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Determination of L-5-Vinyl-2-thiooxazolidone from Plant Material and Milk

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A quantitative micromethod for the determination of L-5-vinyl-2-thiooxazolidone in different parts of plants, in silage, and in milk, is presented. The method is based on the separation of this substance by paper chromatography and its spectrophotometric determination. The absorption maximum is at 240 mμ.

Astwood *et al.*[1] have presented a quantitative method for the determination of the L-5-vinyl-2-thiooxazolidone (VTO) contents in the seeds and the roots of cruciferous plants. No method for the determination of the small amounts of VTO formed enzymatically in the green parts of cruciferous plants has, however, been described earlier. The formation of this substance in the green parts of many cruciferous plants was in fact found in this laboratory only two years ago [2]. Later Long *et al.*[3] also isolated VTO from cabbage. The development of a method for the determination of VTO in milk, in which it can be present only in extremely low concentrations [4], has met with considerable difficulties.

EXPERIMENTAL

I. VTO in green parts of plants.

Equipment (*cf.* Fig. 1). 1. A Mojonnier flask (12 ml); the volume of the lower part 2 ml and that of the upper part 10 ml; glass plug.
2. A conical flask for vacuum evaporation (100 ml) and a glass suction adapter.
3. Chromatography equipment for ascending development; a. A glass cylinder, ø 11 cm, height 26 cm, b. Paper, Whatman No. 1.

Reagents. 1 Water saturated ethyl acetate.
2. Phosphate buffer, pH 6.5, M/15 KH_2PO_4 + M/15 Na_2HPO_4.
3. Ethyl ether-methanol, 1:1. Ether shaken peroxide-free with $FeSO_4$ and distilled.

Determination. 10 g of minced plant material is allowed to stand for 7 h at room temperature in a beaker covered with a polytene membrane. The beaker with its contents is heated on a water bath for 5 min, the plant material is transferred to a piece of cloth (gauze) in which it is tightly pressed. 1.5 ml of the filtered press juice is measured into a Mojonnier flask as well as 0.5 ml of phosphate buffer. The mixture, the pH of which is 6.3–6.5, is extracted with 5 ml of water saturated ethyl acetate by thorough

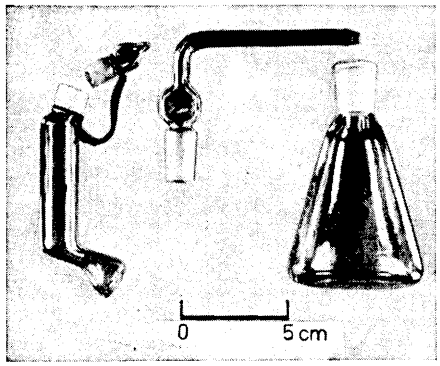


Fig. 1. Equipment for the determination of VTO. A Mojonnier flask (1), a glass adapter (2), and a 100 ml vacuum evaporation flask.

shaking for 20 sec. The clarified extract is poured into a 100 ml conical flask. A second extraction is performed with another 5 ml amount of ethyl acetate. The combined extracts are evaporated to dryness *in vacuo* (water pump). To prevent overheating, the liquid in the conical flask has to be kept in rotatory motion. Using water bath heating as well, evaporation can be effectively speeded up. The residue is now dissolved in 0.5 ml of hot water and 250 μl of the extract obtained is used in two-dimensional paper chromatography, first in methanol-water and then in methanol-ether. Ascending development is used.

On the papers dried at room temperature the boundaries of the spots are drawn using UV-light. The spot of VTO is first cut off and then cut into small slips which are extracted twice with 2.5 ml of hot water in a test tube on a boiling water bath. The combined extracts are estimated photometrically with a Beckmann Spectrophotometer at 220, 240, and 260 mμ.

In calculating the results the method reported by Astwood *et al.*[1] is used. The average of the extinction values at 220 and 260 mμ are subtracted from the extinction value at 240 mμ and the obtained difference is multiplied with the factor 1.16. The correction is justified since the VTO separated by paper chromatography is not yet quite pure. A calibration curve is constructed with pure VTO. The μg-values of the solutions to be investigated are read from this curve, and the results are multiplied with the dilution factor. The results are given in μg/ml of press juice.

In calculating the results *Lamber-Beer*'s law can also be used, in which case the *E*-value obtained by the correction made according to Astwood is multiplied with the factor 7.5. The concentration of the solution to be measured is obtained in μg/ml. The coefficient is equivalent to log *E* 4.23 of the VTO isolated from the seeds of pure big-leafed turnip, rutabaga (Bangholm), and winter turnip rape (Lembke).

II. VTO in silage.

From silage the analysis is made in much the same way as is reported above in connection with the investigation of green parts of plants. Since silage is acid (pH in good silage 3—4) the superfluous acid has to be neutralized first. Such an amount of 1 N NaOH is then added to the juice, obtained from the silage by pressing, that the pH reaches the value 5.5—6. 1.5 ml of the press juice thus treated is pipetted into a Mojonnier flask as well as 0.5 ml of M/15 phosphate buffer, whereafter the analysis is continued in the normal way.

III. VTO in seeds.

1.0 g of finely ground (Willey laboratory mill) seeds are moistened with 5.0 ml of water in a 30 ml beaker, covered with a polytene membrane, and allowed to stand for 9 h at room temperature. The beaker with its contents is heated on a boiling water

bath for 5 min and filtered. The residue on the filter is washed twice with in all 3 ml of hot water. 1.0 ml of the combined filtrates is pipetted into a Mojonnier flask, and the analysis is then continued in the same way as is reported for the analysis of green parts of plants. This time a smaller amount, *e.g.* 100 μl, will, however, suffice in the paper chromatography run.

IV. VTO in milk.

A. Whole milk.

Equipment. Equipments 2 and 3 from section I, and in addition:

4. 3 l and 60 ml separatory funnels.
5. An all-glass vacuum evaporating apparatus with a 1 l flask.

Reagents. 1. Water saturated ethyl acetate.
2. 1 % ammonia.
3. 18 % and 0.5 % acetic acid.
4. Methanol-ether, 1:1.
5. Water-saturated chloroform.

Determination. 300 ml of milk, heated to 85°C and rapidly cooled, is shaken twice with 500 ml of ethyl acetate in a separatory funnel. The combined extracts are evaporated almost to dryness *in vacuo* by slight warming. The residue is treated with 5 ml of 1 % ammonia by gentle shaking and warming in a water bath simultaneously. The ammonia treatment is repeated four times in all. The ammonia extracts obtained are combined and neutralized with 18 % acetic acid to pH 6.5—7.5. Milk fat is removed by treating the extract twice in a separatory funnel with 5 ml of petroleum ether. To avoid emulsion this extraction has to be performed very carefully. The water phase, the volume of which is about 20 ml, is extracted in a small separatory funnel twice with 30 ml of chloroform. The chloroform extract is evaporated to dryness *in vacuo* by slight warming in a 100 ml conical flask. The residue is dissolved in 0.5 ml of hot water and 0.3 ml of it is used in the chromatography run. In the ascending two-dimensional run 1) 0.5 % acetic acid, and 2) methanol-ether, are used as solvents.

A control solution for spectrophotometric measurement is prepared thus that beside the spot of VTO an equally large piece of filter paper is cut out, which piece is then treated in the same way as the spot. The spectrum is measured with a Beckman Spectrophotometer at 220—290 mμ. In calculating the results the extinction value between the maximum point at 240 mμ and the straight line connecting the points at 225 and 260 mμ is used. (E_0, *cf.* Fig. 2).

This extinction value is multiplied with the correction factor 1.16 and the product further with the constant 7.5. The content of VTO in the measuring solution is thus given in μg/ml. The content of the substance to be determined in the milk is obtained when the product is multiplied with the dilution factor.

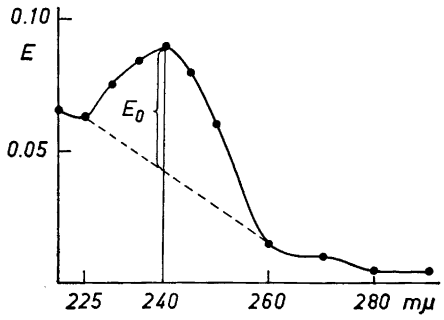


Fig. 2. Extraction curve of the VTO excreted from milk. E_0 used in the calculations, *cf.* text.

B. Skim milk.

This method has been used by Virtanen *et al.*[4]

Equipment and reagents. Equipments 1, 2, and 3 from section I and a 3 l separatory funnel as well as an all-glass vacuum evaporating apparatus with a 1 l flask. Reagents 1, 2, and 4 from section IV and 0.5 % acetic acid.

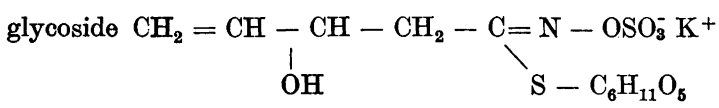
Determination. 500 ml of skim milk is extracted twice in a separatory funnel with 500 ml of ethyl acetate. The acetate is evaporated to dryness *in vacuo* by slight warming, and the residue is treated with 5 ml of 1 % ammonia, and after that also twice with 5 ml of hot water. The extracts are evaporated to dryness in a 100 ml conical flask (vacuum). The residue is washed quantitatively with 2 ml of 0.5 % acetic acid in small amounts into a Mojonnier flask and are shaken twice with 5 ml of ethyl acetate. The ethyl acetate extracts are evaporated to dryness in a 100 ml conical flask (vacuum) and dissolved in 0.5 ml of water. In paper chromatography a two-dimensional ascending run is made from as large a part of the extract as possible in 0.5 % acetic acid and methanol-water. The place for the VTO spot is examined in UV-light whereafter it is cut out from the paper and dissolved in 5 ml of hot water (2 × 2.5 ml). The UV-spectrum is measured at 220—290 mμ. Distilled water is used as control solution. In calculating the results the method described in the section for green parts of plants is used.

RESULTS

Green parts of plants.

Paper chromatograms. In the paper chromatographic run a chromatogram with several spots is obtained from green parts of plants. Fig. 3 shows such a chromatogram. The spot of VTO is marked with S. In the seeds the share of interfering substances is very small although the concentration of VTO is very high.

Enzymatic formation of VTO. VTO is rapidly formed [5] from the progoitrin glycoside



The progress of the reaction can be seen in Fig. 4 which shows experiments performed with (1) rape seeds, and (2) green parts of rape plants.

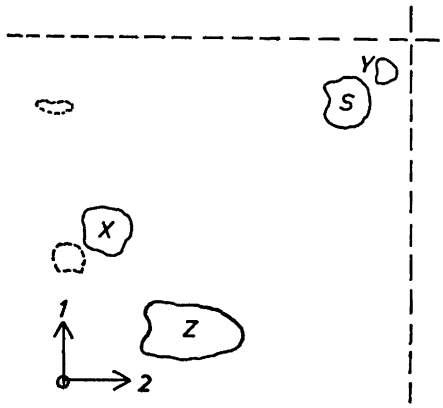


Fig. 3. A two-dimensional paper chromatogram of green parts of winter turnip rape. Solvents used in direction 1: methanol-water, and in direction 2: methanol-ether. Spot S is VTO

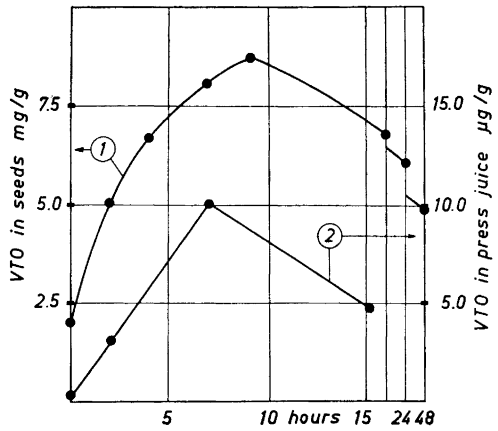


Fig. 4. Enzymatic liberation of VTO at room temperature (1) in crushed seeds and (2) in crushed green parts of Liho rape.

A very rapid enzyme action is obtained when higher temperature is used. In the experiments the maximum value has been obtained after an incubation time of 2 h at 42°C.

The accuracy of the determination of VTO in green parts of plants and seeds. To test the accuracy of the method known amounts of pure VTO was added to the water extract of crushed seeds and to the press juice of fresh as well as preserved green parts of plants. The results of the tests can be seen in Table 1.

The table shows that the developed analytical method worked quantitatively. In the column for µg-amounts in the table pure VTO could also be recovered quantitatively in a determination performed with reagents only.

VTO in the plant material. Using the above methods of determination, the seeds of various cruciferous plants, their green parts, and silage prepared

Table 1. L-5-Vinyl-2-thiooxazolidone contents in water extracts of green parts of rape, silage, and rape seeds as well as the values found after the addition of VTO.

Material	VTO in the extract µg/ml	VTO addition µg/ml	VTO total 1 + 2 µg/ml	VTO found µg/ml	VTO found %
	1	2	3	4	5
Green parts of rape	3.9	1.2	5.1	5.2	102
	3.9	2.7	6.6	6.5	99
Silage *	23.5	15.0	38.5	38.6	100
Rape seeds	15.8	2.3	18.1	17.9	99
	25.6	6.8	32.4	32.4	100
	25.6	22.5	48.1	46.9	98

* Prepared by the AIV method using NH_4HSO_4 as a preservative.

Table 2. VTO values found in crushed seeds of cruciferous plants.

Species	Samples	VTO	
		Ranges of variation mg/g	Average mg/g
Spring rape	8	2.3 — 4.2	3.16
Winter rape	11	7.7 —12.3	8.96
Spring turnip rape	3	0.2 — 0.8	0.46
Winter turnip rape	16	0.04— 0.7	0.27
Big-leafed turnip	9	0.2 — 3.5	1.16
Turnip	2	0.5 — 0.9	0.70
Swede	9	3.2 —14.5	6.70
Marrow kale	1	—	1.07
Radish	1	—	0.04

Table 3. VTO values of green parts of cruciferous plants. Samples from field cultivations and greenhouse. Contents given in μg/ml press juice.

Species	Samples	VTO	
		Ranges of variation μg/ml	Average μg/ml
Spring rape V *	1	—	4
» » K **	8	2— 40	14
Winter rape V	9	7— 73	33
» » K	4	23—135	69
Spring turnip rape K	2	3— 60	32
Winter » » K	1	—	24
Turnip K	1	—	72
Big-leafed turnip V	4	11— 55	25
Marrow kale V	3	0— 8	3
» » K	1	—	9

* V = sample from field cultivation.
** K = sample from greenhouse.

Table 4. VTO values of silage prepared from cruciferous plants by the AIV-method. Contents given in μg/ml press juice from silage.

Raw material	Samples	VTO	
		Ranges of variation μg/ml	Average μg/ml
Spring rape	4	0— 8	3
Fodder rape	22	0—37	5
Winter rape	15	0—56	21
Big-leafed turnip	5	0—33	11
Swede (tops)	3	9—80	28

from cruciferous plants by the AIV method, were investigated. Part of the plants were grown in a greenhouse, but the bulk of the samples investigated came from field cultivations, however. The results of the tests are collected in Tables 2, 3, and 4.

VTO in milk.

Whole milk. Preliminary experiments showed that VTO very rapidly disappears in the milk. To eliminate this factor which hampered the determination, milk into which a known amount of the substance was added under different test conditions was investigated. Heating of the milk appeared to be of most decisive importance for the stability of VTO. Also by deep freezing the milk the stability could be improved. An artificial lowering of the pH again had a harmful effect on the yield. The effect of heating and the test temperature is shown in Tables 5 and 6. From the results shown in the tables

Table 5. The effect of heating on the yield of VTO in whole milk. 300 ml of milk heated immediately after the addition of the substance and cooled rapidly.

Treatment °C	VTO		
	Added μg	Yield	
		μg	%
Unheated	48	22	46
60°	48	29	60
85°	48	37	77
100°	48	37	77
Unheated	30	15	50
85°	30	19	63
100°	30	23	77

Table 6. The stability of VTO at + 20°C and −15°C in unheated whole milk and in whole milk heated to 85°C. The milk hot treated before the addition. Milk amounts 300 ml.

Treatment	Time of keeping h	Addition μg	Found			
			+20°C		−15°C	
			μg	%	μg	%
Unheated	0	43	28	65	—	—
	24	43	3 *	7	13	30
	48	43	2.5 *	6	10	23
	144	43	—	—	8	19
Heated to 85°C	0	65	48	74	—	—
	24	65	—	—	49	75
	48	65	40	62	—	—
	96	65	26	40	48	74
	192	65	—	—	46	71

* sour milk.

it appears that about 75 % of the VTO added to milk is recovered, and that hence about 25 % remains as a loss of analysis. A considerable part of this loss is due to the chloroform extraction used in the method, which causes a loss of about 10 %. When the milk is heated to 85°C and is kept at −15°C, the substance to be determined is very stable and even when kept at room temperature it disappears comparatively slowly.

In the experiment with reagents only, 154 μg of VTO were recovered from the 170 μg eluted into 300 ml of distilled water, corresponding to 90.5 % of the amount added.

Skim milk. In order to test the accuracy of the method 110 μg of VTO was added to 500 ml of skim milk the fat content of which was 0.06 %. Of the amount added 84 % were recovered in the analysis. The result corresponds to the yield in the method for whole milk when the methodical loss of 10 % in this method is taken into consideration. When, in addition to fat, casein is also removed from the milk recovery is not improved.

The determinations performed on skim milk or whey do not yet show with certainty the real content of VTO in the milk used in the experiment. Because the substance is fat soluble it can, for instance, be retained in the milk fat to a large extent. This is also indicated by an experiment in which melted milk fat was mixed into a water solution of VTO. When analyzing the water phase after shaking, it did not contain more than 80 % of the original amount of VTO.

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REFERENCES

1. Astwood, E. B., Greer, M. A. and Ettlinger, M. G. *J. Biol. Chem.* **181** (1949) 121.
2. Virtanen, A. I. *Suomen Kemistilehti A* **31** (1958) 96 (*lecture given at the meeting of Societas Biochemica, Biophysica et Microbiologica Fenniae*, March 19, 1958); *Schweiz. Z. allgem. Pathol. u. Bakteriol.* **21** (1958) 970.
3. Altamura, M. R., Long, Jr. L. and Hasselström, T. *Paper presented at the 134th National Meeting of the American Chemical Society Division of Agricultural and Food Chemistry*, Chicago, Illinois, 7−12 September 1958.
4. Virtanen, A. I., Kreula, M. and Kiesvaara, M. *Suomen Kemistilehti A* **32** (1959) 45 (*communication at the meeting of Societas Biochemica, Biophysica et Microbiologica Fenniae*, October 27, 1958); *Acta Chem. Scand.* **12** (1958) 580.
5. Greer, M. A., Ettlinger, M. G. and Astwood, E. B. *J. Clin. Endocrinol.* **9** (1949) 1069; Greer, M. A. *J. Am. Chem. Soc.* **78** (1956) 1260; Kjær, A., Gmelin, R. and Jensen, R. B. *Acta Chem. Scand.* **10** (1956) 432; Schultz, O.-E. and Wagner, W. *Arch. Pharm.* **289/61** (1956) 597; Ettlinger, M. G. and Lundeen, A. J. *J. Am. Chem. Soc.* **78** (1956) 4172; ibid. **79** (1957) 1764.

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