EESTI NSV TEADUSTE AKADEEMIA TOIMETISED. XVIII KÖIDE
BIOLOOGIA. 1969. nr. 1

ИЗВЕСТИЯ АКАДЕМИИ НАУК ЭСТОНСКОЙ ССР. ТОМ XVIII
БИОЛОГИЯ. 1969, № 1

https://doi.org/10.3176/biol.1969.1.05

*U. MARGNA, E. MARGNA*

# A SUITABLE CHROMATOGRAPHIC METHOD FOR QUANTITATIVE ASSAY OF RUTIN AND FLAVONE C-GLYCOSIDES IN BUCKWHEAT SEEDLINGS

Recently we have shown that four of the five flavonoid compounds occurring in buckwheat (*Fagopyrum esculentum* Moench) cotyledons are flavone C-glycosides, two of them being identical with luteolin C-glycosides orientin and homo-orientin, and the remaining pair of compounds with vitexin and saponaretin, the corresponding analogues of apigenin (Margna et al., 1967). The fifth flavonoid derivative of cotyledons as well as the only flavonoid besides anthocyanins in hypocotyls is known to be quercetin-3-rhamnoglucoside rutin (Troyer, 1955). The co-occurrence of different types of flavonoids in one and the same tissue together with the fact that flavone C-glycosides under consideration are present in cotyledons only, but not in the leaves or other organs of buckwheat plant, has markedly increased the importance of buckwheat seedlings as suitable model plants for different flavonoid studies. In connection with this, an urgent need has arisen for a reliable method of analysis permitting simultaneous quantitative determination of each of the separate flavonoid derivatives present in seedlings and at the same time ensuring a sufficient degree of precision of measurements.

In all of the known attempts hitherto undertaken to solve this problem, the principles of chromatographic technique have been applied exclusively. None of the earlier authors, however, has proposed a method in all aspects satisfactory for complete quantitative analysis of buckwheat flavonoids. Thus, by a circular filter paper chromatography procedure utilized by a number of authors (Nöll, 1955; Bassler, 1957), only two of the five flavonoids of cotyledons could be determined separately, while the remaining three of the compounds, including rutin, had to be assayed together. Harraschain and Mohr (1963) have succeeded in achieving a complete separation of the flavonoid complex of buckwheat cotyledons by two-dimensional paper chromatography, but they did not use this technique for quantitative purposes. The cotyledonary flavonoids were determined by them as a sum of all of the five substances by means of a simple spectrophotometric measurement of the absorbance of the methanolic extracts of cotyledons in the UV-region of the spectrum. More recently, Scherf and Zenk (1967) have fitted for buckwheat studies a thin-layer chromatography method originally developed by Hess and Meyer (1962) for the separation of anthocyanins in Petunia flowers. However, the authors used it for the determination of anthocyanins and rutin in hypocotyls only, and nothing is known about the fitness of the method concerning the assay of flavonoids in cotyledons.

Certain attention deserves the possibility of using direct absorptiometry of paper chromatograms. Troyer (1956a) has shown that this technique is satisfactory for relative measurements on rutin and four other flavonoid substances present in buckwheat cotyledons and has employed it to follow the changes in approximate quantitative levels of the five buckwheat flavonoids during seedling development (Troyer, 1956b). Unfortunately, the precision of determinations made with this procedure is not as good as might be desired, and the method must in fact be regarded as semiquantitative in nature.

In the present paper, an improved method for complete quantitative analysis of buckwheat flavonoids is proposed, involving a procedure of repeated one-dimensional paper chromatography for hypocotyl assay and two-dimensional paper chromatography for the assay of flavonoid substances in cotyledons. The method has been successfully applied in our laboratory and may be recommended as a convenient tool for simultaneous determination of each of the flavonoid substances present in buckwheat seedlings.

## Experimental

All the methodical work was carried out on buckwheat seedlings of the local Estonian variety 'Jõgeva' (*Fagopyrum esculentum* Moench) grown under artificial illumination from white fluorescent tubes.

For flavonoid extraction, the freshly collected plant material was ground in a mortar to a homogeneous mass and then extracted with two portions of 95 per cent ethanol. The chromatographic check-up proved that this procedure is sufficient for exhaustive extraction of rutin and other flavonoids from seedling tissue. The resulting alcoholic extracts were directly used for chromatography (cotyledons), or previously evaporated to dryness with subsequent dissolution of the residue in a small volume of a mixture of ethanol and water (hypocotyls).

All chromatograms were run by ascending technique at room temperature. Two solvent systems were employed, the upper phase of the mixture of isoamyl alcohol-petrol ether-acetic acid-water (3:1:3:3, v/v) (IPAW) being used for the first development and 3 per cent acetic acid for the second. For rutin assay in hypocotyls, both the solvents were used in the same direction, whereas for the resolution of flavonoids from cotyledons, the technique of two-dimensional paper chromatography was employed. By these procedures, complete separation of flavonoids from each other as well as from concomitant anthocyanins and other related substances was achieved (Fig. 1).

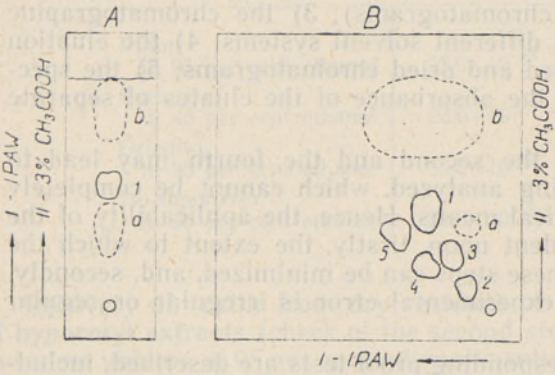


Fig. 1. Chromatograms of buckwheat seedling flavonoids. *A* — hypocotyls; *B* — cotyledons; 1 — rutin; 2 — orientin; 3 — homoorientin; 4 — vitexin; 5 — saponaretin; *a* — anthocyanins; *b* — the zone of hydroxycinnamic acid derivatives.

The chemicals used for the composition of chromatographic solvents were obtained commercially and were of the best available grade. Throughout the work, the Filtrak FN-11 chromatographic paper manufactured by the VEB Spezialpapierfabrik Niederschlag/Erzgeb. (German Democratic Republic) was used. Flavonoid spots on chromatograms were located by ultraviolet light.

All spectrophotometric measurements were conducted on a Soviet SF-4A ultraviolet spectrophotometer using 10 mm prismatic quartz cuvettes. The quantities of flavonoids were determined directly by the ultraviolet absorbance of their ethanolic eluates without any use of colour reac-

tions with chelating or other reagents. The measurements were made at wavelengths corresponding to the absorption maxima in Band I of the separate derivatives: rutin — at 360 nm, orientin and homo-orientin — at 350 nm, vitexin and saponaretin — at 335 nm (Margna *et al.*, 1967). The absorbance of eluates was measured against a blank solution obtained by the eluation of either the corresponding area on blank chromatograms developed with the same solvents or the blank area adjacent to the corresponding flavonoid on the same chromatogram. The absorbance of the solutions of standard substances was measured against the pure solvent as the blank.

As standard substances, the authentic preparations of rutin manufactured by the "Chemapol" (Czechoslovakia) and the preparations of orientin and iso-saponarin (saponaretin-4′-glucoside) were used. The samples of the two last substances were obtained as a generous gift from V. Litvinenko (The Chemical and Pharmaceutical Research Institute, Kharkov). The evaluation of the suitability of the proposed method as well as of the different intermediate procedures was accomplished by the statistical techniques of regression analysis (Youden, 1949; Bailey, 1959) and of analysis of variance (Snedecor, 1957).

## Results

In the method presented here, the following procedures are necessarily involved: 1) the extraction of flavonoids from hypocotyls or cotyledons with the subsequent liberation of the extracts from tissue fragments; 2) the evaporation of extracts to dryness followed by a treatment of the resulting residue for taking it up into a solution of suitable volume sufficiently reduced to be convenient for chromatography (this procedure is omitted when cotyledons are analyzed — the content of flavonoids in this material is sufficiently high to allow direct application of a portion of the initial extract on paper chromatograms); 3) the chromatographic separation of flavonoids in two different solvent systems; 4) the eluation of flavonoid spots from developed and dried chromatograms; 5) the spectrophotometric measurement of the absorbance of the eluates of separate flavonoids.

Of these steps of analysis, the second and the fourth may lead to certain losses of flavonoids being analyzed, which cannot be completely avoided by technical or methodical means. Hence, the applicability of the whole method is greatly dependent upon, firstly, the extent to which the possible losses arising during these steps can be minimized, and, secondly, the fact whether the resulting experimental error is irregular or regular in nature.

Below the results of the corresponding proof-tests are described, including the linearity check-up of the absorbance of solutions of the standard substances rutin, orientin and iso-saponarin, the latter being used as a standard for apigenin C-glycosides.

**The absorbance of the standard solutions of various concentrations.** The plot of spectrophotometer readings against micrograms of flavonoids per millilitre and the fitted straight lines through the origin (Fig. 2) clearly showed that over the range of quantities tested the optical density (OD) of solutions was strictly proportional to their concentration, irrespective of whether the solutions of rutin or those of the two other standard substances were assayed. The comparative trials with rutin indicated

that the proportionality was maintained in both 95 per cent and diluted ethanol. Thus the absorption of solutions followed Beer's law. This fact, together with the sensitivity attainable by the test, suggests that the direct measurement of the ultraviolet absorbance of substances in ethanolic solutions may be successfully applied for quantitative assay of microgram amounts of buckwheat flavonoids, securing a high degree of precision of determinations.

The slopes of the separate regression lines resp. standard curves and the extinction coefficients calculated from the values of the corresponding slopes are characterized by the following estimates (Table 1).

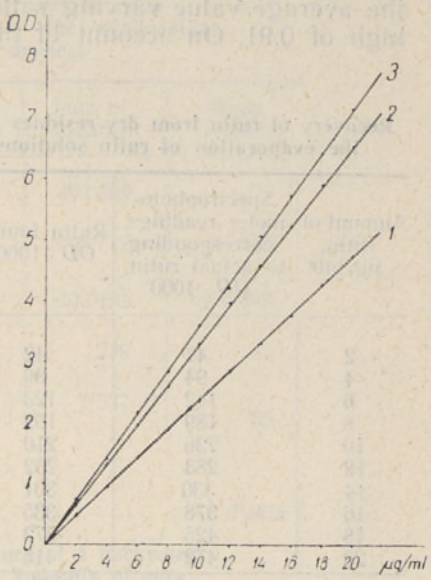


Fig. 2. Standard curves of rutin (1), iso-saponarin (2), and orientin (3) in 95 per cent ethanol.

*Table 1*

**The slopes of standard curves and the corresponding extinction coefficients of rutin, orientin and isosaponarin in ethanolic solutions**

| Compound | Slope, *b*, *OD*/µg per ml | Extinction coefficient, *E*, cm²/Mol |
|---|---|---|
| Rutin, in 95 per cent ethanol | $23.6 \cdot 10^{-3}$ | $E_{360\ nm} = 1.44 \cdot 10^7$ |
| Rutin, in 45 per cent ethanol | $23.0 \cdot 10^{-3}$ | $E_{360\ nm} = 1.40 \cdot 10^7$ |
| Orientin, in 95 per cent ethanol | $35.5 \cdot 10^{-3}$ | $E_{350\ nm} = 1.59 \cdot 10^7$ |
| Iso-saponarin, in 95 per cent ethanol | $32.6 \cdot 10^{-3}$ | $E_{335\ nm} = 1.94 \cdot 10^7$ |

**Recovery of rutin from dry residues of rutin standard solutions and of hypocotyl extracts** (check of the second step of the method). Aliquots of the same volume of 95 per cent ethanolic solutions of ten different concentrations of pure rutin were evaporated to dryness, the residue was taken up into a known volume of 95 per cent ethanol and then subjected to spectrophotometric determination. Each concentration was tested in three replications, using the standard procedure of analysis that will be described in a later section. The results of these tests were compared with the data obtained from direct absorption measurements of the same test solutions prior to evaporation (Table 2).

From this comparison it becomes evident that the dissolution of rutin from dry residue remaining after evaporation of the solvent is not complete and that a recovery of approximately 90 per cent can be achieved only. However, the separate ratios of the amount of rutin found to the

amount actually applied reveal only slight discrepancies as compared to the average value varying within narrow limits from a low of 0.88 to a high of 0.91. On account of this relative constancy, there was a good reason to assume that the experimental error arising during this procedure is not random, but is linearly related to the actual amount of rutin present in sample.

*Table 2*

**Recovery of rutin from dry residues remaining after the evaporation of rutin solutions to dryness**

| Amount of rutin, μg/ml* | Spectrophotometer readings, corresponding to actual rutin, $OD \cdot 1000$ | Rutin found, $OD \cdot 1000$ | Ratio rutin found/actual rutin |
|---|---|---|---|
| 2 | 47 | 42 | 0.894 |
| 4 | 94 | 86 | 0.915 |
| 6 | 142 | 123 | 0.866 |
| 8 | 189 | 169 | 0.894 |
| 10 | 236 | 210 | 0.890 |
| 12 | 283 | 252 | 0.890 |
| 14 | 330 | 301 | 0.912 |
| 16 | 378 | 335 | 0.886 |
| 18 | 425 | 373 | 0.878 |
| 20 | 472 | 415 | 0.879 |
| | | | Av. 0.890 |

Statistics for the fitted regression line of the form $y=a+bx$:

Slope, $b=0.881$

Intercept, $a=1.90$

Standard deviation of the slope, $s_b=0.008$

Standard deviation of the intercept, $s_a=2.46$

$t_b=110.12$ $t_a=0.77$

* Here and in the corresponding column of Tables 4 and 5 the amount of rutin is expressed as the theoretical concentration of the substance in the final solution for spectrophotometric measurement.

To check this hypothesis, the known and the found rutin contents of the sample were taken as the $x$ and $y$ variables correspondingly, and a straight line was fitted for these ten pairs of observations. The parameters of the fitted regression line together with the relevant statistics are presented in the lower part of the Table 2.

As it can be seen from the data, the slope of the line is in close agreement with the average ratio found empirically and has a small standard deviation resulting in a large value for the corresponding Student's $t$. Alternatively a low value for the $t$ of the intercept as well as the negligibility of the intercept itself indicate that the line may be considered to pass through the origin. Thus, the linearity of rutin recovery in this procedure is held reasonably well.

Another argument for the hypothesis is obtained by estimating the significance of deviations of recovery data from linearity using a technique of the analysis of variance recommended by Snedecor (1957).

The results of the analysis clearly demonstrate (Table 3) that the slight deviations from linear regression observed in these recovery experiments are merely due to random variation, but cannot be regarded as a regular tendency worth to be taken into account.

Recovery of rutin from dry residues of buckwheat hypocotyl extracts to which extra amounts of pure rutin were added, lent further support to the hypothesis of linearity under discussion. In all cases the amount of rutin recovered was found to be linearly dependent upon the amount of the substance actually added, the recovery being always of approximately the same magnitude as it was when the solutions of pure rutin were tested (Table 4).

Thus, though the suggested analytical procedure is not free from experimental error, nevertheless it yields a recovery relationship which is linear to the actual amount of rutin and has a slope equal to 0.881 as the best estimate. The linearity is maintained in the range 1 to 20 μg/ml at

*Table 3*

**Analysis of variance of the recovery of rutin from dry residues remaining after the evaporation of rutin solutions to dryness**

| Source of variation | Degrees of freedom | Sum of squares | Mean square |
|---|---|---|---|
| Total | 27 | 402 309 | — |
| Concentration of rutin solutions | 9 | 400 772 | 44 530 |
| Including: | | | |
| Linear regression | 1 | 400 493 | 400 493 |
| Deviations from linearity | 8 | 279 | 35 |
| Variation within replications (random error) | 18 | 1 537 | 85 |

$F = 35/85 = 0.41$

*Table 4*

**Recovery of rutin from dry residues of a buckwheat hypocotyl extract to which extra amounts of pure rutin were added**

| Amount of extra rutin, µg/ml* | Spectrophotometer readings, *OD* · 1000 corresponding to | | | Ratio rutin found/rutin added |
|---|---|---|---|---|
| | extra rutin actually added | total rutin found in test extract (together with extra rutin) | recovery of extra rutin | |
| 0 | 0 | 263 | — | — |
| 2 | 47 | 305 | 42 | 0.894 |
| 6 | 142 | 392 | 129 | 0.908 |
| 10 | 236 | 472 | 209 | 0.886 |

* See footnote in Table 2.

least (up to 1000 micrograms of rutin in starting plant material respectively) which is broad enough to cover the needs of any of the experimental work with buckwheat seedlings and to realize the whole procedure suitable for practical use.

**Recovery of rutin and flavone C-glycosides from chromatograms** (check of the fourth step of the method). Eluation process necessary for removing separated flavonoids from chromatograms into a solution for quantitative measurement is generally not exhaustive, the recovery obtained varying usually within the range 60 to 90 per cent depending on the nature of the compounds as well as on the properties of the chromatographic paper and solving reagents used. This disadvantage could not be eliminated in the present work with buckwheat flavonoids, either.

In order to evaluate the degree of removal of the substances from the developed chromatograms attainable by a single eluation, ten known amounts of rutin were chromatographically run, using the standard proce-

dure recommended for general use in hypocotyl analysis (see later). The resulting spots of rutin were eluated with 45 per cent ethanol, which was found to be the best eluating solvent for this flavonol glycoside, and the amounts of rutin recovered in eluates were compared with the amounts actually applied. Each amount of rutin was tested in three replications.

Results of these recovery trials are presented in Table 5. As can be seen, the removal of rutin from chromatograms was of the order of 85 per cent of the total amount of rutin actually applied. Taking into account the well-known difficulties connected with the attempts to obtain more effective eluation, this degree of recovery can be considered highly satisfactory, and the more so, since the eluation process itself followed strict proportionality. The plot of the amounts of rutin recovered from chromatograms by a single eluation with 45 per cent ethanol vs. amounts of actual rutin resulted in a fitted straight line which passed through the origin (the hypothesis that the intercept $a=0.10$ is significantly different from zero was rejected at a high level of probability; see the lower part of the Table 5) and had a slope equal to 0.868. The latter value proved to be practically identical with the average ratio rutin found/actual rutin, yielding a clear-cut evidence for a linear relationship. The evaluation of deviations of recovery data from linearity by means of analysis of variance advanced further confirmation that the amount of rutin recovered from chromatogram by a single eluation is strictly linear to the amount of the substance actually applied (Table 6).

*Table 5*

**Recovery of rutin from chromatograms by a single eluation with 45 per cent ethanol**

| Amount of rutin actually applied, µg/ml* | Spectrophotometer readings corresponding to actual rutin, $OD \cdot 1000$ | Rutin found in eluates, $OD \cdot 1000$ | Ratio rutin found/actual rutin |
|---|---|---|---|
| 2 | 46 | 40 | 0.870 |
| 4 | 92 | 82 | 0.891 |
| 6 | 138 | 123 | 0.891 |
| 8 | 184 | 154 | 0.837 |
| 10 | 230 | 198 | 0.861 |
| 12 | 276 | 235 | 0.851 |
| 14 | 322 | 285 | 0.885 |
| 16 | 368 | 324 | 0.880 |
| 18 | 414 | 357 | 0.862 |
| 20 | 460 | 399 | 0.867 |
| | | | Av. 0.870 |

Statistics for the fitted regression line of the form $y=a+bx$;

Slope, $b=0.868$

Intercept, $a=0.10$

Standard deviation of the slope, $s_b=0.012$

Standard deviation of the intercept, $s_a=3.29$

$t_b=72.33$ $t_a=0.03$

* See footnote in the table 2.

Essentially analogous were the results of the corresponding recovery tests with orientin. The eluation of this substance also yielded a recovery relationship linear to the amount of the substance actually applied on the chromatograms. In the present case it was found that the best estimate of the slope of the corresponding regression line is equal to 0.750. The main difference as compared with the eluation of rutin is that the best removal of the substance from chromatogram was achieved by using 95 per cent ethanol as the eluating reagent and that the degree of removal in itself is somewhat smaller — 75 per cent only, as it could be seen already from the slope of the recovery relationship mentioned above. This degree of recovery is still sufficiently high to meet the requirements of the present method and to consider a single eluation with 95 per cent ethanol acceptable for the assay of flavone C-glycosides.

*Table 6*

**Analysis of variance of the recovery of rutin from chromatograms by a single eluation with 45 per cent ethanol**

| Source of variation | Degrees of freedom | Sum of squares | Mean square |
|---|---|---|---|
| Total | 29 | 327 248 | — |
| Amount of rutin applied on chromatograms | 9 | 325 491 | 36 166 |
| Including: | | | |
| Linear regression | 1 | 325 140 | 325 140 |
| Deviations from linearity | 8 | 351 | 44 |
| Variation within replications (random error) | 20 | 1 757 | 88 |

$F = 44/88 = 0.50$

Thus, notwithstanding the removal of both rutin and flavone C-glycosides from chromatograms cannot be accomplished quantitatively, the eluation procedure with appropriate solvents yields a recovery relationship which is linear to the actual amount of the substances and, therefore, warrants its use for the purposes of quantitative measurements.

## Concluding remarks

To sum up the results of the recovery-tests described above, it must be concluded that within the range 50 to 1000 micrograms of separate substances per a portion of plant material, the different analytical procedures involved as well as the suggested method as a whole are applicable for quantitative determination of flavonoids in buckwheat seedlings reasonably well. In spite of the fact that experimental losses of the substances cannot be entirely avoided during the analytical procedures, the method yields a strict linear relationship between the final results obtainable by employing it and the amounts of substances actually present in the plant material. The experimental losses, therefore, do not appear to interfere with the reliability of the scientifical information attainable by this method, at the same time do not practically affecting also the precision of determinations.

The average degree of precision estimated here in terms of standard deviation as expressed in relative units of the mean value of the sample consisting of five replicate determinations was found to be equal to 3.8 per cent. That is, the average random error of the method does not exceed the limits of the natural variation normally existing within the biological material. Bearing in mind the great number of intermediate steps of the method and the overall duration of the determinations, this degree of precision may be considered highly satisfactory for practical use.

## Procedure recommended for general use

The following technique, which can be applied to samples consisting of up to 30 seedlings, is recommended for general use in routine analysis of buckwheat flavonoids:

Remove roots from harvested seedlings, dissect the remaining part of seedlings into separate portions of hypocotyls and cotyledons, and grind the two portions of the fresh material separately in a porcelain mortar by means of glass sand until the material is completely homogenized. Add 10 ml of cold ethanol, mix carefully, transfer the mixture together with tissue fragments quantitatively into a centrifuge flask and allow it to stand there for 30 minutes at room temperature, now and then stirring the contents of the flask with a glass rod. Centrifuge the mixture 15 minutes at 7000 rpm, pour the clear supernatant off from sediment, wash the sediment with 5 ml of ethanol, repeat the centrifugation, and add the clear washing to the initial extract obtained after the first centrifugation.

The sequence of further manipulations depends upon whether hypocotyls or cotyledons are examined.

Hypocotyls: Pour the joined ethanolic extract obtained above into a porcelain dish and allow it to stand open at room temperature until the solvent is completely evaporated. Treat the remaining dry residue for 1 minute carefully with 1 ml of distilled water, add 1 ml of 95 per cent ethanol and continue the treatment for another 1-minute period. Take with a micropipette a 0.2 ml portion of the resulting solution, spot it on the starting line of a sheet of chromatographic paper and develop the chromatogram by ascending technique in the upper phase of the mixture of isoamyl alcohol-petrol ether-acetic acid-water (3:1:3:3, v/v) until the front of the solvent reaches the 40 cm mark. Dry the developed chromatogram in a current of air in a fume cupboard and repeat the development in the same direction, now in a 3 per cent acetic acid solution. Then subject the chromatogram to final drying, locate the spot of rutin on it by ultraviolet light, and cut the spot out for subsequent eluation.

Cotyledons: Transfer the ethanolic extract obtained above quantitatively into a calibrated test-tube, add some ethanol or allow the test-tube to stand open for removing the excess solvent so as to adjust the volume of the extract to 15 ml, take with a micropipette a 0.3 ml sample of the adjusted solution, and spot it on a sheet of chromatographic paper for two-dimensional chromatography. Develop the chromatogram using the upper phase of the mixture of isoamyl alcohol-petrol ether-acetic acid-water (3:1:3:3, v/v) for the first direction and a 3 per cent acetic acid solution for the second direction, dry the developed chromatogram, mark the position of the spots of flavone C-glycosides and rutin on it under an ultraviolet lamp, and cut the spots out for eluation.

The eluation procedure and the final absorption measurements are to be carried out as follows:

Cut the flavonoid spot into small pieces, introduce them into a 50 ml flask, pour the pieces over with 5 ml of 45 per cent ethanol (for rutin spot) or with the same amount of 95 per cent ethanol (for the spots of flavone C-glycosides) and allow the flask to stand firmly stoppered for 3 hours, now and then vigorously shaking the contents of the flask to favour eluation. Pour the eluate completely off from the pieces, filter it through the filter paper, and subject the clear eluate immediately to spectrophotometry, measuring its optical density at 360 nm (rutin), 350 nm (orientin and homo-orientin) or 335 nm (vitexin and saponaretin). Adjust the result of the measurement to one of the following formulas and calculate the amount of the flavonoid examined:

$$F_{\text{Hypocotyls}} = \frac{OD \cdot 5 \cdot 10}{b \cdot c_1 \cdot c_2}, \text{ and} \qquad (1)$$

$$F_{\text{Cotyledons}} = \frac{CD \cdot 5 \cdot 50}{b \cdot c_2 (\text{or } c_3)}, \quad (2)$$

where $F$ — total absolute amount in micrograms of the flavonoid in the sample examined;

$OD$ — the optical density of the eluate of the flavonoid spot examined;

$b$ — slope of the corresponding standard curve (see Table 1);

50 and 250 — dilution coefficients for hypocotyls and cotyledons respectively;

$c_1 = 0.881$ — correction factor for recovery of rutin from dry residues remaining after evaporation of hypocotyl extracts to dryness in the second step of the analytical procedure;

$c_2 = 0.868$ — correction factor for recovery of rutin from chromatograms;

$c_3 = 0.750$ — correction factor for recovery of flavone C-glycosides from chromatograms.

In practical work, the best estimates of the correction factors $c_1$, $c_2$ and $c_3$ found in the present study can be successfully replaced by approximate values of 0.90, 0.85 and 0.75 respectively.

## Acknowledgements

The authors wish to express their sincere appreciation to V. Litvinenko for the authentic samples of orientin and isosaponarin.

## REFERENCES

Bailey N. T. J., 1959. Statistical methods in biology. The English Universities Press Ltd. London.

Bassler R., 1957. Der Einfluß ökologischer und ontogenetischer Faktoren auf die Flavone von *Fagopyrum sagittatum* Gilib. Pharmazie **12** : 758—772, 834—841.

Harraschain H., Mohr H., 1963. Der Einfluß sichtbarer Strahlung auf die Flavonoid-Synthese und Morphogenese der Buchweizenkeimlinge (*Fagopyrum esculentum* Moench.) II. Flavonolsynthese und Hypokotylwachstum. Z. Bot. **51** : 277—299.

Hess D., Meyer C., 1962. Dünnschichtchromatographiie von Anthocyanen. Z. Naturforsch. **17b** : 853—854.

Margna U., Hallop L., Margna E., Tohver M., 1967. Chromatographic and spectrophotometric evidence for the occurrence of luteolin and apigenin C-glycosides in the cotyledons of buckwheat seedlings. Biochim. Biophys. Acta **136** : 396—399.

Nöll G., 1955. Untersuchungen über den Einfluß verschiedener Faktoren auf den Flavonolgehalt von *Fagopyrum esculentum* (L.) Moench und *Fagopyrum tataricum* (L.) Gärtner. Pharmazie **10** : 609—615.

Scherf H., Zenk M. H., 1967. Der Einfluß des Lichtes auf die Flavonoidsynthese und die Enzyminduktion bei *Fagopyrum esculentum* Moench. Z. Pflanzenphysiol. **57** : 401—418.

Snedecor G. W., 1957. Statistical methods applied to experiments in agriculture and biology. The Iowa State College Press. Ames, Iowa.

Troyer J. R., 1955. The distribution of rutin and other flavonoid substances in buckwheat seedlings. Plant Physiol. **30** : 168—173.

Troyer J. R., 1956a. Quantitative determination of buckwheat flavonoid substances by direct absorptiometry of paper chromatograms. Plant Physiol. **31** : 71—74.

Troyer J. R., 1956b. Quantitative changes in buckwheat flavonoid substances during seedling development. Plant Physiol. **31** : 75—78.

Youden W. J., 1947. Technique for testing the accuracy of analytical data. Analyt. Chem. **19** : 946—950.

*Academy of Sciences of the Estonian SSR,*
*Institute of Experimental Biology*

Received
April 4, 1968

*U. MARGNA, E. MARGNA*

## PABERKROMATOGRAAFILINE MEETOD RUTIINI JA FLAVOON-C-GLÜKOSIIDIDE KVANTITATIIVSEKS MÄÄRAMISEKS TATRAIDANDEIS

*Resümee*

Esitatakse uus, täiustatud meetod rutiini kvantitatiivseks määramiseks tatraidandite hüpokotüülides ning rutiini ja flavoon-C-glükosiidide orientiini, homoorientiini, viteksiini ja saponaretiini üheaegseks määramiseks tatraidandite idulehtedes. Meetod baseerub uurtavate ühendite paberkromatograafilisel lahutamisel, kusjuures kasutatakse kahekordset voolutamist: esiteks, vahekorras 3:1:3:3 valmistatud isoamüül-alkoholi-petrooleetri-äädikhappe-vee seguga (ülemine faas) ning teiseks, 3%-lise äädikhappega. Rutiini määramisel hüpokotüülides voolutatakse mõlema solvendiga ühes ja samas suunas, idulehtede flavonoidse kompleksi analüüsimisel aga kahemõõtmelise kromatograafia põhimõttel. Nende solventide kasutamine tagab flavonoidide täieliku lahutamise nii üksteisest kui ka tatraidandeis esinevaist antotsüaanidest ning hüdroksükaneelhappe derivaatidest. Analüüsitavate flavonoidide hulga lõplik kindlaksmääramine pärast nende kromatograafilist lahutamist toimub vastavate laikude etanoolsete eluaatide optilise tiheduse otsese spektrofotomeetrilise mõõtmise teel spektri ultraviolettpiirkonnas (rutiini puhul 360 nm, orientiini ja homoorientiini puhul 350 nm ning viteksiini ja saponaretiini puhul 335 nm juures). Hoolimata sellest, et analüüsi käigus ei ole võimalik vältida mõningaid kadusid, on meetodi kasutamisel saadavate tulemuste reprodutseeritavus rahuldav ning vähemalt vahemikus 50—1000 mkg flavonoidi proovi kohta täielikult tagatud määramise lõpptulemuste ning ühendi tegeliku sisalduse vaheline lineaarsus. Meetodi keskmine juhuslik viga on alla 4%, mis ei ületa bioloogilisele materjalile omase loomuliku varieeruvuse piire. See näitab, et meetod on edukalt rakendatav usaldusväärse informatsiooni saamiseks eri flavonoidide sisalduse ning kvantitatiivsete vahekordade kohta tatraidandeis. Artiklis kirjeldatakse üksikasjalisemalt üht selle meetodi varianti, mida tatraidandite flavonoidide biokeemilisel uurimisel soovitatakse standardmodifikatsioonina kasutusele võtta.

*Eesti NSV Teaduste Akadeemia*
*Eksperimentaalbioloogia Instituut*

Saabus toimetusse
4. IV 1968

*У. МАРГНА, Э. МАРГНА*

## ХРОМАТОГРАФИЧЕСКИЙ МЕТОД ДЛЯ КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ РУТИНА И ФЛАВОН-С-ГЛИКОЗИДОВ В ПРОРОСТКАХ ГРЕЧИХИ

*Резюме*

Предложен усовершенствованный метод для количественного определения рутина в гипокотилях, а также для определения рутина и флавон-С-гликозидов ориентина, гомоориентина, витексина и сапонаретина в семядольных листочках проростков гречихи. В основе метода лежит хроматографическое разделение изучаемых веществ с помощью смеси изоамиловый спирт—петролейный эфир—уксусная кислота—вода (3:1:3:3, верхняя фаза) и 3%-ной уксусной кислотой. При анализе гипокотилей оба растворителя применяются поочередно в одном и том же направлении, при анализе же флавоноидного комплекса семядольных листочков — по технике двухмерной хроматографии. Использование указанных растворителей обеспечивает полное отделение отдельных флавоноидов проростков гречихи как друг от друга, так и от сопутствующих антоцианов и производных оксикоричных кислот. Окончательное определение количества отдельных флавоноидов производится путем прямого спектрофотометрического измерения оптической плотности этанольных элюатов соответствующих пятен в ультрафиолетовой области спектра, при 360 *нм* в случае рутина, 350 *нм* в случае ориентина и гомоориентина и 335 *нм* в случае витексина и сапонаретина. Несмотря на некоторые экспериментальные потери, неизбежные в ходе анализа, воспроизводимость результатов при использовании предложенного метода удовлетворительна, причем по крайней мере в пределах 50—1000 *мкг* отдельных флавоноидов в изучаемой пробе полностью сохраняется линеарная зависимость между окончательными результатами анализа и истинным количеством веществ в материале. Средняя случайная ошибка метода — около 4%, что не превышает границ естественного варьирования в биологическом материале. Эти параметры показывают, что метод может быть успешно применен для получения достоверной информации о содержании и количественных соотношениях отдельных флавоноидов в проростках гречихи. В статье дано подробное описание одного из вариантов метода, рекомендуемого в качестве стандартной модификации к применению в лабораторных исследованиях по изучению флавоноидов проростков гречихи.

*Институт экспериментальной биологии*
*Академии наук Эстонской ССР*

Поступила в редакцию
4/IV 1968