High-performance reversed-phase liquid chromatography (HPLC) of favism-inducing factors in *Vicia faba* L.[1]

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Summary. A HPLC assay for L-Dopa, convicine, and vicine in broad-bean extracts has been developed. The distribution of these compounds in the different organs of the plant has been studied, especially in the seeds, where their presence may cause favism if the beans are used as food.

The high protein content of broad-beans (*Vicia faba* L.) has interested many researchers in the field of plant breeding because of the possibility of obtaining good quality proteins from this legume. However, the beans contain toxic compounds, which can induce the haemolytic disease of favism in individuals with a hereditary deficiency of red cell glucose-6-phosphate-dehydrogenase (G-6-PD)[2,3]. This reduces the possibilities for the utilization of this protein in some regions of the Mediterranean and Middle East areas[3-6]. Therefore, in investigating the quality of different broad-bean cultivars, not only the content of protein and other nutritional factors, but also the content of L-Dopa, vicine, and convicine (favism-inducing factors) must be considered.

Materials and methods. A Perkin Elmer liquid chromatograph series 2, equipped with spectrophotometric detector LC-55, was used. The column was a stainless-steel tube (30 cm × 4 mm I.D.) packed with μBondapak C_{18} (Waters Ass., Milford, Mass., USA), having an average particle size of 10 μm. A stainless-steel precolumn (25 ml/unit), packed with μBondapak C_{18}/Corasil (37-50 μm), was used. Conditions used were as follows: flow rate = 1.5 ml/min, pressure drop = 1200 psi, detector regled at 280 nm, recorder at 5 mV and 0.02 A, chart speed = 5 mm/min, eluent was water (HPLC grade). Injected volume = 5 μl.

L-Dopa (Fluka AG, Buchs, Switzerland), vicine (Roth, Karlsruhe FRG), and convicine (purified by Ist. Naz. Nutrizione, Roma, Italy) were dissolved in water.

Dry seeds of *Vicia faba* L. var. major Harz., cv. S. Pantaleo (weight of 1000 seeds = 2350 g) were first milled. The powder was extracted for 30 min with methanol/water (1:1) (50 ml/2 g seed), by refluxing, and the extract filtered through a Schleicher & Schüll membrane filter (pore size 0.45 μm). Residues were extracted twice in a similar way. The combined filtrates were concentrated in vacuo at 30 °C and the final volume adjusted at 50 ml by methanol/water (1:1). The solvent and the extraction scheme are the ones employed by other authors and the aim here is to produce data which can be compared with those already recorded in the literature[5,7-9].

Results and discussion. Table 1 shows the retention time of the standard compounds considered. Under the experimental conditions used, phenolic compounds which may exist in the methanolic extracts (benzoic and cinnamic acid derivatives were tested) do not interfere as they had not eluted before 15 min. The chromatograms in the figure show the separation of L-Dopa and alkaloids in vegetable extracts, prepared from seedlings and seeds (cotyledons only).

The calibration curves for vicine and convicine at 10 concentrations over the range of 250 ppm to 25 ppm were determined. Plots of peak height were linear for the 2 compounds. When 5 μl of standard solution were injected, the error was less than 1%. When the calibration curve for L-Dopa was prepared in the same way the error was higher because of the tendency to oxidize: for this reason the stock solution of L-Dopa was prepared daily and stabilized with a drop of concentrated HCl. The calculated error on methanolic extracts of *V. faba* L., injecting 5 μl, was about 3%.

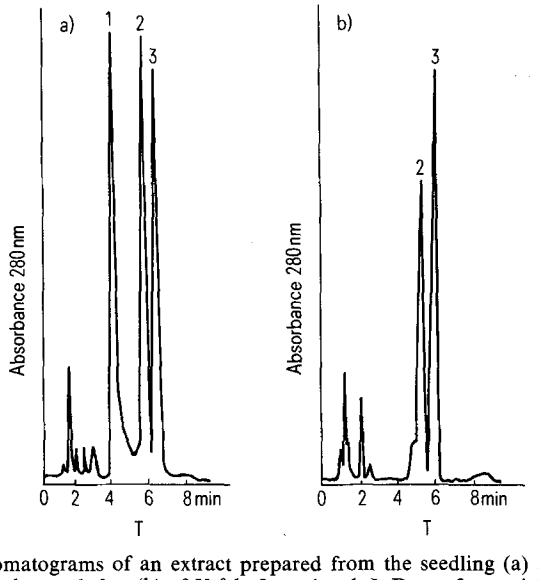
L-Dopa, vicine and convicine content changes in different organs of the fababean plant. Generally, L-Dopa content is very high in leaves (from 1.4% of d.m. to 4.9% of d.m.) and pods (about 4% of d.m.); as regard the seeds it can only be found in very young (less than 1 cm long) seeds (0.5% of d.m.), because L-Dopa disappears during their development.

Table 1. Chromatographic separation of standard compounds

Compound	t_R (min)
L-Dopa	3'54"
Convicine	5'36"
Vicine	6'06"

Table 2. Vicine and convicine content of *Vicia faba* L. major seeds at various stages of development

Seeds weight (g)	d.m. (%)	Vicine (% on d.m.)	Convicine (% on d.m.)	Vicine (mg/seed)	Convicine (mg/seed)
0.13	14.72	3.13	1.03	0.6	0.2
0.68	15.76	2.14	1.26	2.3	1.3
1.10	16.49	1.83	1.15	3.3	2.1
1.50	17.56	1.69	1.10	4.4	2.9
1.93	17.70	1.50	1.16	5.1	4.0
2.47	19.16	1.40	0.91	6.6	4.3
2.89	16.28	1.63	1.13	7.7	5.3
4.27	22.76	1.06	0.45	10.3	4.3
4.95	36.60	0.28	0.16	5.1	2.8
4.96	40.54	0.26	0.16	5.3	3.1



Chromatograms of an extract prepared from the seedling (a) and from the cotyledon (b) of *V. faba* L. major. 1, L-Dopa; 2, convicine; 3, vicine.

Vicine and convicine are present especially in seeds, whereas in senescent leaves they are only 0.26% and 0.11% of d.m. respectively.

Table 2 shows that vicine content, as a percentage, decreases rapidly during seed development (from 3.13% to 1.83% of d.m.); it becomes almost constant in ripe fresh seed (1.5-2.5 g of weight), and then drops during the drying phase. Convicine has a different behavior: values, as percentages, were always uniform until the dry phase starts, then a remarkable decrease was observed.

The table also shows that the production of alkaloids in seeds (mg/seed) steadily increases during development and ripening. Then it decreases during the drying stage, when the color of the cotyledons changes from green to pale yellow (4.5 g of weight). Up to now, it seems impossible to explain the causes of this decrease during the drying phase, as the functions of these compounds in the general metabolism of the plant (perhaps as a nitrogen reserve, or as growth regulators) are unknown. Similar variations have been observed in other cultivars of *V. faba* L., even when the alkaloid content was different.

These results can be considered in relation to the favism problem, a disease related to the use of broad-bean seeds in the human diet. As concerns the oxidization of glutathione (GSH) to GS-SG, vicine, convicine and L-Dopa have not the same activity 'in vitro'; L-Dopa activity is, probably, lower than that of the 2 alkaloids[12]. For this reason the variation of the 2 alkaloids, in relation to the seed age, was studied. In the literature, studies of the variation of the 3 favism-inducing factors, considered together, were not found. At this time it is not clear whether the absence of L-Dopa in ripe fresh seeds is a cultivar characteristic or a general phenomenon of all broad-bean seeds. Longo et al.[10], carrying out a screening in different organs of *V. faba* major, *V. faba* minor, *V. sativa* and *V. narbonensis* plants, found traces of L-Dopa in green seed only and not in dry ones. It must be added, in agreement with what has been observed up to now, that L-Dopa is a precursor in many biosynthetic reactions of the plant; therefore its content falls during seed development. The 2 alkaloids, which are final products of a metabolic process, behave in a different way; vicine and convicine are always present in the seed at various ages. Previous reports[7,8] concerning the vicine and convicine variation in seeds at different physiological stages do not agree with each other, even though they all agree that unripe seeds have a significant toxicity (oxidization 'in vitro' of GSH).

Conclusions. HPLC gives a rapid method for the quantitative determination of the favism-inducing factors in *V. faba* L. Preparation of samples for this procedure is simple, and it is sensitive. The quickness of the method will prevent oxidization and alkaloid precipitation. Furthermore, the high sensitivity makes it possible to obtain more reliable data for the study of antinutritional factors.

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Unstable genetic system in *Drosophila melanogaster:* I. Instability at the *cinnabar* locus

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Summary. A mutant strain containing a *cinnabar* allele *(cn^{rbr}, rojo brillante)* is reported, that produces wild-type revertants at the *cinnabar (cn)* locus. In cn^{rbr}/cn heterozygotes the rate of mutation is highly increased. The presence of a mutator agent acting premeiotically is indicated.

This paper reports studies on an unstable mutation at the *cinnabar* locus of *Drosophila melanogaster,* which arose in a *vestigial* stock with wild-type eye colour, obtained from the Department of Genetics of the Universidad Mayor de San Marcos de Lima (Peru) in January 1970, and since maintained in mass culture in our laboratory. In June 1970, a few flies appeared whose red eyes were more red than those of the wild type; the ocelli were colorless, but the eye color darkened with age. I isolated these flies and demonstrated, using appropriate crosses, that this color was due to the presence of a recessive mutant belonging to the *cinnabar (cn)* series[1] and I called it[2] *rojo brillante*, symbolized by cn^{rbr}.

The behavior of the *rojo brillante* strain was normal. Using a wild strain, I removed the new mutant from the original strain, with the *vestigial* mutant, and I obtained a cn^{rbr} strain on a wild type background. This new strain was maintained in mass cultures but, in some vials, flies with wild-type eye color spontaneously appeared. This wild-type eye color is due to the presence of an allele which is dominant over both cn^{rbr} and *cn.* I thought this allele originated from a spontaneous reversion of the cn^{rbr} allele and named it *normal espontáneo* symbolized by cn^{+e} (the *e* in the symbol indicates its spontaneous origin).

I have studied the spontaneous appearance of wild-type individuals (the reverse mutation from cn^{rbr} alleles to cn^{+e}