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Growth and Germination Inhibitors in Durum Wheat Mature Grain Endosperms*)

By

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With 9 Figures

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Summary

Spanò C., Meletti P. & Floris C. 1994. Growth and germination inhibitors in durum wheat mature grain endosperms. – Phyton (Horn, Austria) 33 (2): 237–248, 9 figures. – English with German summary.

Soluble germination and growth inhibitors occur in the ripe endosperm of *Triticum durum* caryopses. The inhibitors extracted in aqueous methanol were partially purified by utilizing dialysis, organic solvent partitioning and high performance liquid chromatography (HPLC). The inhibitory activity, partitioned in two different phases (phase I and phase II), was evaluated with both lettuce seed and after-ripe wheat seed bioassay. The partially purified inhibitors depressed seed germination and seedling growth. While the inhibiting substances of phase I didn't co-elute with abscisic acid (ABA), in phase II most of the inhibitory activity was present in a fraction which eluted similarly to ABA. The localization of the inhibiting substances was also studied. Inhibitory activity was mostly localized in the bran and little occurred in the starchy endosperm fraction.

Zusammenfassung

Spanò C., Meletti P. & Floris C. 1994. Wachstums- und Keimungshemmer im Endosperm von reifen Hartweizenkörnern. – Phyton (Horn, Austria) 33 (2): 237–248, 9 Abbildungen, Englisch mit deutscher Zusammenfassung.

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Lösliche Keimungs- und Wachstumshemmstoffe treten im reifen Endosperm von *Triticum durum* Karyopsen auf. Die Hemmstoffe werden in wäßrigem Methanol extrahiert und zum Teil durch Dialyse, Trennung mit organischen Lösungsmitteln und HPLC gereinigt. Die in zwei Phasen auftretende Hemmaktivität wurde sowohl mit Salat als auch mit Weizensamen getestet. Die teilweise gereinigten Hemmstoffe unterdrücken sowohl die Keimung als auch das Wachstum von Samen. Während die Hemmsubstanzen von Phase I nicht mit Abscisinsäure gemeinsam eluiert werden können, ist in Phase II die größte Hemmaktivität in einer Fraktion zu finden, die ähnlich ABA zu eluieren ist. Weiters wurde auch die Lokalisation der Hemmsubstanzen untersucht. Der größte Teil der Hemmaktivität konnte in der Kleie lokalisiert werden und nur zum geringen Teil in der Stärke führenden Endospermfraktion.

1. Introduction

The plant hormone abscisic acid (ABA) has long been associated with seed dormancy, but factors other than ABA are probably also involved in the control of seed germination (ZEEVART & CREELMAN 1988).

Besides ABA, many germination inhibitors have been found in dormant seeds: for the most part, these natural inhibitors are relatively simple organic molecules of low molecular weight, such as aldehydes, phenolics, alkaloids, unsaturated lactones such as coumarin (KETRING 1973) and aminoacids such as tryptophan (MORRIS & al. 1988).

Triticum is generally characterized by a relative dormancy (BORRIS 1941, MELETTI 1964, BEWLEY & BLACK 1982) and ABA seems particularly involved in preventing precocious germination (KING 1976, QUATRANO & al. 1983, MORRIS & al. 1991), while its direct role in dormancy after maturation and desiccation is uncertain (WALTON 1980/81, PAULSEN & HEYNE 1983, WALKER-SIMMONS 1987).

Endogenous inhibitors which are different from ABA, such as catechin and catechin tannin (STOY & SUNDIN 1976, MC CREATE & al. 1982), and tryptophan, isolated from *T. aestivum* and located in the bran (MORRIS & al. 1988, MORRIS & PAULSEN 1988), have been found.

In *T. durum* cv. Cappelli, which shows a relative dormancy of 45-60 days after full ripening, MELETTI (1964) found diffusible substances, occurring either in the embryo or the endosperm depending on the stage of maturation, which were capable of inhibiting germination and growth. The presence of these inhibiting substances was tested by experiments with embryo transplant and by utilizing ionizing radiations (MELETTI 1964, MELETTI & al. 1964). Methanolic extracts from isolated embryos at different stages of maturation have revealed the presence of substances which inhibit the germination of seed and the growth of seedling (GRILLI & al. 1975). An as yet unidentified active substance which depresses growth of *Triticum* seedlings and induces chromosome breaking in root meristems, was isolated from after-ripened endosperms (FLORIS & al. 1972). Finally, in methanolic extracts from after-ripened endosperms the presence of an

inhibiting substance, different from ABA, has been shown (MELETTI & FLORIS 1978).

The present study was specifically undertaken to isolate and characterize the inhibiting substances in methanolic extracts from fully ripe endosperms of *Triticum durum* cv. Cappelli.

2. Materials and Methods

Grains of *Triticum durum* cv. Cappelli (crop 1989) were harvested when fully ripe: they had an 11% moisture content. Grains were at the phase of full dormancy and were 35% germinated at 72 hours with a germination energy (G.E.) value of 13.6. Embryos were separated by a sharp gauge from endosperms which have been stored at -20° C until extraction.

Germination test

T. durum cv. Cappelli grains were placed on one layer of Whatman No.1 filter paper in Petri plates. The plates were irrigated with distilled water and incubated at 23° C± 1° C in the dark. Germination counts were made every 24 hours until 72 hours after imbibition had started.

Assay of biological activity of extracts and fractions.

The lettuce germination test was made. For each replicate, 25 achenes of lettuce (*Lactuca sativa,* var. Great Lakes, kindly furnished by Asgrow Italia) were placed on one layer of Whatman No.1 filter paper in a Petri plate to which 250 µl of test solution or of distilled water (control) were added. Petri plates were incubated in the dark at 23° C ±1° C. There were three replications per treatment.

The hypocotyl growth test was also used. Lettuce achenes were placed on one layer of Whatman No.1 filter paper in Petri plates that were irrigated with distilled water. They were incubated at 23° C ±1° C, in the dark until 48 hours after imbibition had started. Lettuce seedlings were then transferred on filter paper irrigated with test solution or with distilled water (control), and allowed to grow at 25° C in the light for 72 hours, at which point the hypocotyl lenght was measured.

The wheat germination and growth test was also performed. After-ripened grains of *T.durum* cv. Cappelli (10 grains for each replication) were placed on one layer of Whatman n.1 filter paper, in a Petri plate which was irrigated with 1ml of distilled water or of test solution. Germination counts were made every 24 hours till 72 hours after imbibition had started, when the coleoptyle and main root were measured.

Extraction of inhibiting substances

Isolated whole endosperms (10000) were milled in a RESTCH MÜHLE mill (2mm sieve) and the powder was extracted in aqueous methanol (80%) at 2° C for three days, on a magnetic stirrer. Every day the extract was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected. Methanolic extract was then evaporated in a rotary evaporator at 35° C to the aqueous phase. The aqueous extract was dialyzed (dialysis membrane 2-18/32″ – Medicell International LTD) against distilled water at 2° C for three days and the volume of the water of dialysis, collected each day, was reduced. The pH of the aqueous phase was adjusted to 8.5 with 5N

NaOH and this phase was partitioned five times with equal volumes of ethyl acetate, the fraction of which was dried under vacuum at 35° C and dissolved in distilled water. The pH of both the aqueous and the ethyl acetate phases was then adjusted to 2.5 with 5N HCl. Both the phases were extracted five times with equal volumes of ethyl acetate and finally two ethyl acetate active phases, indicated as phase I and phase II (as aqueous solutions) were obtained.

HPLC

Phase I and phase II were dried under vacuum and the residues were dissolved in bidistilled water and filtered through a 0.45 μm pore size filter. Phase I (500 μl) was injected into a Waters high performance liquid chromatograph equipped with a Macherey Nagel Nucleosil 7C18 column (250 × 10mm), dual pumps (Waters Assoc., Model 510), a detector for UV absorbance at A_{254} (Waters Assoc., Model 441), an injector Rheodyne (Model 7125), and directed by an IBM personal computer. A 50-min linear program at a flow rate of 4 ml/min from 20% to 100% of aqueous methanol was used. HPLC fractions, collected every 2.5 min, were assayed with the lettuce germination test. The biologically active fraction was injected into the HPLC system with a Macherey Nagel Nucleosil 10 C18 column (250 × 4 mm); a 25-min linear isocratic program (36% of aqueous methanol) at a flow rate of 1.4 ml/min was developed. Phase II (500 μl) was injected into the HPLC system with the Nucleosil 10 C18 column equipped with a pre-column with the same stationary phase as the column. A 50-min linear program at a flow rate of 1.4 ml/min from 0% to 100% of aqueous methanol was used. The chromatograms obtained were compared with those obtained using cis-trans ABA (Fluka) as a standard.

Localization of the inhibiting activity

Isolated whole endosperms (6000) were milled in a rotary drum mill (Mulino Maionchi- Lucca) to achieve the isolation of bran (aleuronic layer and coats) and meal (mostly starchy endosperm); the yield in weight was 26.7%. The quality of the bran and the meal was verified under the microscope. The extraction and purification steps were those utilized for whole endosperms. The resulting phase I and phase II were then fractionated by HPLC as above.

3. Results

Crude extracts from ripe grains of *Triticum durum* cv. Cappelli significantly reduced lettuce seed germination. The strength of inhibition was proportional to the concentration of the extract utilized (Tab. 1). Ethyl acetate partition procedures permitted us to isolate two phases (phase I and II) which were assayed, as aqueous solutions, to evaluate their biological activity by the lettuce germination test (Fig. 1). The pH of the assayed solutions ranged from 4 to 6 and in this pH range lettuce seed germination in water was unaffected. Both phases significantly reduced lettuce germination compared to water control. Moreover, their activity was tested on after-ripe *Triticum* seed germination and seedling growth. The results (Fig. 2 and Tab. 2) showed that phase I and phase II inhibited both

Table 1

Percentage inhibition in lettuce seed bioassay 15,18, 24, and 39 hours after imbibition had started. Extract at concentration of 0.1 endosperm/µl was utilized.

	% inhibition			
	hours of imbibition			
µl of extract	15	18	24	39
25	100	100	100	85
50	100	100	100	100
100	100	100	100	100

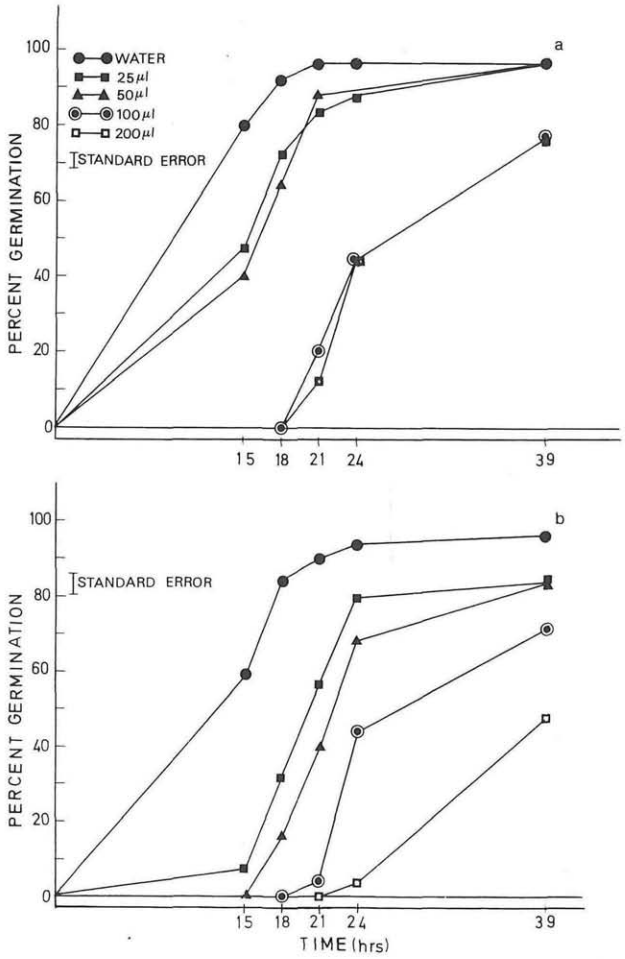

Fig. 1: The effect of various volumes of phase I (a) and phase II (b) at the concentration of 0.3 endosperm/µl on the germination of *Lactuca sativa* var. Great Lakes. Each point is the mean of three replications.

Table 2

The effect of 0.25 (1), 0.5 (2), and 1 (3) ml of phase I and phase II on the coleoptyle (C) and radicle (R) elongation of *Triticum durum* cv. Cappelli. Measurements were made 72 hours after imbibition had started.

		PHASE I			PHASE II		
	H_2O	1	2	3	1	2	3
C	19.1± 0.1[a]	16.4±1	10.3±0.5	6±0.2	10.1±0.3	7±0.4	3.5±0.2
R	47.7±	32.1±1.6	29.8±0,8	16.2±0.5	35.6±1.6	22.7±0.7	5.6±0.7

[a] Values expressed as mean ± SE.

germination and growth. Both the phases, fractionated by HPLC, were collected in different fractions (Fig. 3) which were assayed for their biological activity by the lettuce germination test. The results of phase I showed that most of the inhibition activity was in a fraction that eluted between 10 and 12.5 minutes (fraction 5). This fraction was further fractionated by HPLC and the peaks were collected (Fig. 4). The peak with a retention time of 4.07 minutes was biologically active both undiluted (×) and diluted with distilled water to $^1/_2\times$ and $^3/_4\times$.

The results of the lettuce germination test are shown in Fig. 5. The lettuce hypocotyl growth test was also employed, by utilizing the water diluted ($^1/_2\times$ and $^3/_4\times$) fraction (Fig. 6).

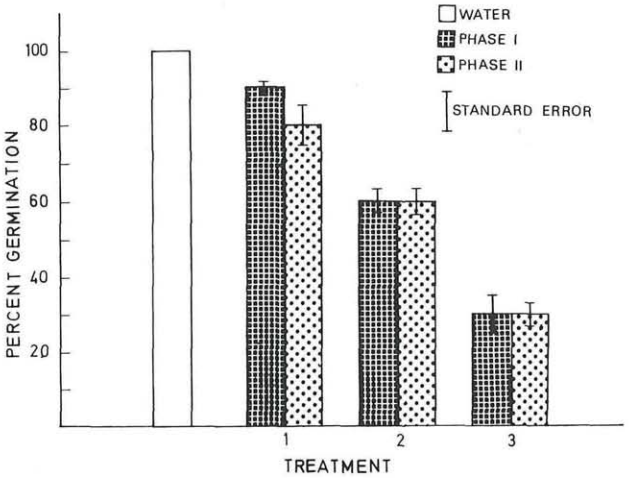

Fig. 2: The effect of 0.25 (1), 0.5 (2), and 1 (3) ml of phase I and phase II on the germination of *Triticum durum* cv. Cappelli 24 hours after imbibition had started. Each point is the mean of three replications.

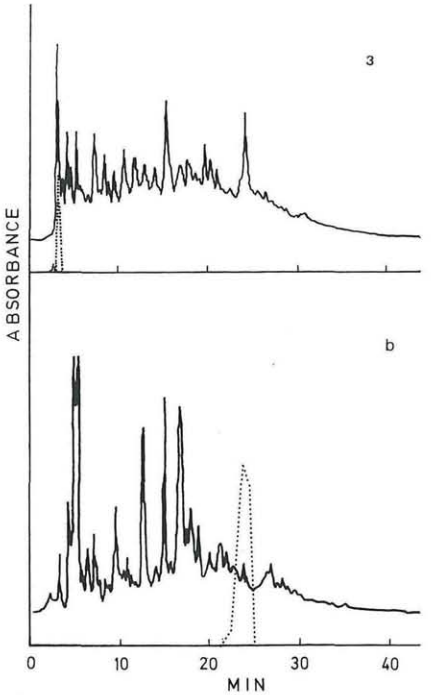

Fig. 3: Chromatogram of phase I (a) and phase II (b). Elution profile of authentic cis-trans ABA is shown as a dotted line.

The main inhibition effect in phase II was due to the fraction that eluted between 22.5 and 25 minutes (fraction 10). By comparing the retention times of the active fractions with the one of standard ABA

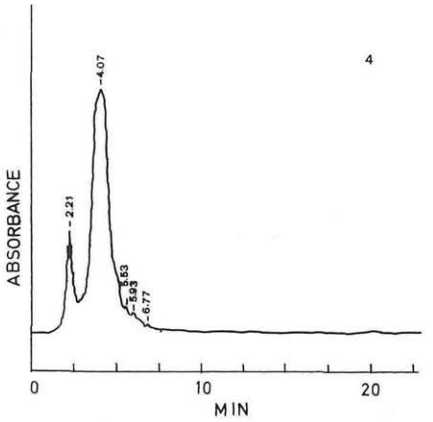

Fig. 4: Chromatogram of fraction 5 with the retention times of the peaks.

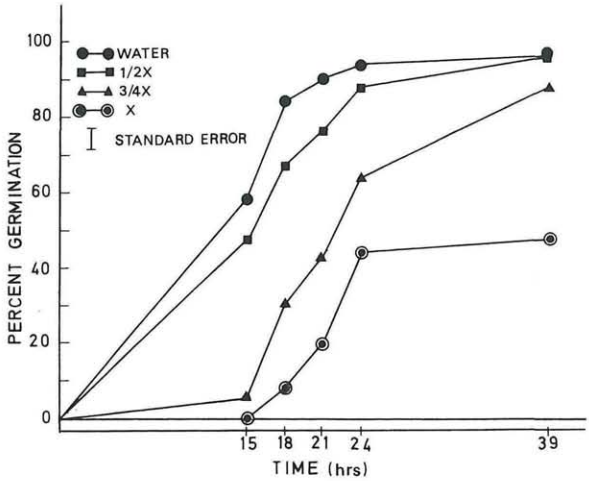

Fig. 5: The effect of various concentrations of the active peak of fraction 5 on the germination of *Lactuca sativa* var. Great Lakes.

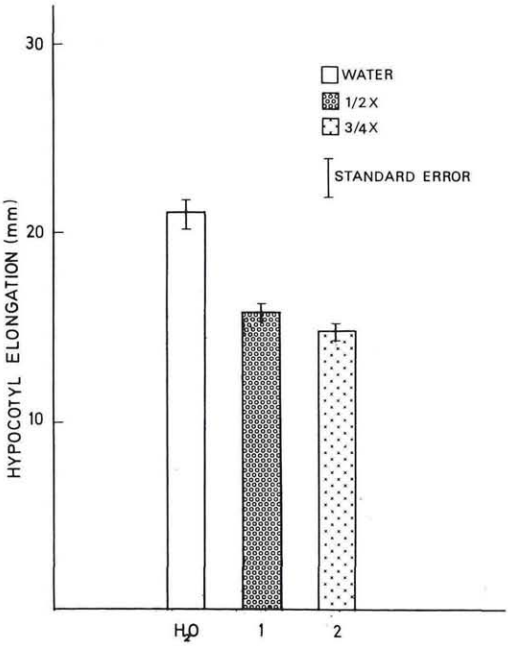

Fig. 6: The effect of various concentrations of the active peak of fraction 5 on the hypocotyl elongation of *Lactuca sativa* var. Great Lakes. Values expressed as mean ± SE.

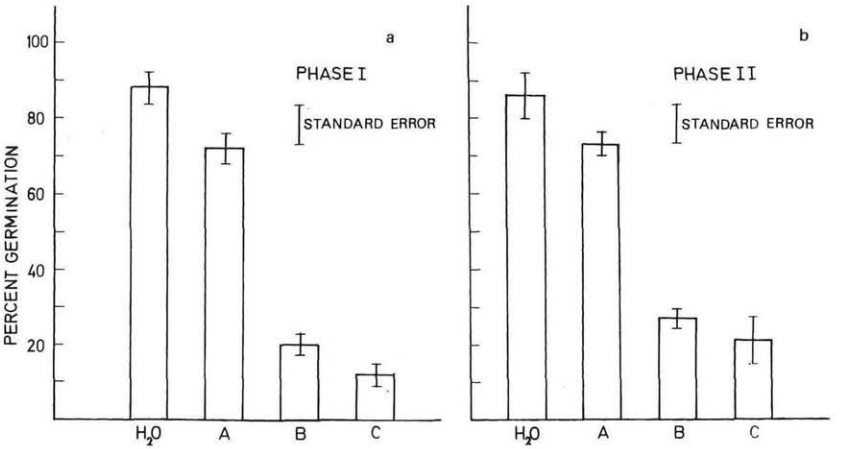

Fig. 7: Percentage germination of *Lactuca sativa* treated with (a) 200 µl of phase I from meal (A) ,bran (B), and whole endosperm (C) and (b) 50 µl of phase II from meal (A), bran (B), and whole endosperm (C), 21 hours after imbibition had started. Each point is the mean of three replications.

chromatographed with similar HPLC conditions, it appears that this inhibitor eluted similarly to fraction 10 and in a different position from fraction 5 (see Fig. 3).

To evaluate the location of inhibiting substances within the caryopses, the phase I and II which had been isolated from the meal and the bran were assayed with the lettuce germination test (Figs. 7a, b). The phase I, obtained from the bran inhibited germination similarly to the phase I from whole endosperms (80% inhibition of bran against 88% inhibition of whole endosperm). The phase I isolated from the meal had a weak inhibition effect. In phase II, most of the inhibition activity was exhibited by the bran (73% inhibition against 27% by the meal). Figures 8 and 9 show chromatograms of phase I and II from the meal and the bran, respectively. The fraction 5 from phase I and the fraction 10 from phase II showed a higher inhibiting activity when they were isolated from bran than from meal (data not shown).

4. Discussion

The present research allowed us to isolate two phases from methanolic extracts of fully ripe grains of *Triticum durum*. These phases contain substances with different characteristics which can inhibit seed germination and seedling growth. The active factor present in phase I is not believed to be ABA, whereas in phase II, part of the inhibition capacity is present in a fraction at the level where standard ABA elutes. ABA would

thus seem to be at least partly responsible for the inhibiting power of this phase, but further studies must be done to test this hypothesis.

ABA is often indicated as the main factor which controls germination, but results obtained in several studies don't seem to confirm its direct role when this growth regulator falls to rather low concentrations in ripe dormant seeds (KING 1976, RADLEY 1979, MORRIS 1989).

Some of the compounds that inhibit the germination of wheat seeds, besides ABA, are catechin and catechin tannins (MIYAMOTO & al. 1961, MC CREATE & al. 1982) and tryptophan (MORRIS & al. 1988).

The fraction responsible for the inhibition of phase I is not very polar and, as previously discussed, doesn't elute similarly to ABA (and to tryptophan, data not shown). This fraction inhibits both germination and growth and it might also occur in the cultivar Creso of *Triticum durum,* as preliminary data seem to indicate. The localization experiments show that the inhibiting fractions are contained mainly in the bran (aleuronic layer and coats), according to data recorded for *T. aestivum,* where inhibiting activity was found in the grain tissues outside the endosperm (MORRIS & PAULSEN 1988).

The simultaneous occurrence of two different substances (or groups of substances), both capable of inhibiting germination and growth in similar way, could be an essential condition to ensure a more refined control of germination. This fact could be particularly important in wet or rainy areas where the water soluble inhibitors might be leached from the seed during ripening phases.

Future work will involve further studies on the chemical nature of inhibiting substances, since their availability will permit us to evaluate their actual influence on the mechanisms which control *T. durum* seed germination.

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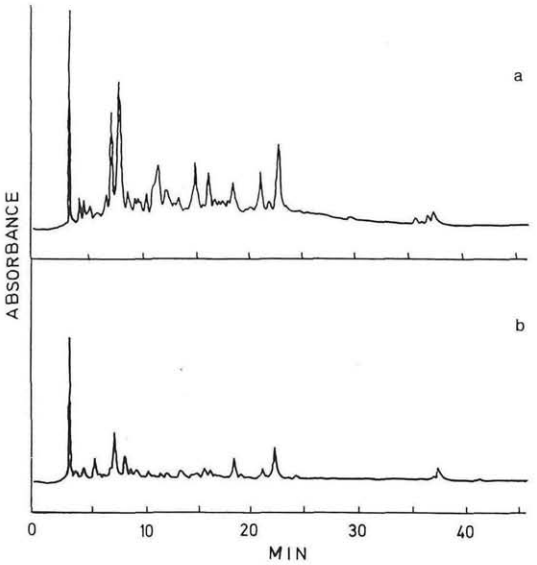

Fig. 8: Chromatograms of phase I from bran (a) and meal (b).

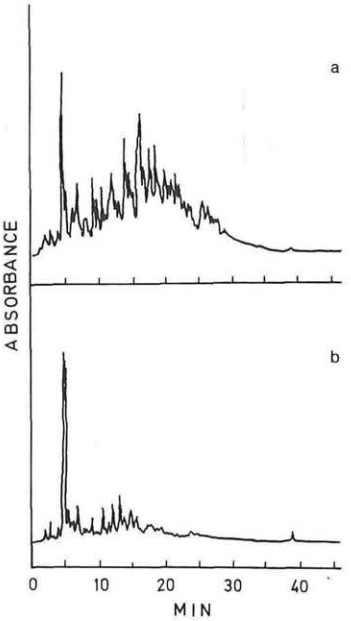

Fig. 9: Chromatograms of phase II from bran (a) and meal (b).

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