extraction No. (arbitrary units)			Amount of compound		
				Found by GC		Label value (mg C/l)
	1	2	3	μmol	mg C/l	
Acetic	1622	1118	827	0.601	1442	1369
Propionic	579	211	73.8	0.045	160.7	180.2
Isobutyric	1226	168	N.D.	0.047	226.3	240.2
Butyric	1193	176	N.D.	0.046	220.5	240.2
Isovaleric	1766	113	N.D.	0.048	290.7	300.3
Valeric	1763	105	N.D.	0.048	290.1	300.3
Isocaproic	2210	73.4	N.D.	0.053	379.1	360.3
Caproic	2252	73.5	N.D.	0.053	381.9	360.3
Heptanoic	2362	80.6	N.D.	0.053	446.4	420.4
Total VFA (mg C/l) (sum of individual compounds)					3838	3771
Total VFA (mg C/l) (using carbon analyzer)					3801	

The amount of compound found was calculated using the first injection data, and the concentrations of the individual compounds are also shown (Label value). N.D. = Not detectable.

The first extraction was incomplete for all the VFAs, particularly the C_2, C_3 and C_4 VFAs. The C_4 VFAs may not be a problem as their slope match the solid line in Fig. 2, and the extractions were approximately 95% or better for the higher VFAs. After the first extraction, 28.6% acetic acid and 64.3% propionic acid were recovered in the organic phase, which corresponds to $P = 0.4$ ($\log P = -0.398$) and $P = 1.8$ ($\log P = 0.255$), respectively. If the slopes of the calibration curves are corrected for 100% extraction efficiency for these two compounds, the values would match the linearity model (▲ in Fig. 2). This approach would suggest that all the extractions for C_2 and C_3 VFAs should be corrected to 100% efficiency, which may add one extra step in data manipulation. However, if the standards are rigorously manipulated in the same way as the samples in the preparation step, and if the precision is consistent and within reasonable limits (in our case <5%), then such a correction factor is not necessary. Therefore, analysts must be aware of these limitations, because the accuracy may suffer, especially if the extractions are not repeatable.

4. Conclusions

Potential limitations on the analysis and the calibration procedures were found with the GC analysis of VFAs using liquid–liquid extractions. This could affect the precision and accuracy of the results. We have reported a consistent precision with good reproducibility (1–5%). We have used the carbon analyzer as an independent method to ensure the accuracy of the calibration, and concluded that our current calibration method is satisfactory. Analysts using this method can calibrate their instruments using liquid–liquid extraction of standards, provided that the samples and the standards are rigorously treated in the same way.

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Short communication

Recoveries of organochlorine compounds (polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans) in water using steam distillation–solvent extraction at normal pressure

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Abstract

A simultaneous steam distillation–solvent extraction (SDE) procedure was used for determining polychrlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCBs, PCDDs and PCDFs) at sub-ppb levels in water samples. Recoveries of 39.8–138.7% and a standard deviation of lower than 10% were achieved for the individual coplanar PCB and the 2,3,7,8-substituted PCDD/F congeners. SDE is a fast, clean, inexpensive and reproducible procedure for the determination of individual PCBs, PCDDs and PCDFs in waters at very low concentrations.

1. Introduction

Polychlorinated biphenyls (PCBs), polichlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) have been recognized as being highly toxic and ubiquitous environmental contaminants [1,2]. Of them, the most toxic are the PCB coplanar congeners (unsubstituted and mono- and di-*ortho*-substituted), with toxicity levels near that of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [3], and the PCDD and PCDF congeners with chlorines in the 2,3,7,8-positions.

The standard analytical procedure for monitoring the concentrations of organochlorine compounds in waste water utilizes either sequential or continuous liquid–liquid extraction [4,5]. Increasing concern about the use of large volumes of organic solvents, however, has resulted in efforts to develop alternative extraction approaches.

Solid-phase extraction (SPE) using many types of adsorbent (XAD-2 macroreticular resin, graphitized carbon, alumina, silica, etc), Sep-Pak C_{18} cartridges and Empore discs is an alternative approach which has received considerable attention. This technology offers several significant advantages over the conventional liquid–liquid extraction techniques typically employed for water analysis. It reduces the volume of solvent

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required for extraction and provides a concentration step without the necessity for removing a large volume of solvent [6,7]. The disadvantages of the adsorption methods are the presence of organic impurities coming from some adsorbents and the long analysis time [8–11].

Steam distillation extraction (SDE) at normal pressure, in which the compounds were first distilled and then extracted into an organic solvent, has recieved much attention in recent years [12]. The small amounts of solvent used (2 ml) and the short analysis time (1 h) have made SDE a very attractive extraction–concentration technique for organochlorine compounds in waste water [13,14]. In addition, when the solvent was compatible with the analytical technique and the concentration of the analytes was high enough, the preconcentration step, before the chromatographic injection, could be avoided.

Since SDE was first used by Likens and Nickerson [12] in 1964, different modifications has been introduced to improve the results and extend the field of its applications, including the environmental field [15–20].

The objective of this study was to evaluate the steam distillation–solvent extraction at normal pressure procedure as an alternative to solid-phase extraction in determining organochlorine compounds (PCBs, PCDDs and PCDFs) in water at sub-ppb levels. For this purpose, a new micro-version of the SDE apparatus was proposed earlier [21]. So far, this SDE method has been found to be highly efficient for the extraction of compounds with different polarity, volatility and solubility from food products [22,23].

2. Experimental

2.1. Standard solutions

PCB standards

The 14 individual PCB coplanar congeners, selected by their abundance in environmental samples or by their toxicity (Table 1), were purchased from Ehrenstorfer (Germany).

PCDD and PCDF standards

EPA 1613 stock native standard solution, chemical purity >98% (Chemsyn Science Labs., Lenexa, KS, USA), consists of a mixture of 2,3,7,8-substituted congeners from tetra- to octa-PCDD and -PCDF, which are the most toxic congeners, at concentrations from 0.4 to 4.0 μg/ml from tetra- to octa-PCDD/Fs.

PCB standard solutions

A working stock standard solution was prepared containing 1 μg/ml of each of the 14 PCB individual congeners in *n*-pentane and stored at 4°C. synthetic mixtures were prepared directly in the extraction flask by adding the appropriate volume of the stock standard solution to 100 ml of water purified in a Milli-Q system (Millipore). Final concentrations of 1, 0.1 and 0.01 ppb (ng/ml) of each PCB were prepared.

PCDD/F standard solutions

The EPA 1613 PCDD/F stock native standard solution was used as a working standard solution. Synthetic mixtures were prepared directly in the extraction flask by adding the appropriate volume of the working standard solution. The congener concentrations ranged between 2.5 and 2000 pg/ml (ppt) from the tetra- to octa-congeners (Table 2).

The extractions of PCBs and PCDD/Fs were carried out separately.

2.2. Simultaneous distillation and extraction

Sample extraction and concentration were carried out using the micro SDE equipment designed by Blanch et al. [21]. This microinstrument was basically a distiller with two distillation arms connected to two separate flasks. The flask containing the sample and that containing the extraction solvent were placed in different heating baths and each of them was distilled through the individual arms. After their vapours had condensed on the cool condenser surface, they were mixed in the separation chamber where the

extraction process took place. The continuous circulation and mixing of both the aqueous and the organic phases allowed efficient and rapid extraction to be achieved.

The new design of this SDE includes an enlarged surface condenser, which allows more effective mixing of the solvent vapour with sample vapour and, the fact that the distillation solvent and distillation sample arms enter the mixing chamber at the same height, allowing the use of both high- and low-density extraction solvents with only one configuration. The main advantage of this system is the high recovery in a very short time even for small amounts of analytes.

The experimental conditions for SDE were chosen according to the results obtained by Blanch et al. [21], who optimized the experimental variables using the modified sequential simplex method [24]. These optimized variables were chosen for a wide range of compounds of different natures, and it can be expected that the values of these variables would be adequate for PCBs and PCDD/Fs. Among the various possible solvents that could be used, *n*-pentane was chosen for the first experiments. The use of *n*-pentane allowed the direct injection of the extract obtained into a high-resolution gas chromatographic–electron-capture detection (HRGC–ECD) system without concentration for the higher concentrations. *n*-Pentane was replaced with dichloromethane in the analysis of PCDD/Fs at the lower concentrations (2.5–20 ppt for tetra- to octa-chlorinated congeners) to obtain better recoveries.

Thus, the experimental variables adopted were as follows: sample heating bath temperature, 149°C; solvent heating bath temperature, 67°C; coolant temperature, 3.4°C; and extraction time, 56 min. A volume of 2 ml of the solvent was used. The final extract was concentrated to 0.2 ml under a stream of nitrogen for the lower concentrations when *n*-pentane was used and 0.8–1.2 μl of the extract were injected to the HRGC–ECD system. When dichloromethane was used it was necessary to evaporate to dryness and change the solvent.

2.3. HRGC–ECD

A Perkin-Elmer (Beaconsfield, UK) Model 8600 gas chromatograph equipped with a ^{63}Ni electron-capture detector was used.

The column was DB-5 (60 m × 0.25 mm I.D., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA). The column temperature was programmed from 60°C (held for 1 min) at 50°C/min to 180°C, then at 4°C/min to 230°C (held for 40 min) and finally at 4°C/min to 270°C (held for 10 min). Nitrogen was used as the carrier gas at a flow-rate of about 22 psi. Samples of 0.8–1.2 μl were injected in the splitless mode.

2.4. Quantitative analysis

Identification of individual PCB, PCDD and PCDF congeners was based on retention times relative to stock standard solution mixtures. The concentrations of individual congeners were determined by comparison of the individual peak-area responses with those for similar concentrations in the stock standard solutions, in the linear range of the detector used. The PCBs with IUPAC Nos. 12 and 209 [25], added before the injection, were used to correct for injection errors and detector fluctuations.

3. Results and discussion

3.1. PCB congeners

Table 1 shows the concentrations of PCB congeners investigated in different assays and their recoveries as an average of three experiments. In all instances the relative standard deviations (R.S.D.) was lower than 10%. Fig. 1 shows a chromatogram of a PCB standard mixture with ppb level concentrations.

The recoveries of all PCB congeners were around 100% for the higher concentrations (1 and 0.1 ppb). For lower concentrations (0.01 ppb), the recoveries were around 100% for all congeners except for PCB 101, 118, 126 and 156, for which they were between 56.8 and 75.5%.

Table 1
Recoveries of the method for PCB congeners (in *n*-pentane) at concentrations of 1, 0.1 and 0.01 ppb (ng/ml) in water

Peak No.	PCB structure	IUPAC No.	Recovery (%)		
			1 ppb	0.1 ppb	0.01 ppb
1	2,2′4,5,5′-P_5CB	101	97.7	95.0	56.8
2	3,3′4,4′-T_4CB	77	93.3	97.5	92.9
3	2,2′3,5,5′,6′-HxCB	151	101.1	113.6	102.0
4	2,3′4,4′,5-P_5CB	118	100.4	100.5	70.4
5	2,2′4,4′,5,5′-HxCB	153	97.9	97.0	114.6
6	2,3,3′,4,4′-P_5CB	105	97.9	92.3	102.6
7	2,2′,4,4′,5-P_5CB	138	99.8	108.2	126.4
8	3,3′,4,4′,5-P_5CB	126	101.9	104.7	68.2
9	2,3′,4,4′,5,5′-HxCB	167	102.6	93.3	104.8
10	2,3,3′,4,4′,5-HxCB	156	100.6	104.7	75.5
11	2,2′,3,4,4′,5,5′-HpCB	180	113.7	102.4	101.7
12	3,3′,4,4′,5,5′-HxCB	169	98.9	113.1	92.6
13	2,2′,3,3′,4,4′,5-HpCB	170	103.4	102.6	111.8
14	2,2′,3,3′,4,4′,5,5′-OCB	194	102.2	108.6	105.7

These values, however, are satisfactory in comparison with those obtained by other extraction methods, such as Soxhlet extraction and sonication [19,26].

The results are in accordance with those of other workers [13,15], who used older versions of SDE in the extraction of organochlorine compounds for water samples at ppb levels.

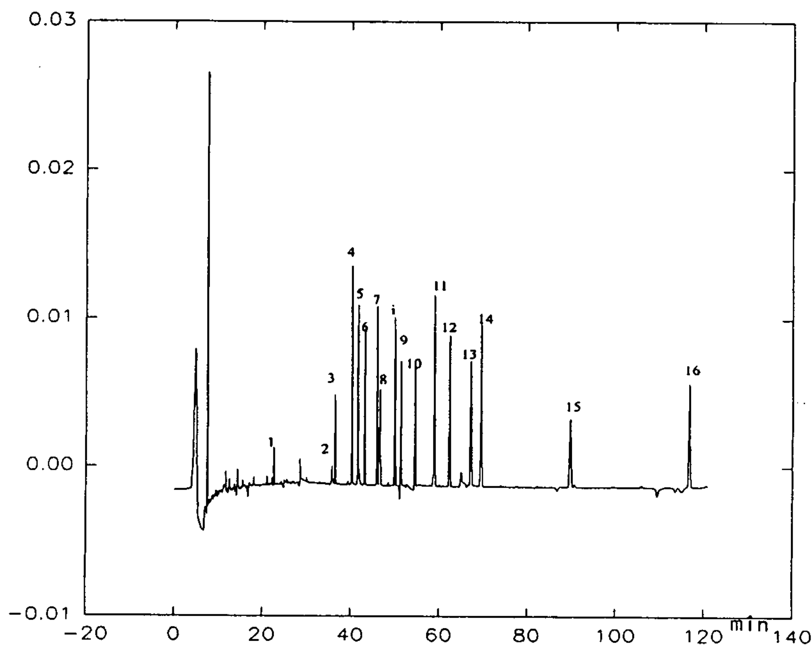

Fig. 1. Separation of 14 PCB congeners included in the standard mixture at ppb levels. Peaks 1 and 16 (PCB-12 and PCB 209) are internal standards; i = unknown. For other peak identifications, see Table 1.

The use of the new version of SDE developed by Blanch et al. [21] together with the implementation of modified sequential simplex optimization of the operating variables allowed the extraction of the individual PCBs from water samples at ppt levels, with high recoveries.

3.2. PCDD/PCDF congeners

Table 2 gives the concentrations of PCDD and PCDF congeners investigated in different assays and their recoveries as an average of three experiments. In all instances the relative standard deviation was lower than 10%. Fig. 2 shows a chromatogram of the PCDD and PCDF standard mixture at ppb levels.

The concentrations of the individual congeners were in the ppt range and the recoveries depended on both the concentration level and the physical characteristics of the different PCDD/F congeners. The recoveries decreased strongly with decreasing concentration of PCDD/F congeners.

When SDE was carried out under the same conditions as for the PCB extractions, good recoveries were found from penta- to hepta-PCDD/F congeners at 0.1 and 1 ppb concentrations, and in general the recoveries decreased as the level of chlorination increased. The recoveries obtained for the lower chlorinated isomers (tetra-PCDD/F) were always lower than those found for penta- to hepta-congeners. This is probably due to the lower concentration of these two isomers in the standard commercial solution, which are four times lower than those of the penta-, hexa- and hepta-congeners. The recoveries of OCDD and OCDF isomers were around 50%. These two isomers have the highest molecular masses and the lowest vapour pressures [26], and probably these characteristics are related to the low recoveries.

The low efficiencies found at lower concentrations (2.5–20 ppt from tetra- to octa-PCDD/

Table 2
Recoveries of the method for PCDD and PCDF congeners down to sub-ppb (ng/ml) concentration levels

No.	Structure	*n*-Pentane solvent						Dichloromethane solvent	
		Recovery (%)	ppb[a]	Recovery (%)	ppb[a]	Recovery (%)	ppb[a]	Recovery (%)	ppb[a]
1	2,3,7,8-TCDF	49.0	0.25	46.9	0.025	55.5	0.0025	138.7	0.0025
2	2,3,7,8-TCDD	76.5	0.25	60.0	0.025	50.1	0.0025	105.6	0.0025
3	1,2,3,7,8-PCDF	94.7	1	70.1	0.1	48.1	0.01	108.7	0.01
4	2,3,4,7,8-PCDF	137.7	1	91.6	0.1	–[b]	0.01	89.6	0.01
5	1,2,3,7,8-PCDD	107.1	1	68.1	0.1	70.8	0.01	111.8	0.01
6	1,2,3,4,7,8-HxCDF	100.6	1	69.2	0.1	39.4	0.01	108.9	0.01
7	1,2,3,6,7,8-HxCDF	95.3	1	71.3	0.1	36.1	0.01	100.4	0.01
8	2,3,4,6,7,8-HxCDF	129.6	1	86.4	0.1	72.5	0.01	132.7	0.01
9	1,2,3,4,7,8-HxCDD	86.8	1	71.4	0.1	35.4	0.01	104.0	0.01
10	1,2,3,6,7,8-HxCDD	80.8	1	77.8	0.1	24.8	0.01	102.2	0.01
11	1,2,3,7,8,9-HxCDD	92.3	1	65.7	0.1	63.2	0.01	107.6	0.01
12	1,2,3,7,8,9-HxCDF	83.7	1	77.4	0.1	67.5	0.01	109.9	0.01
13	1,2,3,4,6,7,8,HpCDF	95.7	1	62.5	0.1	47.4	0.01	70.8	0.01
14	1,2,3,4,6,7,8-HpCDD	72.7	1	84.3	0.1	39.5	0.01	74.7	0.01
15	1,2,3,4,7,8,9-HpCDF	81.6	1	83.2	0.1	38.8	0.01	95.7	0.01
16	OCDF	40.5	2	39.1	0.2	42.4	0.02	55.9	0.02
17	OCDD	39.5	2	42.9	0.2	37.9	0.02	49.4	0.02

[a] Concentration of each PCDD/F congener in water.
[b] Not detected.

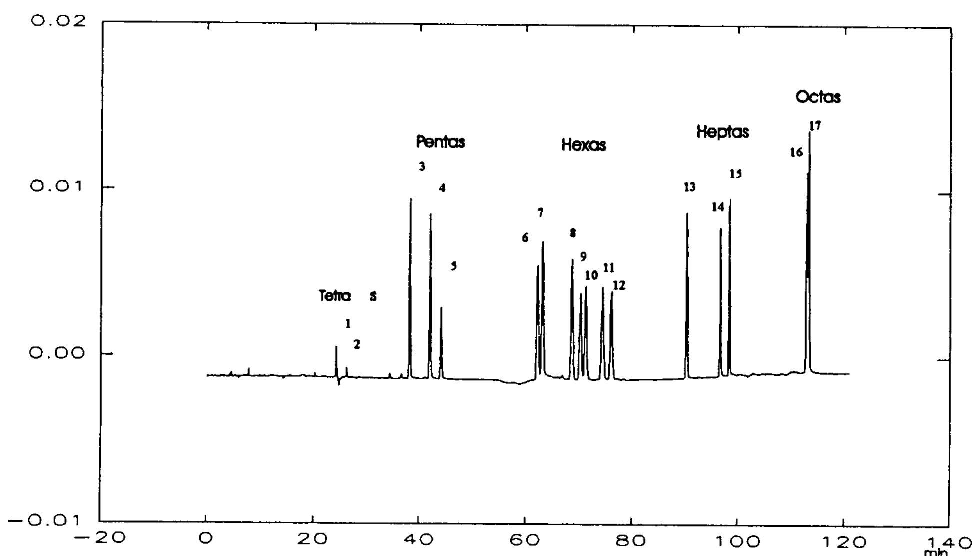

Fig. 2. Separation of the 2,3,7,8-substituted congeners of PCDD and PCDF standard mixture at ppb levels. For peak identifications, see Table 2.

PCDFs), usually lower than 50%, suggested the necessity for optimizing some of the experimental variables. The use of a stronger solvent with a higher affinity for organochlorine compounds, such as dichloromethane, gave better results (Table 2). It can be observed that the recoveries increased significantly, being almost 100% for all the isomers except for octachlorine isomers. Therefore, the use of dichloromethane is advisable when the PCDD/F concentrations in water are lower than 100 ppt, even when an additional step, changing to an electron-capture detector-compatible solvent, was necessary before the chromatographic analysis.

4. Conclusions

The SDE technique allows the determination of organochlorine compounds as individual PCDD, PCDF and PCB congeners at very low concentrations. The SDE apparatus offers, with some exceptions, excellent recovery efficiencies for the investigated compounds in the pg/ml or ng/ml range. In addition, it is inexpensive, fast and, as it needs only a small amount of solvent (2 ml), ecologically beneficial.

The good results obtained for the extraction of organochlorine compounds in water together with the versatility of the micro-SDE apparatus proposed, which allows the use of sample volumes five times larger than those previously used, without any modifications, suggest the possibility of applying this method to real water samples and other environmental samples. These aspects will be the topic of forthcoming works.

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Supercritical fluid extraction of diosgenin from tubers of *Dioscorea nipponica*

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Abstract

Supercritical fluid extraction (SFE) was used to extract diosgenin following acid hydrolysis from *Dioscorea nipponica* tuber. Diosgenin determinations were carried out using capillary GC of trifluoroacetate derivatives. Highest diosgenin yields were obtained using 3100 p.s.i. (1 p.s.i. = 6894.76 Pa) pressure for 70 min, but >82% of this yield was extracted by 40 min. Higher recoveries were obtained than by extraction using light petroleum (b.p. 40–60°C) extraction.

1. Introduction

The tuber of *Dioscorea nipponica* is extensively used in China for extraction of diosgenin sapogenin and its glycoside dioscin [1], for use as steroid intermediate in the pharmaceutical industry. The industrial extraction of diosgenin involves mineral acid hydrolysis of chopped tuber, followed by extraction using petrol [2]. This latter stage is expensive and wasteful of solvent; also low diosgenin recovery is achieved.

Analytical-scale extraction of diosgenin is usually carried out using acid hydrolysis and petrol extraction, but again the extraction stage has disadvantages. This time, the light petrol Soxhlet extraction takes much time, particularly when screening large sample numbers. Acid hydrolysis of dioscin is tedious, but essential, either before or after removal from the plant material. No reports have appeared concerning diosgenin extraction using supercritical fluid extraction (SFE), but it was considered that both analytical- and industrial-scale (SFE) extractions would save time and solvents. Previous workers have used SFE for extraction of a wide range of natural products such as oil products [3], essential oils [4], triglycerides [5] and Chinese herbal drugs [6].

2. Experimental

2.1. Materials

Tuber of *Dioscorea nipponica* was collected from natural habitat in the Hubei province, China, and identified by the Hubei Province Institute of Drug Identification. The tuber (8 g) was cut into small pieces and hydrolysed by refluxing with 50 ml of 2 *M* HCl for 3 h. It was next filtered (Whatman No. 1), washed with 5% Na_2CO_3 and water until neutral, and then dried

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at 80°C. Dried, hydrolysed tuber was then powdered prior to extraction.

2.2. Supercritical extraction

The custom-built supercritical fluid extractor [7] was operated at variable temperatures and pressures of CO_2. Samples were extracted in a 2-ml stainless-steel Upchurch short HPLC column (Anachem, Luton, UK) and the CO_2 extraction solvent (BOC) was made supercritical by pumping at 1.5–3.6 ml/min (LC pump T-414, Kontron). Samples of 0.05 g were extracted over a range of temperatures and pressures for differing periods of time. Constant extraction pressures were maintained by modification of flow-rates of the LC pump. Extracts were vented into 2 ml of $CHCl_3$ via a 20 cm × 50 μm I.D. fused-silica capillary restrictor to collect the extracted diosgenin. The extraction cell, restrictor capillary and collection solvent were maintained at constant temperature in an oven. Solvent loss by evaporation was compensated for by periodic addition of $CHCl_3$ to maintain the original volume. Sample extracts (0.5 ml) were derivatised with 0.3 ml of trifluoroacetic anhydride (TFA) (Sigma) at room temperature for 30 min, and then evaporated to dryness in a stream of nitrogen [8]. Sample residues were re-dissolved in a given volume of $CHCl_3$ containing a standard concentration of 1,8-dihydroxyanthraquinone TFA derivative (1 mg/ml) as internal standard. Diosgenin TFA standards were made from commercial diosgenin (Koch-Light) which had been re-crystallised from $(CH_3)_2CO$, and reacted with TFA as for the samples.

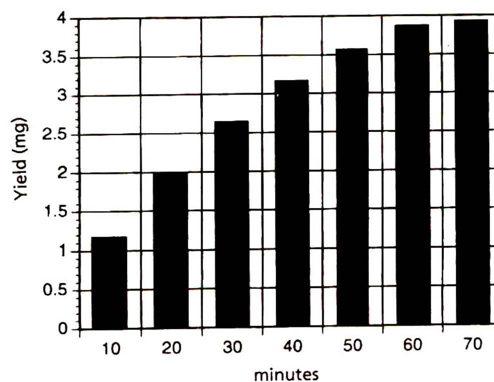

Fig. 1. SFE yields after extraction times up to 70 min (0.05 g sample, 44°C, 3100 p.s.i.).

2.3. Solvent extraction

A 200-mg amount of hydrolysed tuber was extracted in a Soxhlet extractor for 4 h with light petroleum (b.p. 40–60°C) using a 5-ml extraction thimble at a syphon rate of 4 cycles/min.

2.4. Gas chromatography

Gas chromatography was carried out using a Hewlett-Packard 5890 GC system fitted with a 5 m × 0.53 mm I.D. OV-1 capillary. Oven temperature was held at 200°C for 2 min, then

Table 1
Comparison of diosgenin yield using light petroleum extraction, with SFE over a range of pressures of CO_2 ($n = 5$ replicate extractions)

	SFE				Light petroleum ($n = 5$)
	CO_2 Pressure (p.s.i.)				
	1890	2250	2700	3100	
Diosgenin (mg/g)	19.33 (S.D. ± 0.36)	30.75 (S.D. ± 1.87)	39.23 (S.D. ± 2.82)	63.16 (S.D. ± 2.29)	47.45 (S.D. ± 5.54)

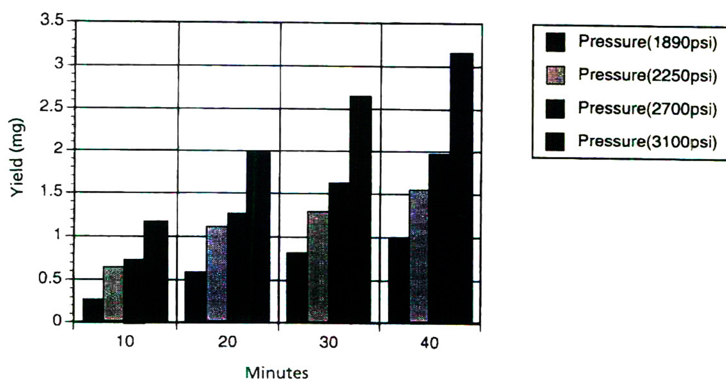

Fig. 2. SFE yields of diosgenin over a range of pressures (0.05 g sample, 44°C).

programmed from 200 to 230°C at 2°C/min; injector and flame ionization detector were kept at 260°C, and eluted components were recorded and integrated using a Hewlett-Packard 3392 A integrator. A linear calibration curve was produced for diosgenin TFA derivative over the concentration range 0.283–1.507 μg/ml, S.D. = 0.9887 ($n = 5$). The identity of diosgenin in sample extracts was confirmed by simultaneous chromatography with standards (diosgenin TFA t_R 14.8 min) and GC–MS (diosgenin TFA chemical ionization MS, M^+ 511 m/z, 397, 282, 139). SFE and solvent extraction both yielded chromatographically pure diosgenin.

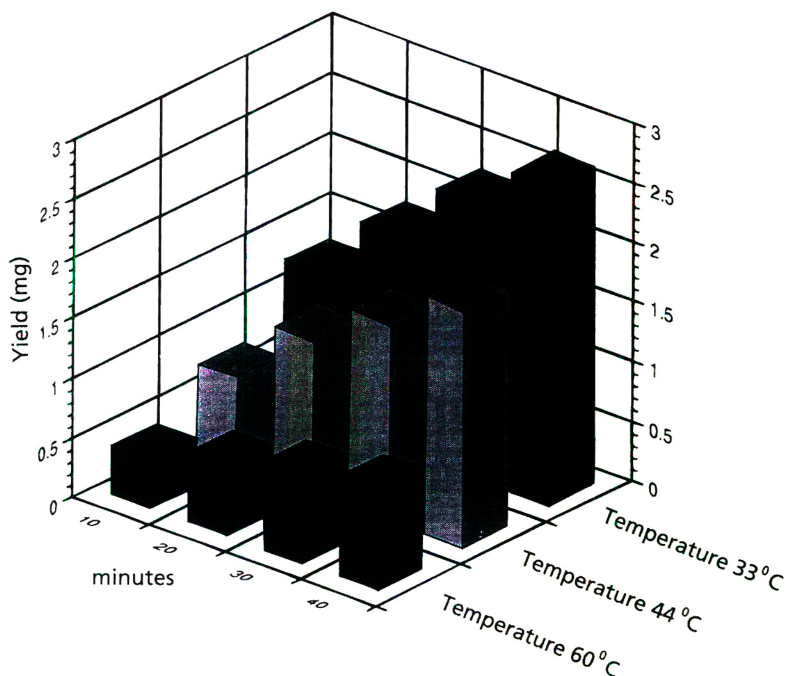

Fig. 3. SFE yields of diosgenin over a range of temperatures (0.05 g sample, 2700 p.s.i.).

3. Results and discussion

Sample amounts of 0.05 g of tuber hydrolysate were extracted over a range of time periods, temperatures, and pressures. Fig. 1 shows the effect of extraction yields over periods extending to 70 min. By 40 min >82% of diosgenin (compared to the yield at 70 min) had been extracted. The effect of extracting hydrolysate for 40 min periods at four different pressures was determined and it can be seen from Fig. 2 that highest yields were obtained at 3100 p.s.i. (1 p.s.i. = 6894.76 Pa). Table 1 shows that extraction with supercritical $C0_2$ at 44°C for 40 min at 3100 p.s.i. produces 33% greater yields than light petroleum (b.p. 40–60°C) extraction.

Fig. 3 shows the effect of extraction temperature on yield of diosgenin. Highest yield occurs at lowest extraction temperature used.

This study shows the possibility that CO_2 SFE could replace organic solvent extraction on at least the analytical scale. It could also be a realistic environmentally sound alternative to petroleum extraction in industry.

Acknowledgements

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JOURNAL OF CHROMATOGRAPHY A

Book Review

Recovery Processes for Biological Materials, edited by J.F. Kennedy and J.M.S. Cabral, Wiley, Chichester, New York, Brisbane, 1993; X + 592 pp; price £110.00; ISBN 0-471-93349-X.

Biological scientists working in areas which have the potential of commercial application are too often unaware of the pitfalls, problems and costs involved in scaling up from laboratory to industrial production. This is the province of the chemical engineer: *Recovery Processes for Biological Materials* is written from the engineering standpoint, and will be a useful source of information for the budding biotechnologist. The editors have brought together contributions, mainly from Britain and the Iberian peninsular, which cover the most important aspects of the industrial extraction and purification of biological products. Additional chapters include analytical methods and small-scale separation procedures, principally for proteins.

The task of coordinating diverse contributions is always difficult, and more so when contributors' first language is not English; it must be said that the result in this case is not entirely satisfactory. Apart from the annoying frequent minor errors of spelling, grammar and missing words (and some factual errors), there has been little attempt to cross-reference, nor to deal with duplication. The latter is particularly evident in the chapters on sorption and on chromatography, which ought to have been combined and co-ordinated.

The overall picture is a collection of articles mainly on the theory of processing biological materials: some authors got a bit carried away with the theory and presented few if any practical examples such as would be expected from a book of this title. However, that does not detract from the large amounts of useful information, and descriptions of industrial machinery that academics constrained to their laboratories may never have realised existed. Particularly useful are the chapters on cell wall disruption, filtration, centrifugation and liquid extraction methods. But this is a personal view, and maybe a chemical engineer would find the chapters on chromatography, electrophoresis and crystallisation more useful, which is an indication of the general appeal of this sort of publication.

An impression is given that the book spent a long time in gestation. Some chapters had no references later than the mid-1980s, and many of the details given (at least those that your reviewer is familiar with) are now irrelevant because of the widespread use of newer technologies. For instance, in the chapter on electrophoresis it is stated that tubular disc gel electrophoresis is now the main method used for analysing proteins. In fact, the "disc" technology virtually disappeared over a decade ago with the development of more convenient slab gels apparatus. And the advantages and reasons for using sodium dodecyl sulphate were not explained (although how to get rid of it was dealt with in extensive detail) despite the fact that sodium dodecyl sulphate–polyacrylamide gel electrophoresis has been the universal method of protein analysis for the past 20 years. And I

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doubt that the lovingly-described Tiselius apparatus has been used in the last 30 years!

One final disturbing feature: in one chapter I had a sense of deja vu while reading it. Substantial amounts were in fact paraphrased from a work of my own, including complete diagrams, without acknowledgment. Perhaps this is flattering, but since it added little to the original, published many years ago, it again demonstrated a lack of modern day relevance for a subject that is so fast moving.

In summary, although this book does contain a useful compilation of subject matter concerning the extraction and processing of biological materials, it would not be so suitable for someone wishing to familiarise him/herself with the latest techniques in biotechnological processing.

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JOURNAL OF CHROMATOGRAPHY A

Book Review

Separations for Biotechnology 3, edited by D.L. Pyle, Royal Society of Chemistry, Cambridge, 1994, XIV + 603 pp; price £92.50, ISBN: 0-85186-724-3.

The book includes the oral and poster presentations at the *3rd International Conference on Separations for Biotechnology* held in September 1994. On account of the broad spectrum of methods applied for separations of biotechnology products, it is not easy to give a short review on the particular papers. Therefore, in following the presented contributions are divided in distinct groups according to the applied methods.

Extraction processes

No common solvent extraction process was presented. The papers discuss modern and novel extraction techniques for protein and metabolite separation, respectively: two papers deal with extraction with microemulsions, seven papers with reversed micelle systems, two with supported liquid membranes, five with liquid ion exchangers as well as two with direct extraction of metabolites from cell suspensions and six with protein extraction by means of two aqueous phase systems. A single paper considers the special use of a centrifugal extractor.

Membrane processes

No standard membrane separation process, only novel membrane applications are presented: a single paper reports on separations with affinity and ion exchange membranes and another on the charged ultrafiltration membranes.

Affinity methods

With ten papers the affinity purification and separation using molecular recognition are one of the central topics of this book. Two reports deal with affinity precipitation.

Chromatographic and adsorptive separation processes

As expected, most reports, altogether twenty, deal with chromatographic separation methods, which include affinity, displacement, size-exclusion and ion-exchange chromatography. Six papers report on adsorption separation methods.

Other separation processes

The book includes several papers which deal with less frequently applied separation techniques: froth flotation and foam separation as well as flocculation, each of them discussed in a single paper. Three reports from the same research group deal with the application of colloidal liquid aphrons. A novel rate zonal centrifugation technique was described by a research group. Classical separation techniques: hydrocylon dewatering of microbial suspensions and spray drying of enzymes are discussed by a single paper as well.

General and particular purification strategies

Five papers deal with general and particular purification strategies and three with integral (product formation-recovery-purification) methods.

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Mixed topics

Several contributions do not belong to the separation techniques, such as: temperature induction of gene expression for production of a fusion protein, protein denaturation by supercritical CO_2, solvent toxicity for microorganisms, particle concentration measurement with photon correlation spectroscopy. Each of these topics is treated in a single paper.

The safety aspects are important for separation and purification processes as well, especially by using recombinant proteins. Expert systems are increasingly used for production processes. Both of these subjects are treated in a single paper.

The book gives an excellent review on the novel separation techniques in the biotechnology in advanced level. It is recommended for all specialists who are dealing with analysis, separation and purification of proteins and metabolites. It is a pity, that because of the rather high price, it is too expensive for students and other persons with a low income. Therefore, scientific libraries should provide this book for them.

Hannover, Germany K. Schügerl

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เอกสารชุดที่ 1

ใส่แฟ้มผู้ป่วย