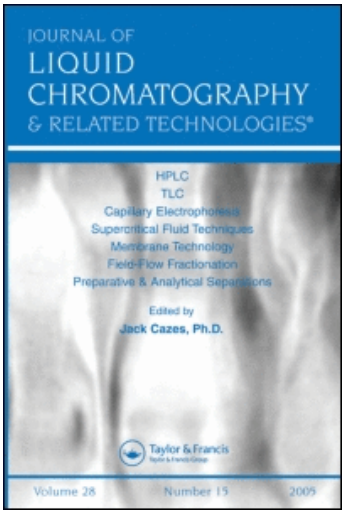

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CHEMICAL HYBRIDIZING AGENT IN WHEAT

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ABSTRACT

A simple HPLC method has been developed for the quantification of chemical hybridizing agent (CHA) in grains, leaves, straw and glumes of traited wheat. The procedure included acido-basic extractions of wheat grains and tissues and derivatization of CHA residue as methyl ester compound. The chromatographic separation was achieved with Spherisorb silica using ethyl acetate-methanol (98:2) and UV detection at 285 nm. Good recoveries of CHA for fortified wheat grains and leaves were obtained. Recoveries of CHA in traited wheat demonstrated accumulation of the agent in reproductive organs.

INTRODUCTION

Several chemical hybridizing agents (CHA, gametocides) have been tested in recent years for inducing pollen sterelity in economic crops. Practical aspects of the development of CHA for commercial use have been reviewed, together with a discussion of their effects on the anther tissues (1, 2, 3).

Fenridazon-potassium (potassium 1-(4-chlorophenyl)-1,4-dihydro-6-methyl-4-oxo-pyridazine-3-carboxylate) is a good wheat hybridizing agent developed by Rohm and Haas (4, 5) as RH0007 or Hybrex. This chemical hybridizing agent (CHA) inhibits wheat pollen formation while maintaining the normal fertility of the female portion of the wheat flower.

Recently, a HPLC analytical method of residue was described (6) for the analysis in wheat grain and straw of the CHA RH0007. The method employed alkaline extraction, copper-activated Chelex 100 column chromatography and then liquid-liquid partition-quantification of RH0007 residue was performed by ion-pair reverse-phase liquid chromatography at 50°C.

This paper describes a new method of extraction of the CHA and easier quantification after derivatization with normal-phase HPLC method at 25 °C.

EXPERIMENTAL

A - Apparatus

The HPLC system consisted of a Gynkotek pump (model 501, Münich, Germany) fitted with a Rheodyne injection valve (20 µl loop; California, US) and a Pharmacia UV detector (Uppsala, Sweden). The detection wavelenght was set at 285 nm.

B - Chromatography

The column used for the separation was Spherisorb silica (150 mm x 4.6 mm I.D.; 5 µm particule size) purchased from SFCC (Neuilly-Plaisance, France). The eluent used was ethyl acetate-methanol (98:2) at a flow rate of 0.8 ml/min.

C - Reagents and chemicals

All organic solvents (from Rhône Poulenc, France) were analytical grade or HPLC grade for the eluent. Water was HPLC grade from a milli-Q purification system. Sodium chloride, hydrochloric acid, ammonium sulfate, sodium hydrogenocarbonate were analytical grade from Rhône Poulenc.

Fenridazon (RH0007) 1 was synthetized in our laboratory (7). Derivatization of fenridazon as methyl ester 1b was performed quantitatively (>99%) with diazomethane. The methyl ester 1b was purified on a silica gel column by medium-pressure liquid chromatography on an AXXIAL apparatus (Axxial Modul Prep, Marseille, France); 200 g of silica Merck 15 μm (Merck, Nogent, France) were packed in a 40 mm x 450 mm inox column; packing pressure = 50 bars; elution = 17 ml/min; eluent: ethyl acetate-methanol (98:2).

Internal standard 2 (see discussion underneath) was synthetized in our laboratory (7). Derivatization of this internal standard in methyl ester 2b was performed with diazomethane and subsequent purification carried out as described for methyl ester 1b. Standard solutions for calibration curves of methyl esters 1b and 2b were prepared by serial dilution of 1b and 2b in the HPLC mobile phase. Standard solutions were chosen in the range estimated from the residue extract (10^{-4} to 10^{-6} M). The results obtained for six concentration values included in these ranges showed good linearity. Calibration was performed by plotting known concentrations of 1a and 2b versus peak area.

D - Plant materials

Wheat (Tritium Aestivum) cultivation and gametocide treatment were performed according procedures described in reference 8. Female and male winter varieties (Pernel and Castan, Festival, Arminda from Verneuil-Semences, France) were sown and grown in fields in a factorial split-splot plan.

Gametocide RH0007 used as potassium salt was dissolved in water along with a wetting agent (Triton x-100 at 0.1%).

Female plants were sprayed before meiosis (between seven and eight stages according to Feekes scale used for estimation of anther developmental stages and vegetative growth (9)).

The gametocide solution was applied as a foliar spray with a manual pressure sprayer at different rates (0.5; 1; 2 kg per ha) and control plants were sprayed with wetting agent solution (a rate of 1 kg/ha corresponded to 1.4 10^{-5} mole CHA/plant). Treatment combinations were completly

randomized and replicated twice in two field blocks. Bags were applied to spikes (4 to 6 spikes by line) prior to anthesis after gametocidal treatment. At maturity open-pollinated and self-pollinated spikes were collected to obtain spike lenght, number of seeds per spikelet and to evaluate degree of male sterility and female fertility.

E - Extraction procedure

A 20 or 10 g wheat grain sample (or 5 to 10 g leaves, straw or glumes sample) was homogenized in a blender at high speed for 2 or 3 min. Petroleum ether (150 or 75 ml) was added and the mixture was sonicated at 0°C for 10 min and then vacuum filtered through a sintered glass frit. The petroleum ether filtrate was discarded (lipophilic constituents). Internal standard 2 (see discussion after) (10^{-7} mole in 0.1 ml $CHCl_3$) was added to the filter cake and a modified "Folch" extraction procedure (10) was then performed: 70 ml of a solution $CHCl_3$-MeOH-brine (1-2-0.8) were added and the mixture was sonicated at 0 °C for 10 min and then centrifugated at 5000 g for 20 min. The supernatant was decanted and saved. Re-sonication was performed with 70 ml of a solution $CHCl_3$-MeOH-HCl 1 M (1-2-0.8) exactly as before and after centrifugation the supernatant was saved. The pellet was again sonicated at 0 °C with 70 ml of CH_2Cl_2 for 15 min and vacuum filtered. The supernatant layers were combined and 50 ml of ammonium sulfate solution (40%) and 5 g of NaCl were added. The mixture was sonicated at 0 °C for 15 min and centrifugated (partial precipitation of proteines to avoid further emulsion). The pellet was discarded and the supernatant transferred to a separatory funnel. The decanted organic phase was evaporated to dryness at 40 °C under reduced pressure. The residue was sonicated at 0 °C for 15 min with 100 ml of 0.1 M $NaHCO_3$ and extracted with 3 x 50 ml of diethyl ether (partial elimination of pigments). The aqueous phase was acidified to pH = 1.0 with concentrated hydrochloric acid added dropwise. The acidified phase was extracted with CH_2Cl_2 (3 x 50 ml) and the organic extract was evaporated to dryness at 40 °C under reduced pressure.

F - Derivatization of the extract

Esterification of residual RH0007 1 and recovered internal standard 2 in the extract was performed with CH_2N_2 in THF according to classical procedure. Diazomethane was freshly prepared from "Diazald" (11) in ether.

TABLE 1

Recovery of RH0007 1 and Standard 2 for Fortified Wheat Grains and Leaves.

Sample type	Fortification Level 1 (mole)	Fortification Level 2 (mole)	% Recovery a) 1	% Recovery a) 2
Grains	$5.0\ 10^{-7}$	$5.0\ 10^{-7}$	97.8 ± 1.6	73.5 ± 1.5
	$2.5\ 10^{-7}$	$2.5\ 10^{-7}$	99.7 ± 2.0	71.2 ± 1.6
	$1.0\ 10^{-7}$	$1.0\ 10^{-7}$	87.2 ± 2.2	67.6 ± 1.8
	$5.0\ 10^{-8}$	$5.0\ 10^{-8}$	81.7 ± 2.2	58.4 ± 1.7
Leaves	$5.0\ 10^{-7}$	$5.0\ 10^{-7}$	81.7 ± 1.8	60.5 ± 1.3
	$2.5\ 10^{-7}$	$2.5\ 10^{-7}$	79.2 ± 2.0	60.9 ± 1.5
	$1.0\ 10^{-7}$	$1.0\ 10^{-7}$	80.0 ± 2.3	57.1 ± 1.7
	$5.0\ 10^{-8}$	$5.0\ 10^{-8}$	76.5 ± 2.4	58.8 ± 1.9

a) average of four replicate experiments for each fortification level.

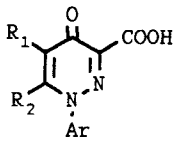
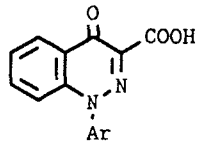
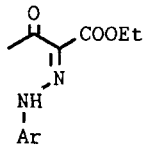
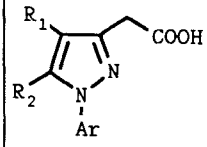
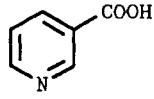
The dry extract was dissolved in THF. The solution was cooled at 0 °C and diazomethane in ether (five times excess) was added dropwise. The mixture was shaken for one hour before the solvent was removed under reduced pressure. The dry residue was dissolved in 2 ml of the HPLC mobile phase and submitted to serial dilutions if necessary.

RESULTS AND DISCUSSION

Before analysis and quantitation of RH0007 residue in treated wheat grains, leaves and straw, it was necessary to control recovery of the chemical hybridizing agent after the different steps of extraction procedure and

TABLE 2

Retention Time of RH0007 Analogues

Compound	X	R_1	R_2	Ar	t_R[a](min)
	1	H	CH_3	p-Cl-C_6H_4	9.5
	2	H	C_6H_{13}	p-Cl-C_6H_4	4.5
	3	H	H	p-Cl-C_6H_4	6.0 [b]
	4	H	CH_3	naphtyl	9.2
	5	H	CH_3	p-HOOC-C_6H_4	9.5
	6	C_2H_5	H	p-Cl-C_6H_4	4.0
	7	H	H	C_6H_5	6.1 [b]
	8	-	-	p-Cl-C_6H_4	4.5 [b]
	9	-	-	p-HOOC-C_6H_4	2.0
	10	H	OH	p-Cl-C_6H_4	2.6
	11	-	-	-	3.1

a) retention time of methyl esters $\underline{X}_b$ synthetized from compounds X. Flow rate: 0.8 ml/min; eluent: ethyl acetate-methanol (98:2). b) X poorly soluble in THF and ether, not suitable for derivatization.

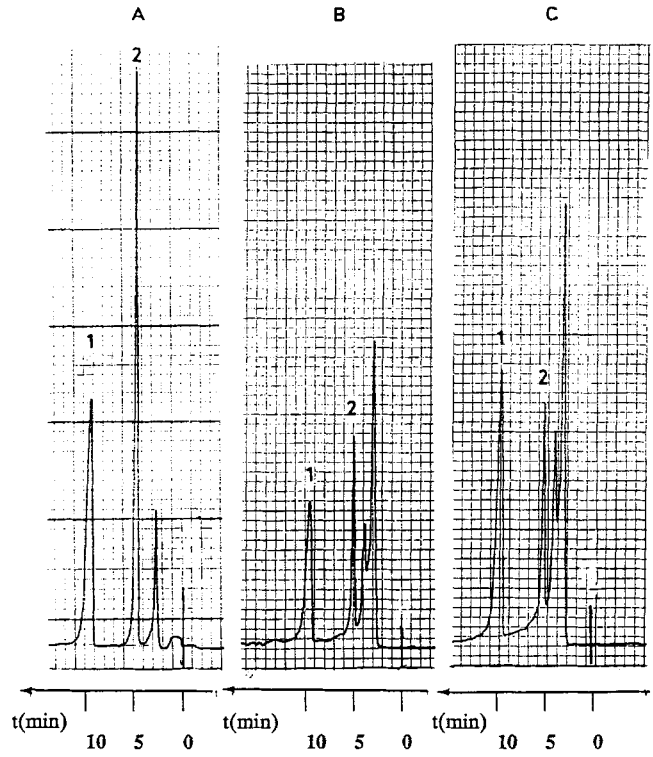


FIGURE 1
Chromatograms of extracts of treated wheat: **A**: Methyl esters of 1 (RH0007) and standard **2** in standard solution. **B**: treated wheat grains (dose of treatment: 2 kg/ha; extract of shrunken grains; 2.10^{-7} mole of internal standard **2** added; extract in 8 ml of eluent). **C**: extract of smooth grains (same set of treated wheat as in B; identical conditions to B).
Conditions given in text.

derivatization. Recovery efficencies were determined by fortifying control wheat grains (or leaves) after the initial petroleum ether extraction with RH0007 ($5\ 10^{-7}$ to $5\ 10^{-8}$ mole in 0.1 ml $CHCl_3$; RH0007 is not soluble in petroleum ether). After derivatization, the chromatograms demonstrated that methyl ester 1b of RH0007 was adequately resolved from any interfering components. Recovery data are summarized in Table 1. Extraction and derivatization procedures exhibited good RH0007 recovery.

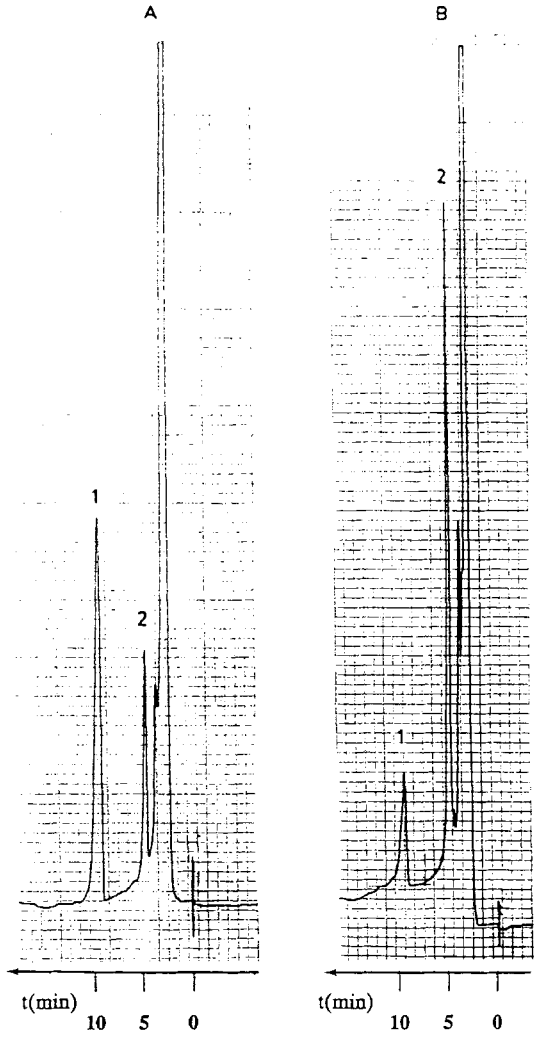


FIGURE 2

Chromatograms of extracts of treated wheat: **A**: treated wheat glumes (dose of treatment: 2 kg/ha; internal standard **2** added: 3.10^{-7} mole; extract in 4 ml of eluent). **B**: treated wheat leaves (dose of treatment: 2 kg/ha; internal standard **2** added: 4.10^{-7} mole; extract in 6 ml of eluent).
Conditions given in text.

TABLE 3

Recovery of RH0007 1 for Treated Wheat Grains

Type of grain	Dose of treatment (kg/ha)	1 recovery a) (mg/g)
smooth	2	$5.2\ 10^{-3} \pm 0.1\ 10^{-3}$
shrunken	2	$26.5\ 10^{-3} \pm 0.4\ 10^{-3}$
smooth	1	$3.3\ 10^{-3} \pm 0.1\ 10^{-3}$
shrunken	1	$17.2\ 10^{-3} \pm 0.5\ 10^{-3}$
smooth	0.5	$2.4\ 10^{-3} \pm 0.1\ 10^{-3}$
shrunken	0.5	$6.6\ 10^{-3} \pm 0.3\ 10^{-3}$

a) average of four extractions for each dose and each type of grain; mg of 1/g of grains

When RH0007 analysis resulted from residue of treated plants, accuracy of extraction and derivatization was controled by addition of an internal standard to the living tissue after the initial petroleum ether extraction. Absolute recoveries could not be determined because there is no method to estimate the *in situ* level of gametocide in the plant sample.

A serie of RH0007 analogues (11 compounds X) was synthetized (7) and methylated with diazomethane as methyl ester derivatives Xb. Retention times of the different compounds are summarized in Table 2. Methyl ester 2b was choosen as internal standard because it was efficiently separated from methyl ester 1b and from extraction plant constituents. Recovery efficencies of ester 2b were determined as for ester 1b by fortifying control wheat grains with known concentrations of 2 (together with RH0007 1) after the initial petroleum ether extraction. Recovery data of 2 are given in Table 1. Hybrex

TABLE 4

Recovery of RH0007 1 for Treated Wheat Tissues [a)]

Tissue extracted	1 recovery [b)] (mg/g)
leaves	$7.7\ 10^{-3} \pm 0.3\ 10^{-3}$
straw (stems)	$3.3\ 10^{-3} \pm 0.1\ 10^{-3}$
glumes	$35.5\ 10^{-3} \pm 0.7\ 10^{-3}$
grains	
smooth	$5.2\ 10^{-3} \pm 0.1\ 10^{-3}$
shrunken	$26.5\ 10^{-3} \pm 0.4\ 10^{-3}$

a) dose of treatment: 2 kg/ha
b) average of four extractions for each tissue; mg of 1/g of tissue

1 displayed better recovery as internal standard 2. Recovery factor of RH0007 1 versus standard 2 was 1.3.

Representative chromatograms for the determination of RH0007 in wheat grains are illustrated in Figure 1.

Figure 2 shows chromatograms of leaves and glumes.

When grains were collected from spikelets of treated plants they were separated in two sets according to their aspect: smooth grains and shrunken hypertrophied grains.

Table 3 represents recovery of RH0007 in grains collected from wheat crop (wheat treated in fields with several amounts of CHA). This table

demonstrates that higher was the initial amont of sprayed CHA, greater was recovered residual RH0007. Recovered CHA reflects excess of gametocide that accumulates in grains. Amount of CHA used in sterility process is not detectable. Shrunken grains retained three to five times as much residual CHA as smooth grains.

We observed that for an equal number of shrunken and smooth grains, wheight of shrunken grains was reduced significantly (three to four times) regard to that of smooth ones. Accumulation of RH0007 in grain could affect synthesis of growth or nutritious substances of walls (polysaccharide, hemicellulose, starch .....) (1, 5).

Table 4 represents recovery of RH0007 in leaves, glumes and straw of plants treated at 2 kg/ha in fields. Comparison of Tables 3 and 4 demonstrates that gametocide accumulates in glumes and grains. Though grains were not still developed at the foliar spray stage of gametocide, it is obvious that ACH migrated rapidly from leaves and stem through phloem to reproductive organs (anthers) and accumulated to mature stage.

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