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ARE STEROIDAL ESTROGENS NATURAL PLANT CONSTITUENTS?

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; kidney bean; *Perilla crispa*; Labiatae; *Chenopodium rubrum*; Chenopodiaceae; goose foot; *Malus pumila*; Rosaceae; apple seeds; *Phoenix dactylifera*; Palmae; date palm seeds; estrogens.

Abstract—Several plant sources were analysed to identify steroidal estrogens. Earlier reports of steroidal estrogens in these plants could not be confirmed by mass fragmentographic analysis. Monoglycerides were identified in the 'estrogen-like' fraction.

INTRODUCTION

It is widely assumed that regulation of flower formation in plants involves a floral stimulus (=florigen or flower hormone) which is formed in the leaves and moves to the receptor bud, but attempts at isolating and identifying the floral stimulus have so far been unsuccessful [1]. However, Kopcewicz [2–4] reported that flower formation in several plants is associated with the appearance of 'estrogen-like' substances in the shoots of these plants. These findings would be in agreement with the hypothesis that steroids may have hormonal functions in plants analogous to those in aminals [5–7], provided that the 'estrogen-like' substances can be identified as steroidal estrogens.

Exogenously applied estradiol caused flowering in *Salvia* [8] and in *Cichorium* [9]. Furthermore, estrone and estradiol were reported to increase stem elongation in a dwarf pea cultivar [10], but this effect of applied estrogens could not be confirmed [11].

Before estrogens can be assigned a physiological role in plants, it is essential to ascertain that they are naturally occurring plant constituents. Although the presence of steroidal estrogens has been reported in several plants [5–7, 12], in none of these reports were these compounds identified by mass spectra. The concentration of estrone in pomegranate seeds, first reported by Heftmann *et al.* [15], was later found to be 4000× lower, if the substance was present at all [16].

In view of the potential significance of Kopcewicz's observations, we have analysed the 'estrogen-like' fraction from several flowering plants by GC–MS. The material studied included some of the plants studied by Kopcewicz [2, 3]. As reference material, we have also analysed by the same method two reported sources of estrone, viz. seeds of apple [17] and of date palm [18].

RESULTS AND DISCUSSION

Efficiency of the extraction method

The 'estrogen-like' fraction was isolated from plant material as described by Kopcewicz [2]. Recovery of added estrone-[4-^{14}C] and estriol-[4-^{14}C] was measured after successive purification steps of an extract from *Phaseolus*. In the presence of crude plant hydrolysate, 16% of the added estrogens was recovered (Table 1). When estrone-[4-^{14}C] was subjected to acid hydrolysis in the absence of plant material, the radioactivity was quantitatively recovered in the toluene and CH_2Cl_2 phases. Following acidification of the N NaOH phase to pH 3, 86% of the estrone-[4-^{14}C] was recovered by extraction with CH_2Cl_2. The efficiency of the extraction procedure was therefore much reduced by the presence of plant material.

Table 1. Efficiency of the extraction method for estrogens from plant material*

Extraction step	cpm × 10^{-6}	% Recovered
Soxhlet extract in toluene–MeOH (3:1)	2.04	100
CH_2Cl_2 phase after acid hydrolysis	1.77	87
CH_2Cl_2 extract from the N NaOH phase after acidification	0.32	16

* Estrone-[4-^{14}C] and estriol-[4-^{14}C] were added simultaneously to a crude *Phaseolus* extract and their recovery was measured after successive extraction steps.

Kober reaction

The 'estrogen-like' fraction from induced *Perilla* shoots and flowering *Phaseolus* shoots was analysed for Kober positive material as described by Kopcewicz [2]. The absorption spectra showed no maximum around 515 nm while the corrected extinction values at 515 nm were negative.

Analysis by GC–MS

The 'estrogen-like' fraction of all investigated plant sources was analysed by GLC after silylation. No peaks were observed with retention times identical with those of the TMSi ethers of estrone, estradiol or estriol. We

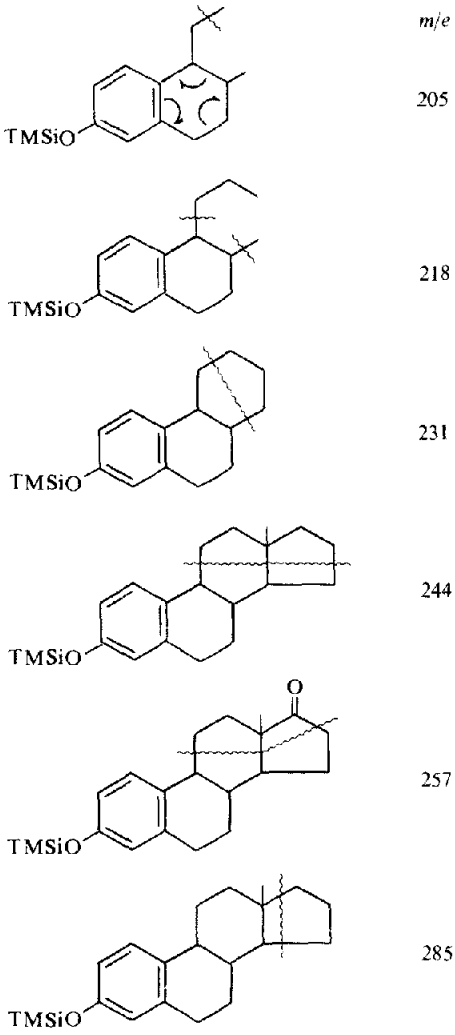

Fig. 1. Characteristic fragments of silylated phenolic steroids. A mass chromatogram of each fragment was recorded, searching for steroidal estrogens or close metabolites in the investigated plant sources.

searched by mass fragmentography for traces of steroidal estrogens or closely related metabolites as indicated by the chosen fragments (Fig. 1). No phenolic steroids were detected in apple seeds and date seeds. Flowering and non-flowering shoots of *Phaseolus* were compared, but no phenolic steroids were found in either. Shoots of *Perilla*, induced for flower formation, were compared with non-induced plants, again without detecting phenolic steroids. The lower detection limit under our experimental conditions was found to be 50 ng of estrone added to an apple seed extract. This corresponded to a lower detection limit of 0.5 μg estrone/100 g apple seeds (uncorrected for losses). The reported level of 13 μg/100 g apple seeds [17] could thus not be confirmed.

Coinciding ion intensity peaks were found for *m/e* 205 and 218 in the MS of a constituent, present in all 'estrogen-like' fractions. Interpretation of the corresponding MS showed that fragments *m/e* 205 and 218 originated from monoglycerides.

When the deuterosilylated MS was compared with the MS of the silylated compound, a shift from *m/e* 147 to 162 was observed. This shift can only be explained by the occurrence of the fragment $TMSiO^+{=}SiMe_2$, characteristic of vicinal hydroxyls [19]. The ethylene glycol structure was confirmed by the formation of an acetonide. The structure of the acetonide could be assigned by its characteristic *m/e* 101 fragment [20]. An accurate measurement of the M^+ of the silylated substance showed the underivatized M^+ to be $C_{21}H_{36}O_4$. This formula could be the monoglyceride of linolenic acid. This monoglyceride was synthesized and its MS after silylation was identical with the MS from *Phaseolus*. The 1-monolinolenin eluted (R_t 14 min) between estradiol (R_t 10 min) and estriol (R_t 19.5 min). More saturated monoglycerides were found in the same GLC peak (Table 2).

Table 2. Monoglycerides observed in the estrogen-like fraction of different plant sources

Plant source	Plant part	Fatty acid of monoglyceride
Chenopodium rubrum	shoots	$C_{18:3}$
Perilla crispa	shoots	$C_{18:3}$, $C_{18:2}$
Phaseolus vulgaris	shoots	$C_{18:3}$, $C_{18:2}$
Malus pumila	seeds	$C_{18:2}$, $C_{18:1}$
Phoenix dactylifera	seeds	$C_{18:2}$, $C_{18:1}$, $C_{18:0}$

The term 'estrogen-like' compounds was assigned by Kopcewicz [2] solely on the basis of a positive Kober reaction. This criterion is not sufficient since the name 'estrogen-like' should be restricted to steroids with a phenolic A-ring. Kopcewicz himself has not been able to confirm the demonstration of Kober positive material in flowering plants and feels that it may have been based upon phenolic compounds, unrelated to estrogens (personal communication). We could not positively identify estrone by MS from extracts of reportedly rich sources of this compound, such as apple seeds and date palm seeds. Preliminary experiments with the more sensitive radioimmunoassay did not confirm the presence of estrone either (unpublished results).

We conclude that before a hormonal function of endogenous steroidal estrogens in plants can be considered, their presence still needs to be established beyond any doubt.

EXPERIMENTAL

Plant materials. Phaseolus vulgaris L., cv Mecosta, *Perilla crispa* (Thunb.) Tanaka, and *Chenopodium rubrum* L., selection No. 374 [21] were grown in a greenhouse maintained at a minimum temp. of 22°. Flower formation in the short-day plants *Chenopodium* and *Perilla* was induced by subjecting the plants to 8-hr days by placing them from 4 p.m. to 8 a.m. in a dark room kept at 23°. *Perilla* shoots were harvested after 83 days when induced plants had been subjected to 28 short days. *Chenopodium* was harvested after 144 days; flowering was induced during the last 10 days. Non-flowering *Phaseolus* plants were harvested within 15 days after planting and flowering ones after 26 days and later. The harvested shoots were frozen in liquid N_2 and lyophilized. Apple seeds were purchased from F. W. Schumacher Co, Sandwich, Mass. Date palm seeds were a gift from Dr. J. B. Carpenter, U.S. Date and Citrus Station, Indio, Calif.

Isolation of the estrogen-like fraction. The method described

by Kopcewicz [2] was applied. Toluene was substituted for benzene to avoid the health risk. The crude methanolic extract was spiked with esterone-[4-^{14}C] (0.5 μCi, 52 mCi/mmol) and estriol-[4-^{14}C] (0.5 μCi, 60 mCi/mmol). Following successive extraction steps, aliquots were taken in duplicate, dried on cellulose and combusted in a sample oxidizer. After combustion, the radioactivity was measured by liquid scintillation counting.

Kober reaction. Reaction conditions optimal for estrone were used [22]. The spectrophotometric measurement of the estrogen content was as described by Kopcewicz [2]. Extinction readings were corrected for unspecific background colouration by applying: $E_{corr} = 2E_{515} - (E_{474} + E_{556})$.

GC–MS. Silylated samples were analysed on a 3% SP-2100 coiled column (183 × 0.3 cm). He at a flow rate of 70 ml/min was the carrier gas. The temp. was programmed from 200 to 270° at a rate of 5°/min. The mass range up to *m/e* 700 was scanned every 8 sec and the spectra were stored in a data acquisition system. Mass chromatograms of selected single ions (Table 2) were subsequently recalled and displayed underneath each other. The MS of coinciding ion intensity peaks were then recalled. In addition, the mass chromatograms of the M^+ of the TMSi ethers of estrone, estradiol and estriol were recorded, since these aromatic ions are intense.

Synthesis of 1-*monolinolenin.* The method described by Bourne *et al.* [23] for the synthesis of glyceryltripalmitate was modified to obtain 1-monolinolenin. A 10-fold excess of glycerol was used to favor the formation of a monoglyceride. The 1-monoglyceride was selectively extracted from the reaction mixture according to the method of ref. [24].

Identification of monoglycerides. The MS of 1-monolinolenin from *Phaseolus* was identical with the MS of the synthetic monoglyceride after silylation: MS 70 eV *m/e* (rel. int.): 506 (M^+, 6), 481 (30), 406 (75), 393 (100), 261 (70), 218 (80). The silyl groups were removed by treatment at room temp. with aq. EtOH overnight. The MS of the underivatized 1-monolinolenin obtained by direct probe introduction was as catalogued [25]. The acetonide of 1-monolinolenin was obtained by treatment with dry $CuSO_4$ in dry Me_2CO [20]; MS 70 eV *m/e* (rel. int.): 392 (M^+, 3), 377 (55), 334 (30), 101 (75). The M^+ characterized the silylated monoglycerides of linoleic acid (508), oleic acid (510) and stearic acid (512).

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