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Development of a new high-performance liquid chromatography method to analyse N-methylcarbamate insecticides by a simple post-column derivatization system and fluorescence detection

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Abstract

A simple chromatographic method is proposed to analyse N-methylcarbamate insecticides by post-column derivatization and fluorescence detection. The new method performs the post-column reaction without the necessity of using any delivery pumps to introduce the reagents after the analytical column in the chromatographic system. The simplification is possible since the hydrolysis (at basic pH) and derivatization (with *o*-phthaldialdehyde and 2-mercaptoethanol) reagents are present in the mobile phase. Analytical columns (which tolerate basic conditions) and pH of the mobile phase were studied. Kromasil-100-C_{18} column and borate buffer were chosen. The length and the temperature of the tubular reactor were also optimised. Results of the validation study with the proposed chromatographic method are shown. The values demonstrated good performance of the complete system, including the post-column derivatization reaction. The column showed constant efficacy for more than 200 injections. © 1997 Elsevier Science B.V.

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1. Introduction

Pesticides belonging to a group of carbamate insecticides were introduced in 1953, since then their consumption has increased notably. The N-methylcarbamates (NMCs) have the highest insecticide activity of this group.

Their thermal instability does not allow analysis of NMCs by gas chromatography (GC) so high-performance liquid chromatography is used. The NMCs have a low ultraviolet absorption, except at very low wavelengths, but in this spectral region, the analysis is not selective. Fluorescence detection could be an excellent sensitive and selective technique but this class of compounds does not have natural fluorescence. That is why derivatization methods have been developed.

The first chromatographic method was introduced by Frei et al. [1] in 1974 using a pre-column reaction with dansyl chloride, but this technique has not been useful due to low performance in the resolution of dansylated compounds. The post-column reaction, however, has become a widely used technique. In this method, introduced by Moye et al. [2] in 1977, the NMCs were hydrolysed with sodium or potassium hydroxide to methylamine, after their separation in a reversed-phase silica column. The hydrolysis reaction had to be performed at high temperature (about 90°C). Immediately, the amine was labelled with *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol (MCE) and the resulting fluorophore was detected on-line with a fluorescence detector (λ_{ex}=340 nm/λ_{em}=460 nm).

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The method proposed by Moye et al. [2] was optimised by Krause [3,4] and is the reference for the majority of chromatographic methods analysing N-methylcarbamate insecticides in crops [5,6] and the environment [7,8] at the present time. The method is also used by the US Environmental Protection Agency (EPA Method 531.1) and accepted as a J. Assoc. Off. Anal. Chem. Official Methods of Analysis [9].

The method requires the connection of one oven and two pumps to a conventional HPLC system. One of the pumps is used to introduce the hydrolysis strong base reagent (NaOH) after effecting the NMC separation in the chromatographic column and immediately, the NMCs are hydrolysed in a tubular reactor in the oven. Afterwards, the second pump introduces the OPA–MCE reagent.

Some chromatographic methods that simplify the classical conditions by the elimination of one pump have been developed, one of which was based on the simultaneous introduction of the hydrolysis and derivatisation reagents with a single pump [10]. The single reagent (NaOH–OPA–MCE) was always introduced after the column and the reaction was performed in a tubular reactor (stainless-steel tube) in the oven at 140°C.

In the search to simplify the carbamate method, Nondek et al. [11] and later De Kok et al. [12] proposed a HPLC method in which the post-column hydrolysis took place in an anion-exchange resin (catalyst) heated to 140°C. Only the mobile phase, a water, acetonitrile and sodium acetate solution flowed through the resin. This method does not use strong base reagents and only a single pump, to introduce the reagent of derivatization (OPA–MCE) after the chromatographic column and the anion resin, is required.

In 1988 Miles and Moye [13] proposed performing the hydrolysis of carbamates with a post-column photolysis using a UV (254 nm) lamp. After this reaction, the single pump introduces the derivatization reagent (OPA–MCE).

Generally, the mobile phase is water modified with acetonitrile or methanol while the stationary phase is normally octyl- or octadecylsilane.

In the present study, a simplification of the carbamate chromatographic method has been developed which obviates the need for reagent delivery pumps, anion-exchange resin and strong base reagents. This is possible because the hydrolysis (borate solution) and derivatization (OPA–MCE) reagents involved in the post-column reaction were present in the mobile phase. The carbamates were separated in a reversed-phase column and the hydrolysis and derivatization reactions were carried out in a tubular reactor in the post-column oven.

2. Experimental

2.1. Reagents and materials

Sodium tetraborate decahydrate of analysis-grade was supplied by Fluka (Buchs, Switzerland).

Sodium hydroxide was obtained from Scharlau (Barcelona, Spain).

OPA for fluorometry and MCE of analysis-grade were obtained from Merck (Darmstadt, Germany).

Acetonitrile and methanol of HPLC-grade were obtained from Carlo Erba (Milan, Italy).

Deionized water, was produced by a Milli-Q system, obtained from Millipore (Molsheim, France).

The tubular reactor was made of stainless-steel tubing (1.59 mm O.D.×0.51 mm I.D.) from Alltech (Deerfield, IL, USA).

Standards of N-methylcarbamates (methomyl, dioxacarb, butocarboxim, aldicarb, propoxur, carbofuran and carbaryl) were supplied by the Institute of Organic Industrial Chemistry (Warsaw, Poland). Stock solutions (1 mg/ml) were prepared in methanol and were stored in a freezer at −25°C. Standard mixture solution was prepared with 1 ml of each NMC stock solution for final concentration of 142 μg/ml. Several dilutions were prepared in the mobile phase without reagents.

2.2. Chromatographic instruments

The HPLC system used was a 600S controller and 616 pump Model equipped with in-line degasser and 717 plus autosampler, all from Waters. A Waters 470 scanning fluorescence detector and a Waters temperature control system (post-column reactor oven) were used.

The post-column reaction system was connected after the column and before the fluorescence detector

and consisted of the tubular reactor enclosed in the post-column reactor oven. The three elements were connected using stainless steel capillaries (0.25 mm I.D.) with low dead volume capillary connectors, from Supelco (Bellefonte, PA, USA).

Millennium Software version 2.00 (from Waters) was used to control the system and to collect and integrate data.

2.3. Chromatographic conditions

2.3.1. Polystyrene–divinylbenzene column

A Hamilton PRP-1 column (250 mm×4 mm I.D., 10 μm particle diameter) from Hamilton (Reno, NV, USA) was used.

A ternary gradient system was used. Mobile phase A consisted of acetonitrile and mobile phase B was water. Mobile phase C (reagent) consisted of 0.15 *M* sodium hydroxide–0.33 mg/ml OPA–0.42 μl/ml MCE. The gradient conditions are given in Table 1.

The flow-rate was 1 ml/min and the injection volume was 20 μl.

The tubular reactor was a stainless-steel tube 1.4 m in length and was thermostatted at 80°C.

The fluorescence excitation and emission wavelengths were set at 340 and 460 nm, respectively.

2.3.2. Octadecylsilane column

A Kromasil-100-C_{18} column (150 mm×4 mm I.D., 5 μm particle diameter) and a Kromasil-100-C_{18} pre-column (10 mm×3 mm I.D., 5 μm particle diameter) from Akzo Nobel (Bohus, Sweden) and packed by Tecknokroma (St. Cugat, Barcelona, Spain) were used.

Chromatographic runs were performed isocratically at a flow-rate of 1 ml/min. The composition of the binary mobile phase was 28% acetonitrile and 72% 0.82 g/l sodium tetraborate decahydrate–0.05 mg/ml OPA–0.06 μl/ml MCE (reagent). The injection volume was 20 μl. The fluorescence excitation and emission wavelengths were set at 340 and 460 nm, respectively.

The length of the tubular reactor and the temperature of the post-column reactor oven were optimised.

Table 1
Gradient conditions used with polystyrene–divinylbenzene column

Time (min)	Acetonitrile (%)	Water (%)	Reagent (%)
0.00	20.00	70.00	10.00
5.00	20.00	70.00	10.00
25.00	80.00	10.00	10.00
28.00	80.00	10.00	10.00
35.00	20.00	70.00	10.00

3. Results and discussion

The basic pH necessary to perform the post-column reaction was incompatible with the conventional reversed-phase silica columns, which have an operating pH range between 2.5 and 7.5. Hence, different basic conditions and stationary phases compatible with these conditions were established in order to allow the utilisation of a basic mobile phase.

3.1. Sodium hydroxide hydrolysis reagent. Polystyrene–divinylbenzene column

Initially, a strong basic pH was tested in our laboratory (0.1 *M* NaOH, pH 13). This is the reagent most commonly used in post-column systems to hydrolyse NMCs to methylamine. However, the presence of sodium hydroxide (0.1 *M*) in the mobile phase does not allow the use of reversed-phase silica columns; only polymeric stationary phases can tolerate this extreme pH. Therefore, the separation was performed in a polystyrene–divinylbenzene column and sodium hydroxide, OPA and MCE (reagents), water and acetonitrile were used as the mobile phase. The post-column reaction was carried out in the tubular reactor at 80°C.

A chromatogram of NMCs solution is shown in the Fig. 1, which demonstrates the possibility of performing the post-column reaction while including the reagents in the mobile phase rather than after the column. Thus allowing the elimination of extra-pumps from the system. However, it was seen that propoxur and carbofuran were coeluted with the chromatographic conditions used. Furthermore, high-performance polymeric columns are more expensive than reversed-phase silica columns and extreme basic conditions in the mobile phase are also undesirable in the long-term use of chromatographic systems.

It was decided, therefore, to adjust the pH in order to use the less expensive and more common re-

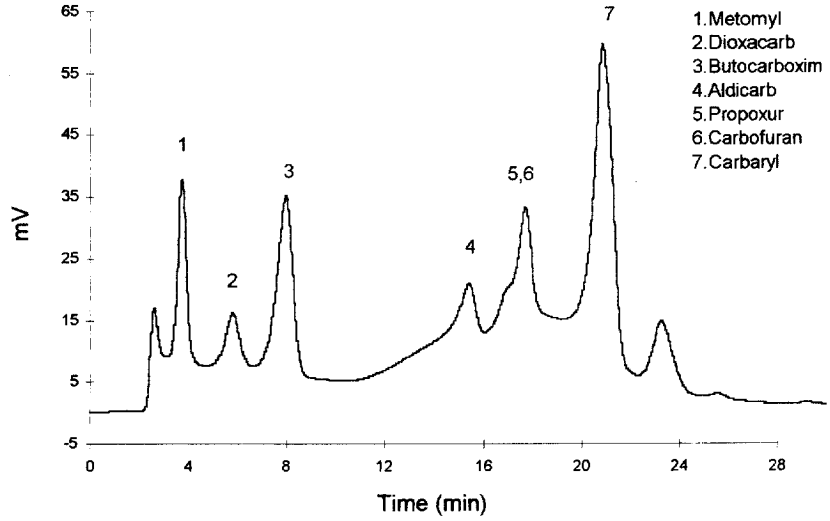

Fig. 1. Chromatogram in gradient conditions of NMCs (14.2 μg/ml) from standard mixture solution diluted in acetonitrile–water (30:70). Stationary phase: polystyrene–divinylbenzene.

versed-phase silica columns. A number of studies have shown their good performance in the resolution of several NMCs [14].

3.2. Borate buffer hydrolysis reagent. Octadecyl silica column

A study of the efficacy of the post-column system operating with a pH less basic than sodium hydroxide was carried out. The temperature of the reaction and the length of the tubular reactor were also optimised.

Two different pH values were tested in the mobile phase to evaluate the post-column reaction efficiency. Borate buffer (0.82 g/l sodium tetraborate decahydrate, pH 9.3) and borate buffer acidified with

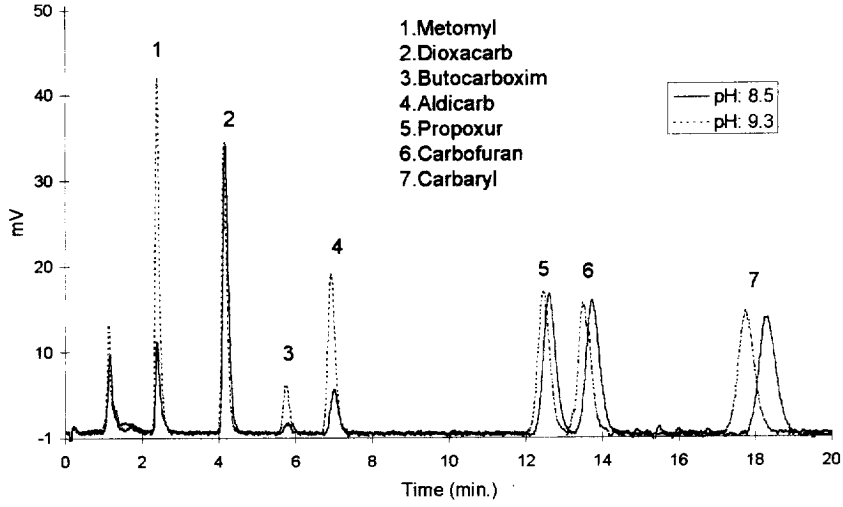

Fig. 2. Chromatograms of NMCs (1.42 μg/ml) obtained with pH values of 8.5 and 9.3 in the mobile phase. Analytical column: Kromasil. Post-column reaction: 1.4 m and 140°C.

0.1 *M* hydrochloric acid (pH 8.5) (72%) combined with acetonitrile (28%) were used as mobile phases. OPA (0.05 mg/ml) and MCE (0.06 μl/ml) were also present. The tubular reactor was initially 1.4 m in length and heated to 140°C.

The incompatibility of these pH values is no longer a difficulty since new generation reversed-phase silica columns (Kromasil) allow the use of mobile phases with pH ranges from 1.5 to 9.5.

Chromatograms of the two pH conditions are shown in Fig. 2. All pesticides were separated using this new type of column. The responses of the aromatic NMCs were similar under both conditions, but the responses of aliphatic NMCs (metomyl, butocarboxim and aldicarb) were higher when pH 9.3 was used. Therefore, the borate buffer (pH 9.3) was chosen as the basic solution for the post-column reaction.

The lengths of the stainless-steel tubular reactor (1.5, 3 and 6 m) and the temperature of the post-column reactor oven (100, 120 and 140°C) were optimised in the following way. A standard of NMCs in acetonitrile–water (30:70, v/v) of 1.45 μg/ml was injected twice under each condition. Signal (area)-to-noise ratios were calculated for all NMC insecticides (Fig. 3). Noise increased slightly with temperature.

An analysis of variance of the results obtained showed significant influence of the temperature, length and the interaction temperature–length ($P<0.01$). For six of the seven NMCs studied, temperature was the most important factor while for carbaryl the length of the tubular reactor was the most significant.

The compounds were seen to have divided into two groups according to their behaviour: the first comprising the aliphatic NMCs (metomyl, butocarboxim and aldicarb) and the second group the aromatic NMCs (dioxacarb, propoxur, carbofuran and carbaryl).

In fact all the carbamates showed had maximum response at 140°C when 1.4 m or 3 m of tubular reactor were used. However, the aromatic NMCs noticeably increased their response between 100 and 120°C, while the aliphatic NMCs did not significantly increase their signal until the temperature reached 120°C. Using 6 m, all the NMCs showed maximum responses at 120°C, except the butocarboxim (at 140°C).

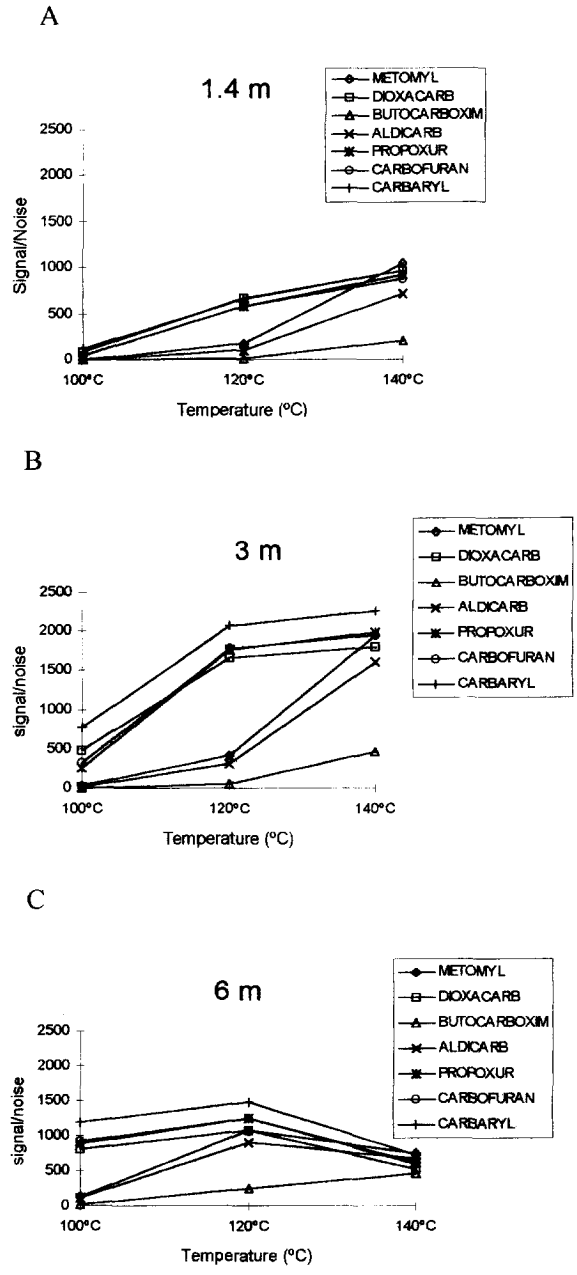

Fig. 3. Signal (area)-to-noise ratio versus reaction temperature of MNCs (1.42 μg/ml) obtained using 1.4 m (a), 3 m (b) and 6 m (c) of tubular reactor.

It is interesting to note the different behaviour shown by six of the seven compounds studied in the 6 m reactor. The increase in response using 1.4 or 3 m when the temperature was raised, changed their

profile when a 6 m tube was used. In this case, the maximum responses were shown at 120°C (except for butocarboxim).

These results may best be explained taking to account that the response of the carbamates depends on the efficacy of the hydrolysis and the thermal stability of the fluorophore derivate [15]. Hydrolysis efficacy increases with temperature while stability decreases.

The increase in response seen using 1.4 m and 3 m tubes can be attributed to the relationship between hydrolysis efficiency and temperature. In the case of the 6 m tube however, the profile shows that at high temperature, the degradation of the fluorofhore derivate begins to take a significant importance.

Comparing the three sets of results, it is clear that signal-to-noise ratio is at a maximum under conditions of 3 m and 140°C, although butocarboxim showed a very slightly elevated response at 140°C and 6 m. Optimum conditions are, then, 3 m and 140°C (Fig. 4).

3.3. Validation of proposed chromatographic method

The method shown above was validated in order to obtain values for repeatability, linearity and the detection limit for the NMCs.

Table 2
Repeatability[1] (R.S.D.) of the chromatographic method

Carbamates	Area	Height	Retention time
Metomyl	0.73	0.94	0.32
Dioxacarb	0.71	1.3	0.34
Butocarboxim	0.66	1.1	0.59
Aldicarb	0.78	1.6	0.58
Propxur	0.76	1.9	0.67
Carbofuran	0.83	2.0	0.65
Carbaryl	0.70	2.1	0.62

[1] Nine replicates (1.42 μg/ml) on the same day were injected.

3.3.1. Repeatability

Peak height repeatability (Table 2), obtained from nine injections on the same day, ranged from 0.94 to 2.1% relative standard deviation (R.S.D.). Peak area repeatability ranged from 0.66 to 0.83% R.S.D. and retention time repeatability ranged from 0.32 to 0.67% R.S.D..

3.3.2. Linearity and detection limit

Several dilutions ($n=10$) corresponding to 0.3–3000 ng absolute injected amounts (AIAs) were

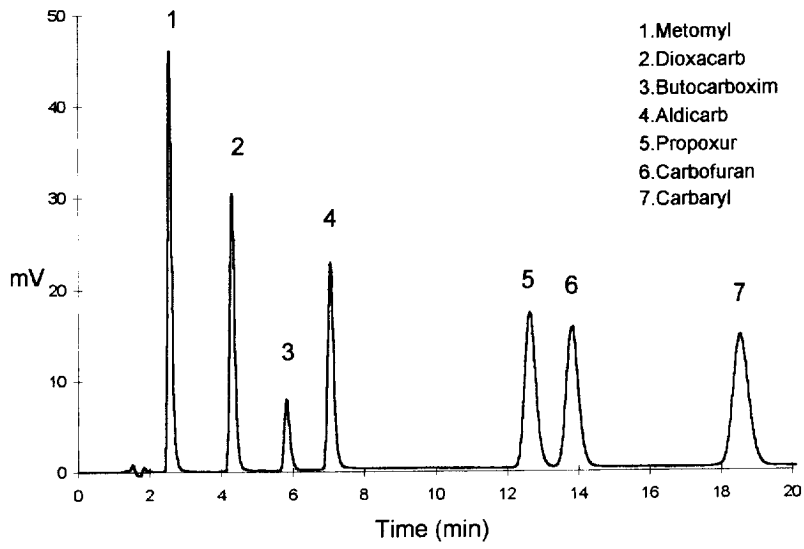

Fig. 4. Chromatogram of NMCs (1.42 μg/ml) run at optimum conditions. Mobile phase: 72% borate buffer-OPA-MCE, 28% acetonitrile; flow-rate: 1 ml/min; length of tubular reactor: 3 m; oven temperature: 140°C.

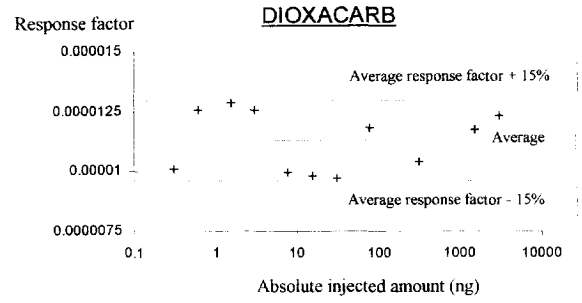

Fig. 5. Response factor (Rf: AIA to peak area) of dioxacarb versus AIA (ng).

assayed in order to study the linearity of the chromatographic method.

Linearity ranges were determined using response factor versus AIA graphs, where response factor is defined as the ratio AIA to peak area (Fig. 5). Out-of-linearity points are those that fall out of the zone delimited by the horizontal lines "response factor mean±15%". Linearity ranges are given in Table 3.

Regression lines (peak area versus AIA) were calculated for all NMCs in the previously-defined linearity ranges. The responses were linear in the wide range of concentrations studied with $r>0.999$ (Table 3).

The detection limits (based on a signal-to-noise ratio 3:1) ranged from 0.2 to 0.7 ng (Fig. 6 and Table 3).

The results of the validation of the proposed method were compared with the results obtained using the McGarvey method [10] in our laboratory and with published values [10,12,16]. The results of the new proposed method are equivalent to these methods.

The analytical column demonstrated constant chromatographic performance for more than 200 injections.

3.4. Recent results and future trends

Future studies will be directed towards the extension of the method to other NMCs and metabolites. Gradient conditions will also be required in order to analyse a large number of carbamates with wide polarities in a short time. Recent experiments carried out by the authors have shown good performance of the proposed chromatographic method using gradient conditions with an organic solvent proportion (acetonitrile) ranging from 30 to 80%.

Finally, the chromatographic method has also recently been tested in the analysis of carbamate residues in foods [17]. Initial results (repeatability, linearity and the detection limit) have shown good performance under the proposed conditions.

4. Conclusions

A new method was developed for determination of carbamate insecticides by post-column derivatization. The method proposed is inexpensive, robust and relatively simple since only requires a conventional HPLC system, a tubular reactor and an oven. Furthermore, removing the need for auxiliary pumps, flow pulsation problems are avoided.

The analytical column used showed good performance during the development of the method,

Table 3
Linear response (area=$A+B\cdot$AIA)[1] and detection limit of the chromatographic method

NMCs	A ($\cdot 10^2$)	B ($\cdot 10^2$)	Mass (ng)[2]	r	Detection limit (ng)
Metomyl	21 368	1027	7–3100	0.9997	0.3
Dioxacarb	9879	816	0.3–3100	0.9997	0.2
Butocarboxim	1194	294	3–3100	1.0000	0.8
Aldicarb	8388	857	0.6–3100	0.9999	0.3
Propxur	19 865	990	7–3000	0.9998	0.4
Carbofuran	18 116	951	7–3000	0.9998	0.5
Carbaryl	23 508	1089	8–3200	0.9998	0.7

[1] Eleven data points. Concentration from 0.0123 to 142 μg/ml.
[2] Linearity range.

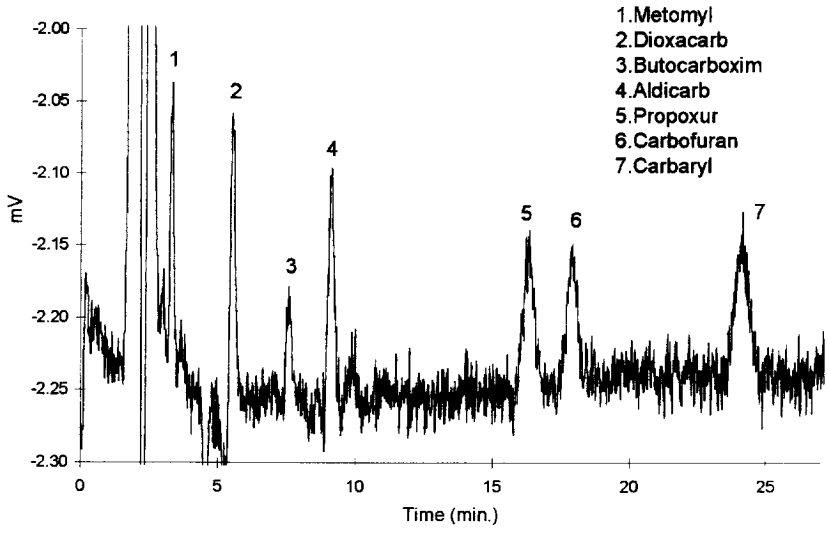

Fig. 6. Chromatogram of NMCs near the detection limit (0.0142 μg/ml) run at optimum conditions.

even when working near the limit of the operating pH range (9.3) during more than 200 injections.

The chromatographic method has excellent repeatability and good linearity and detection limits to apply in the analysis of carbamate residues.

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