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The Quantitative Determination of 2(3)-Benzoxazolinone from Rye Seedlings

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A method for the quantitative determination of the antifungal substance, benzoxazolinone (BOA), found in rye seedlings, is described. The method is based on the determination of the absorption at 275 mμ in an ethanol solution in which BOA shows a strong maximum. Before the determination of the absorption a fraction has to be prepared from the rye extract where BOA is found in its entirety, and from which disturbing substances are removed. The purification is performed in a cellulose powder column using *cyclo*hexane-ethanol-water as solvent.

After we had discovered the occurrence of benzoxazolinone as an antifungal factor in rye seedlings [1] it was important to develop a quantitative method for its determination. The method developed by us and used in this laboratory since the beginning of 1956 is reported in this paper.

PRINCIPLE

2(3)-Benzoxazolinone (BOA) is extracted from a homogenized sample with boiling water, shaken into ether, the ether is evaporated, the evaporation residue is chromatographed on a cellulose powder column using *cyclo*hexane-ethanol-water as a solvent. The fraction containing BOA is diluted with absolute ethanol to a fixed volume, and its ultraviolet absorption spectrum is measured. The 275 mμ extinction of the maximum is compared with an extinction curve determined with known amounts of BOA, by which procedure the BOA content of the sample to be investigated is obtained.

Reagents: Absolute ethanol. *Cyclo*hexane (Merck's or May & Baker's *cyclo*hexane): The ultraviolet absorption spectra of different batches of *cyclo*hexane differed noticeably. When necessary, *cyclo*hexane was purified by silica gel-aluminum oxide treatment [2]. Several batches could, however, be used as such without special purification. Ether: The ether was purified by shaking with acid ferrosulphate solution 4 to 5 times, by washing with water, and finally by distillation. Cellulose powder (Whatman, "Ashless",

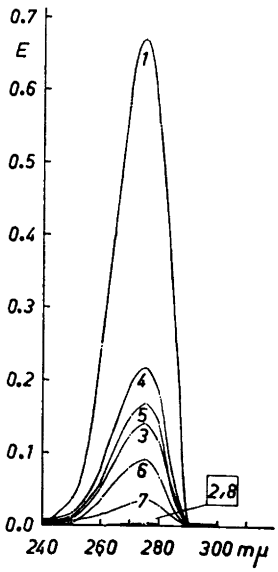

Fig. 1. Elution of BOA from the cellulose powder column with *cyclo*hexane-ethanol-water (*cf.* text). 200 μg pure BOA was used in the experiment.

Curve	1.	Fraction	between	5.6	and	9.0	cm	206	μg	BOA
»	2.	»	»	5.6	»	6.0	»	~0	»	»
»	3.	»	»	6.0	»	6.5	»	44	»	»
»	4.	»	»	6.5	»	7.0	»	68	»	»
»	5.	»	»	7.0	»	7.5	»	52	»	»
»	6.	»	»	7.5	»	8.0	»	28	»	»
»	7.	»	»	8.0	»	8.5	»	11	»	»
»	8.	»	»	8.5	»	9.0	»	~0	»	»
								203	μg	BOA

W. & R. Balston & Co.). Benzoxazolinone: The product used for determination of the extinction curve was synthesized from *o*-aminophenol and urea; m. p. 141°C. Glass-wool: Merck's *pro analysi* glass-wool or ordinary glass-wool washed with acid, ethanol, and water.

Preparation of cellulose powder columns: The columns used in chromatography were prepared from cellulose powder in glass tubes, 20 cm high and 9 mm in diameter. The lower end was drawn into a short beak. The diameter of the tube should not deviate much from 9.0 mm.

Glass-wool is placed into the lower end of the tube and stopped as tight as possible with a glass rod. Cellulose powder (1.70 g) is placed on the top of the wool in 5 or 6 portions. After each addition the tube is shaken in vertical position until the powder ceases to sink, and then it is packed tight with a glass rod. The height of the finished cellulose powder column has been 5.0 ± 0.2 cm. Distilled water is absorbed through the column from below, after which distilled water is placed on top of the column and sucked through with a vacuum pump. The column should not be allowed to dry. The column is now washed once with ethanol (94 %), and twice with the solvent *cyclo*hexane-absolute ethanol-water (400:80:1). Even cellulose powder columns are obtained more quickly when using centrifugation.

PROCEDURE OF THE DETERMINATION

0.3 to 4 g of rye seedlings, homogenized in a mortar, is weighed in a Pyrex-glass centrifuge tube, 15 ml of distilled water is added, and the suspension is mixed. The tube is kept in boiling water for 20 min under occasional shaking. After that the tube is immediately centrifuged and the separated solution is filtered into a 250 ml centrifuge tube. The residue is treated four times in the same way. The combined water extract in the centrifuge tube is shaken four times with peroxide free ether. The ether solution, separated by centrifugation, if necessary, is evaporated to dryness in a glass bowl, and the residue

is transferred to a 4 ml test tube by small amounts of ether. The ether is evaporated to dryness, and the residue dissolved in *cyclo*hexane-ethanol-water solution (1.00 ml) in which it dissolved completely. 0.200 ml of the solution obtained is placed on top of the column when there is no more solvent. The solution is allowed to run into the column, 3 drops of solvent are carefully added with a pipette, and when these drops have run into the column the tube is filled to the 0 mark on a centimetre scale fastened behind the tube. A small glass tube is placed upside down on the column to prevent evaporation. When the surface of the solvent has sunk 5.6 cm, the liquid emerging from the column is collected into a 10 ml volumetric flask. BOA emerges from the column between 5.6 and 9.0 cm as could be shown in experiments with pure BOA (Fig. 1). This fraction is taken up in a volumetric flask which is then filled to the mark with absolute ethanol, and the ultraviolet absorption spectrum is measured in a Beckman spectrophotometer.

EXTINCTION CURVE

The BOA content of the solution measured is obtained from the extinction curve, or by using multiplication by a coefficient calculated from the curve. The extinction curve was obtained with the following amounts of BOA: 50, 100, 200, and 400 μg/10 ml. The same amount of solvent was placed in a 10 ml volumetric flask as when chromatographing the proper samples to be investigated. The above-mentioned amounts of BOA, dissolved in absolute ethanol, were pipetted into this solution and the flasks were filled to the mark with absolute ethanol. The blank, made by simply running the solvent through the column, showed that if the *cyclo*hexane used is pure, the effluent has no absorption, and that the measurements can as well be made using absolute ethanol in the blank.

The extinction at 275 mμ follows Lambert-Beer's law. The extinction using 1 cm quartz cells has to be multiplied by 317.0 to establish the amount of BOA contained in 10 ml solution. The logarithm of the molar extinction of the substance is 3.63. The spectrum in the region 240—300 mμ is always measured in order to find out if there is an absorption caused by foreign substances. From young rye seedlings, the BOA content of which is high, a curve nearly corresponding to that of the pure substance is generally obtained, the absorption at 245—255 mμ and 285—300 mμ being, however, somewhat higher than with pure BOA solution. Especially at 245—255 mμ this absorption has every now and then become considerably high. The BOA content has been calculated by subtracting the calculated unknown absorption from the extinction measured at the absorption maximum at 275 mμ. If the foreign absorption is high, the accuracy of the method is low.

Control of the method: 1) In order to find out if BOA in the extract from rye seedlings emerges completely from the column at the range 5.6—9.0 cm, the following experiment was performed with the rye-varieties Ensi and Kungsråg II, which in regard to winter hardiness represent extreme types among the varieties used:

Ensi, 20 seedlings, 7 days old, fresh weight 2.138 g
Kungsråg II, 21 seedlings, 7 days old, fresh weight 2.380 g

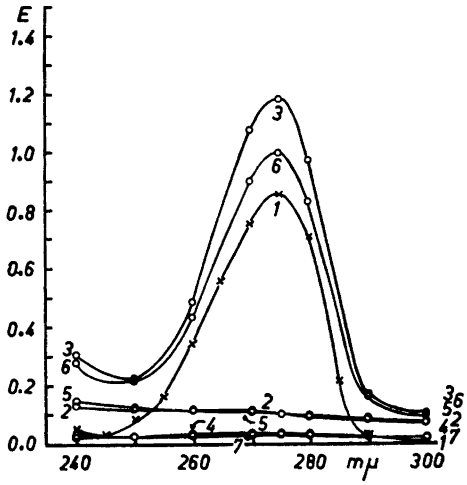

Fig. 2. BOA-Determination of Ensi and Kungsråg II rye seedlings. BOA-fraction (5.6—9.0 cm), prefraction (4.6—5.6 cm), and afterfraction (9.0—10.0 cm). E = E/g fresh weight. 1. Pure BOA. 2. Prefraction (Ensi rye). 3. BOA-fraction (Ensi rye). 4. Afterfraction (Ensi rye). 5. Prefraction (Kungsråg II rye). 6. BOA-fraction (Kungsråg II rye). 7. Afterfraction (Kungsråg II rye).

Determination and chromatography were performed in the usual way. In addition to the proper fraction containing BOA, 1 cm of solvent was taken before and after this fraction. Fig. 2 shows the spectra of pre- and afterfractions as well as the spectrum of the fraction containing BOA. The absorption maximum of BOA is completely lacking in pre- and after-fractions. It can thus be concluded that BOA has emerged entirely at the range mentioned.

Table 1. Recovery of BOA added to the rye extract.

Run	Extinction 275 mμ	μg BOA found	Recovery %
200 μl (1 μg BOA/μl)	0.628	199	99.5
100 μl BOA solution + 100 μl rye solution	0.596	189	102.5
200 μl rye solution	0.551	173	

Table 2.

Determination	BOA μg/g fresh weight	Deviation % from mean value
1	467	2
2	442	3
3	438	5
4	455	0
5	480	5
	Mean value 456	

Table 3. Parallel determinations of BOA from different varieties of rye (field experiments).

Variety of rye	BOA μg/g fresh weight	Mean value	Deviation % from mean value
Ensi 1	334	323	3
2	312		
Onni 1	456	486	6
2	516		
Pekka 1	490	476.5	3
2	461		
Visa 1	211	211	0
2	211		
Toivo 1	458	438.5	4
2	419		

This is in accordance with the results obtained with pure BOA (*cf.* Fig. 1). Similar curves were also obtained with Pekka, Visa, and Greus rye.

2) Recovery of BOA added to the plant extract. 70 g of rye was extracted. The residue of the ether extract was dissolved into 8.00 ml solvent. The determinations were carried out as described above; details of the experiments and results in Table 1.

3) Differences between parallel determinations. The following determinations were made on August 16, from young rye seedlings (Pekka variety) cultivated in pot cultures in sand. The same solution was used in all determinations (Table 2). Some results obtained in parallel determinations on rye seedlings from field experiments are shown in Table 3.

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