Determination of Sterol and Triterpene Content of *Ocimum basilicum* and *Salvia officinalis* at Various Stages of Growth

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The sterol (β-sitosterol) and triterpene (ursolic acid I and/or II and oleanolic acid) content of *O. basilicum* and *S. officinalis* L. was determined quantitatively by a colorimetric method in various organs of the mature plants and in at least one prior stage of growth. In both plants the acidic triterpenes were absent from the seeds, but like the sterol, present in all stages of growth examined. There was an increase in concentration of both sterol and triterpene with increasing maturity of the plants, with leaves and flower parts containing the highest concentration.

IN THE COURSE of studying the incorporation of labeled precursors into sterols and pentacyclic triterpenes of higher plants it seemed of interest to determine quantitatively the sterol and triterpene content of the various organs as growth proceeds. Although it may generally be assumed that sterols occur at all stages of growth in higher plants (1) the same cannot as yet be said for the pentacyclic triterpenes, and the quantitative distribution of the latter within any particular plant has received comparatively little attention.

The isolation of β-sitosterol, ursolic acid II, and oleanolic acid from *O. basilicum* has been previously described (2). β-Sitosterol has also been isolated from *S. officinalis* L.[1] The ursolic acids (I and II) and oleanolic acid have also been reported present in *S. officinalis* L. (3). These plants and several others of the Labiatae family therefore seem suitable for study of sterol and pentacyclic triterpene biosynthesis. In approaching this problem with *O. basilicum* and *S. officinalis* L. it seemed of merit to (*a*) develop techniques satisfactory for separating the sterol and acidic triterpenes on a small scale and (*b*) determine the quantitative relationship between sterol and triterpene, based on the use of these techniques.

EXPERIMENTAL

Materials.—All seeds were obtained from the Vaughn Seed Company, Chicago, Ill., and all plant material was grown from these seeds. The ethyl ether was A. R. grade; all other solvents were a highly purified grade and were distilled before use. The petroleum ether and ligroin had boiling points of 30–60° and 90–120°, respectively. The sterol and triterpene standards were obtained from sources previously outlined (2). For the colorimetric determinations either a Beckman DU or Coleman model 14 spectrophotometer proved satisfactory.

Preparation of Plant Material for Quantitative Estimation of Sterol and Triterpene.—Eight full-flowering *O. basilicum* plants were uprooted, washed, and sectioned into roots, stems, leaves, flowers, and flower stalks. Stems consisted of that portion about 5 cm. above ground level to the level of the lower flowers. Leaves were removed close to the stem and consisted of blade and petiole. Stipules were removed close to the stem and discarded. Flowers consisted of all flower parts including the greenish calyx; they were carefully dissected from the flower stalks with scissors and tweezers. A number of flowering *S. officinalis* L. plants were similarly treated, except that the roots were discarded. There were no flower stems present on these plants.

Seedlings were obtained from flats grown under greenhouse conditions. All portions were dried in a cool area for several weeks, then finely ground. Preliminary tests with *O. basilicum* indicated a possible difference in concentration of sterol and triterpene in leaves depending upon the position of the leaf on the plant. Therefore the leaves from the flowering *O. basilicum* plants were divided into two portions, one consisting of only those extending downward about 8 cm. from the top of the plant ("upper leaves") and those extending upward 8 cm. from the lowest leaf producing area ("lower leaves").

Preparation of Extracts for Determination of Sterol and Triterpenes.—Each plant part was exhaustively extracted with 95% ethanol in a Soxhlet extractor, with several changes of ethanol over at least a forty-hour extraction period. The ethanol was distilled off *in vacuo* and the residue was subjected to the fractionation shown in Fig. 1. This flow sheet was established by trial and error and found to give nearly quantitative recovery of microgram quantities β-sitosterol and the acidic triterpenes from aqueous mixtures or when added to plant extracts low in the sterol or triterpene.

Determination of Sterol.—The nonsaponifiable fractions (Fig. 1) were dissolved quantitatively in acetone-absolute ethanol. To aliquots of this solution digitonin was added exactly as described by Sperry and Webb (4). The precipitated digitonides were washed as for "total" cholesterol digitonide (4) and the β-sitosterol determined quantitatively

Received June 30, 1960, from the University of Kansas Medical Center, Department of Obstetrics and Gynecology, Kansas City.
Accepted for publication October 28, 1960.
This investigation was supported by a research grant from the National Institutes of Health, U. S. Public Health Service.

[1] Unpublished observations. Analytical data obtained were similar to that for β-sitosterol from several other Labiates (2): free compound, m. p. 138–140°; $[\alpha]_D^{18}$ −29 ($CHCl_3$); acetate, m. p. 126–127°; $[\alpha]_D^{18}$ −34 ($CHCl_3$); benzoate, m. p. 147–148°; $[\alpha]_D^{18}$ −12 ($CHCl_3$).

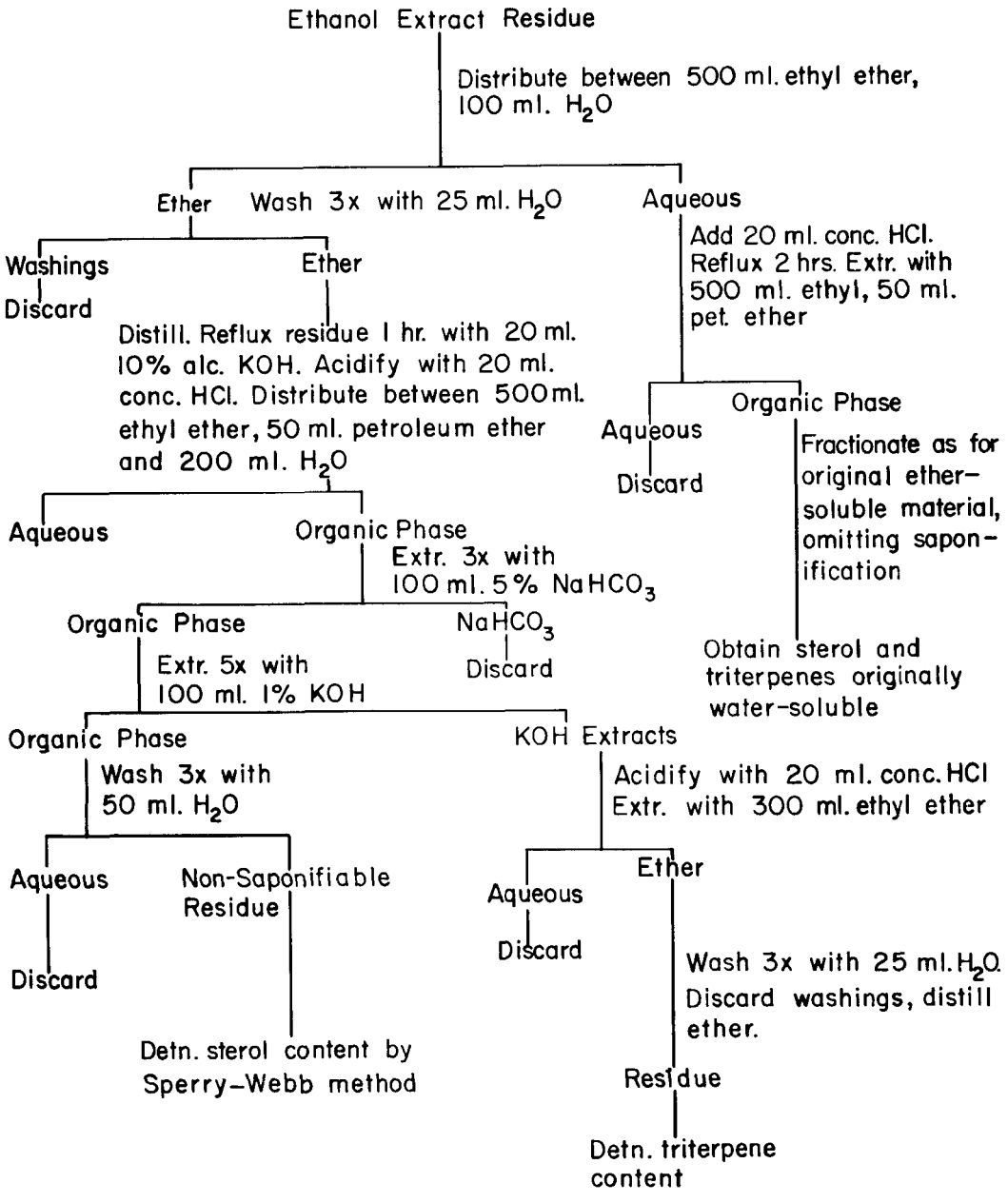

Fig. 1.—Flow sheet for quantitative fractionation of sterol and acidic triterpenes from plant extracts.

on dried digitonide residues with the Liebermann-Burchard reagent. β-Sitosterol digitonide (or sitosterol) exhibits maximum absorption at 620 mμ within twenty minutes with this reagent. The concentration-absorbance curve is linear up to 0.1 mg., and the quantity of β-sitosterol in the plant fractions was determined by comparison of absorbance readings with those from a sitosterol digitonide standard curve. Based on our earlier isolation studies (2), β-sitosterol is the principal sterol present in *O. basilicum*. It should be borne in mind, however, that trace 3-β-hydroxy sterols not detected in this previous work and responding to the Liebermann-Burchard reagent will also be included in the above determination.

Separation and Determination of the Triterpenoid Acids.—The Bush solvent system ligroin 10, methanol 8, water 2 (5) was found to separate oleanolic acid and ursolic acids I and II satisfactorily. The triterpene acid fractions (Fig. 1.) were quantitatively dissolved in absolute ethanol and aliquots were spread on a starting line 7 cm. from the edge of a 17 × 47 cm. strip of Whatman No. 1 paper previously washed with water and ethanol (and thoroughly dried). A current of warm air was necessary for distributing large volumes of aliquots. After overnight equilibration at 37° decending chromatography was begun with the upper phase of the Bush mixture as the developing phase. Under these conditions oleanolic

TABLE I.—CONCENTRATION OF β-SITOSTEROL AND URSOLIC ACID II IN *O. basilicum* ORGANS AT VARIOUS GROWTH STAGES

Plant Part	Sample Wt., Gm.	Micrograms per Gm. of Dried Plant Part[a] β-Sitosterol	Ursolic Acid II	Oleanolic Acid
Seeds	14.22	529	0	0
Seedlings, whole				
1 wk. old	3.79	230	...	...
5 wks. old	3.96	284	64	...
Preflowering plant				
Stems	4.67	535	Trace	...
Roots	2.40	469	Trace	...
Leaves	11.29	1062	454	...
Mature, flowering plant				
Upper leaves	4.81	1,709	1,143	...
Upper leaves	4.86	1,767	1,195	...
Upper leaves	4.54	1,762	1,299	...
Lower leaves	4.13	899	412	...
Lower leaves	3.06	937	547	...
Main stem	35.50	846	106	...
Roots	16.67	409	...	...
Flower stems	3.24	324	154	...
Flowers	6.28	1,053	1,274	1,300

[a] Each determination is the average of at least two values checking within 5%.

TABLE II.—CONCENTRATION OF STEROL AND ACIDIC TRITERPENES IN *S. officinalis* AT VARIOUS GROWTH STAGES

Plant Part	Sample Wt., Gm.	Micrograms per Gm. of Dried Plant Part β-Sitosterol	Ursolic[a] Acid	Oleanolic Acid
Seedlings, whole, 3 wks. old	3.0	Trace	Trace	Trace
Mature, flowering plant				
Leaves	9.8	2,450	1,260	790
Stems	4.2	1,220	200	400
Flowers	3.2	2,060	1,300	140

[a] Includes ursolic acid I and II since these are not separated by the paper chromatography used.

acid moved almost with the solvent front while ursolic acids I and II remained at the starting line. Pure oleanolic acid moved as a discrete spot with slight tailing in quantities of 100 mcg. or less; quantities greater than this tended to spread as a smudge. For detection or quantitative determination of oleanolic acid, the lower end of the paper was tapered and led into a small beaker into which the overflow was collected. Separation was allowed to proceed for forty-eight hours. For ursolic acid I or II, estimation strips 2 cm. above and 6 cm. below the whole length of the starting line were cut from the paper into small pieces. The latter were boiled three times with 25-ml. quantities of absolute ethanol; the solvent was then decanted through glass wool into flasks from which the ethanol was evaporated *in vacuo*. The residue was then diluted to fixed volume with absolute ethanol; the overflow material, freed of solvent, was similarly treated. Aliquots of these solutions were freed of solvent *in vacuo* and the triterpene content determined quantitatively on the residue with the Liebermann-Burchard reagent, as described by Sperry and Webb (4) for cholesterol determination except for the following time intervals for color development: oleanolic acid, ninety minutes; ursolic acids I or II, sixty minutes. The compounds exhibited a linear relationship between concentration and absorbance up to 0.1 mg. Quantitative determination was made by comparison with standard curves prepared from the free, analytically pure compounds.

A preliminary qualitative survey for the triterpenoid acids using 3–5 Gm. of dried plant part was made by spraying the chromatographed papers with 10% antimony pentachloride in chloroform, whereupon a gradually developing pink-red color indicated the presence of triterpene. As little as 10 mcg. of ursolic acid I or II and 50 mcg. of oleanolic acid were detected visually by this method. Oleanolic acid was best detected by respotting the forty-eight-hour overflow material on a second paper, followed by the antimony pentachloride spray. A similar qualitative determination was made for sterol, using as the detection method formation of a digitonide precipitate according to Sperry and Webb (4).

RESULTS AND DISCUSSION

Preliminary qualitative tests indicated that in *O. basilicum* sterol, ursolic acid II, and oleanolic acid were present in one week-old seedlings, with the triterpenoid acids present both in the "aqueous fraction" (presumably conjugated as glycosides) and as the "free," ether-soluble triterpene. No triterpene acid and only a trace of sterol was detected in the "aqueous fractions" of ethanol concentrates from all parts of the mature plant. Apparently with increasing age, in this plant at least, there is a shift of conjugated triterpene to the nonconjugated form.

Some quantitative values for sterol and triterpene at several periods in the life cycle of *O. basilicum* and *S. officinalis* are shown in Tables I and II, respectively. Values shown are only for the ether-soluble portion of alcohol concentrates. Since the water-soluble form of sterol and triterpene in very young plants was indicated in only trace amounts by the preliminary qualitative survey, the primary conclusions drawn from these figures would appear to be valid; namely, that there is an increased concentration of both sterol and triterpene with increasing maturity of the plant. In the mature plants both sterol and triterpene were concentrated in the flower parts and leaves and in *O. basilicum* were higher in the upper portions of the leaves. The reason for the latter is not apparent at present. In general, it appears that within both plants the distribution of sterol and triterpene follows a similar quantitative pattern. Both acidic triterpenes were conspicuously absent from the seeds.

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