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MEASUREMENT OF XANTHOXIN IN HIGHER PLANT TISSUES USING ^{13}C LABELLED INTERNAL STANDARDS

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; *Phaseolus vulgaris*; Leguminosae; *Zea mays*; Gramineae; *Helianthus annuus*; Compositae; xanthoxin; 2-*trans*-xanthoxin; isomerization; ^{13}C-labelled internal standards; GC-MS selected ion monitoring.

Abstract—A new procedure for the reliable measurement of xanthoxin from plant tissues has been developed. This accounts for both its breakdown and its isomerization to 2-*trans*-xanthoxin, and relies on the use of ^{13}C-labelled internal standards, HPLC purification and analysis by GC-MS SIM. The antioxidant *tert*-butylated hydroxyquinoline was found to reduce the amount of isomerization to 2-*trans*-xanthoxin during extraction. Levels of xanthoxin present in all tissues examined were much lower than previously thought, and 2-*trans*-xanthoxin: xanthoxin ratios much higher.

INTRODUCTION

Xanthoxin (Xan; **19**) has frequently been proposed as an abscisic acid (ABA; **1**) precursor in higher plants, most often as an intermediate between a carotenoid such as violaxanthin (**3**) and ABA [1, 2]. However, the evidence for such a role remains circumstantial. Xan, and its geometrical isomer 2-*trans*-xanthoxin (*t*-Xan; **18**) can be formed via carotenoid cleavage *in vitro* [3, 4], both isomers have been identified in extracts from a wide variety of plants [5], and the conversion of Xan to ABA has been demonstrated in detached tomato and dwarf bean leaves [6–8]. It now seems likely that there exists a universal ABA biosynthetic pathway in higher plants, and that this is an indirect pathway involving carotenoids [2, 9]. In etiolated bean seedlings, 9-*cis*-violaxanthin (**4**) and 9′-*cis*-neoxanthin (**5**) appear to be the major ABA precursors [10; A. D. Parry, M. J. Babiano, R. Horgan, unpublished results]. By implication Xan should be the 'missing link' between these xanthophylls and ABA-aldehyde (**2**), the immediate precursor of ABA [11, 12]. The ability to detect and measure Xan is therefore very important.

Difficulties associated with the measurement of Xan levels have in the past hindered research into the relationship between it and ABA, and also into the possible roles of Xan in physiological processes such as phototropism and gravitropism [13, 14]. This paper discusses these problems and describes a new procedure, involving the use of ^{13}C-labelled internal standards and GC-MS of underivatized Xan and *t*-Xan, which allows reliable measurements of both isomers to be made. Results obtained using this technique are compared to previous findings.

*Abbreviations: DDC, sodium diethyldithiocarbonate; PLC, preparative thin layer chromatography; SIM, selected ion monitoring; TBHQ, *tert*-butylatedhydroxyquinoline; *t*-Xan, 2-*trans*-xanthoxin; Xan, xanthoxin.

RESULTS AND DISCUSSION

Preliminary studies on the behaviour of Xan *in vitro* showed that the major problem associated with its measurement was the facile interconversion that took place between it and *t*-Xan during the extraction procedure. Exposure of methanolic solutions of Xan or *t*-Xan to light (sunlight or UV) led to rapid isomerization (up to 50% Xan→*t*-Xan after 30 min) with an equilibrium of *ca* 3:2 *t*-Xan:Xan being reached within 2 hr. A similar ratio was obtained by Shen-Miller *et al.* [14]. Little isomerization occurred in the dark, even after 3 days at 20°. Base-(1:1 1.0 M KOH–MeOH; pH 13.5) catalysed isomerization was swiftly effected even in the dark, with an equilibrium ratio of 4:1 *t*-Xan:Xan being reached within 15 min. This is consistent with the interconversion of Xan and *t*-Xan proceeding via an enolate intermediate. Samples of pure Xan or *t*-Xan were taken through the entire purification procedure used for plant extracts and the ratios of *t*-Xan:Xan observed after HPLC and GC-MS are given in Table 1. The variation in rate of isomerization probably reflects changes in ambient light levels between replicate extractions. Breakdown of Xan also occurred upon exposure to light but at a slower rate than isomerization. After 5 hr of sunlight, HPLC (system B) revealed that 20% of total Xan plus *t*-Xan had been lost from a methanolic solution, concurrent with increases in two unknown compounds. These were purified (HPLC system C) and full mass spectra obtained. One was identified as 3-hydroxy-5,6-epoxy-β-ionone (**6**) by comparison with the authentic compound while the second was tentatively identified as 3-hydroxy-β-ionone (**7**). Both compounds are known to occur naturally in plants [16], and in this study 3-hydroxy-β-ionone was extracted from tomato leaves at levels equivalent to those of *t*-Xan (*ca* 100–200 ng/g fr. wt).

It was clear from the properties of Xan in plant extracts that reliable measurements would require the use of a suitable internal standard. Given the possible synthetic

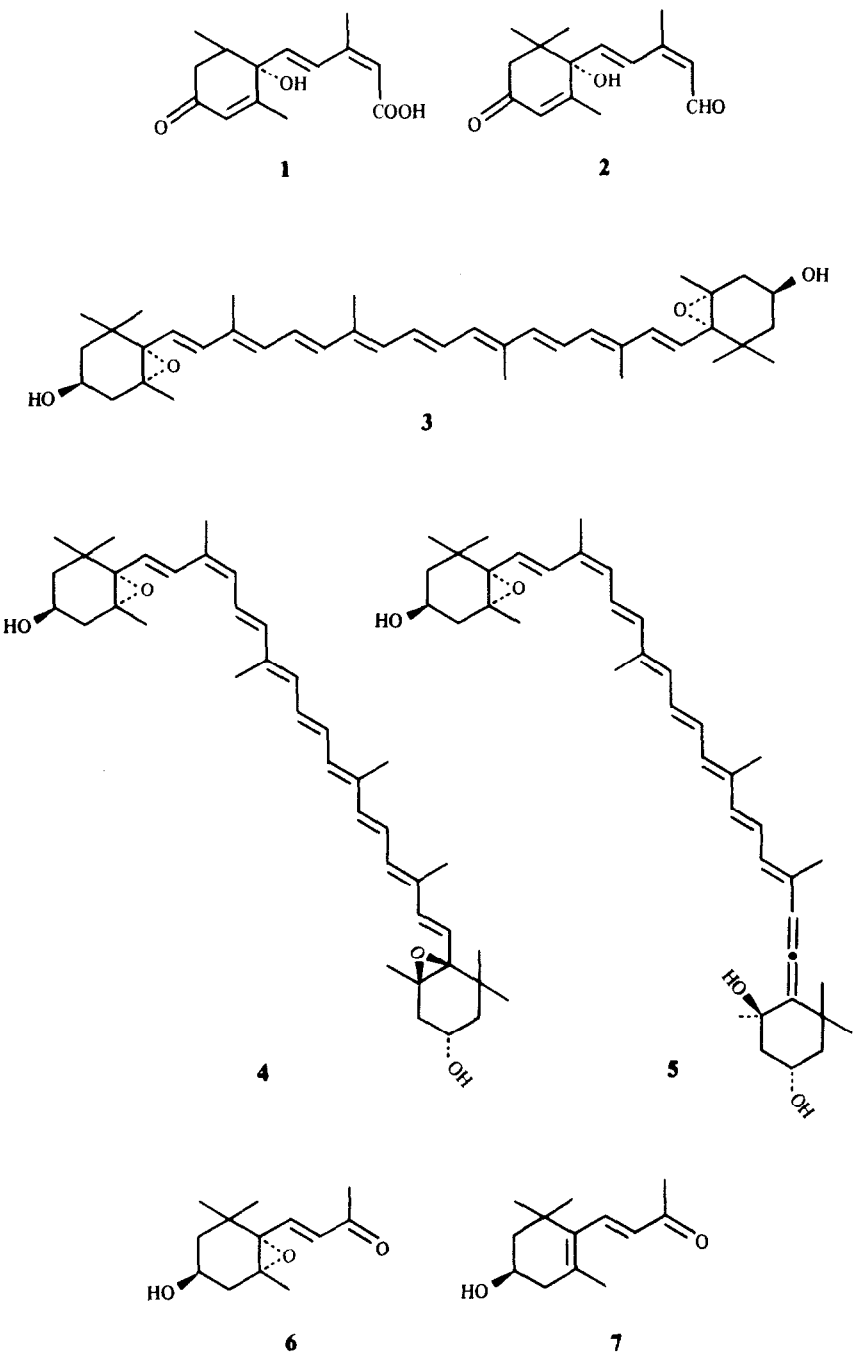

Table 1. Isomerization of [^{13}C]Xan and *t*-Xan standards during extraction procedure used for plant extracts

Isomer 'extracted'	*t*-Xan:Xan		
	t = 0	ad. HPLC	GC-MS/SIM
t-Xan	98:2	98:2	92:8
t-Xan	98:2	98:2	84:16
t-Xan	98:2	91:9	87:13
Xan	4:96	61:39	56:44
Xan	4:96	57:43	51:49

routes to Xan it was decided that [1, 2-^{13}C] Xan would be most suited to this purpose.

The need to introduce the ^{13}C label late in the synthesis led to the development of a route which was essentially a combination of the published methods of the Hoffman-La Roche group [17, 18] and Taylor and Burden [6]. The route is outlined in Fig. 1. Safranal (**8**) was stereospecifically converted to (3*R*)-3-hydroxy-β-cyclogeranial (**10**). Reaction of this with acetone gave (3*R*)-3-hydroxy-β-ionone (**11**). This was epoxidized with *m*-chloroperbenzoic acid to give a mixture of (3*S*,5*S*,6*R*) (**12**), and (3*S*,5*S*,6*S*)-3-hydroxy-5,6-epoxy-β-ionone (**13**) which was separated by flash chromatography. From the known

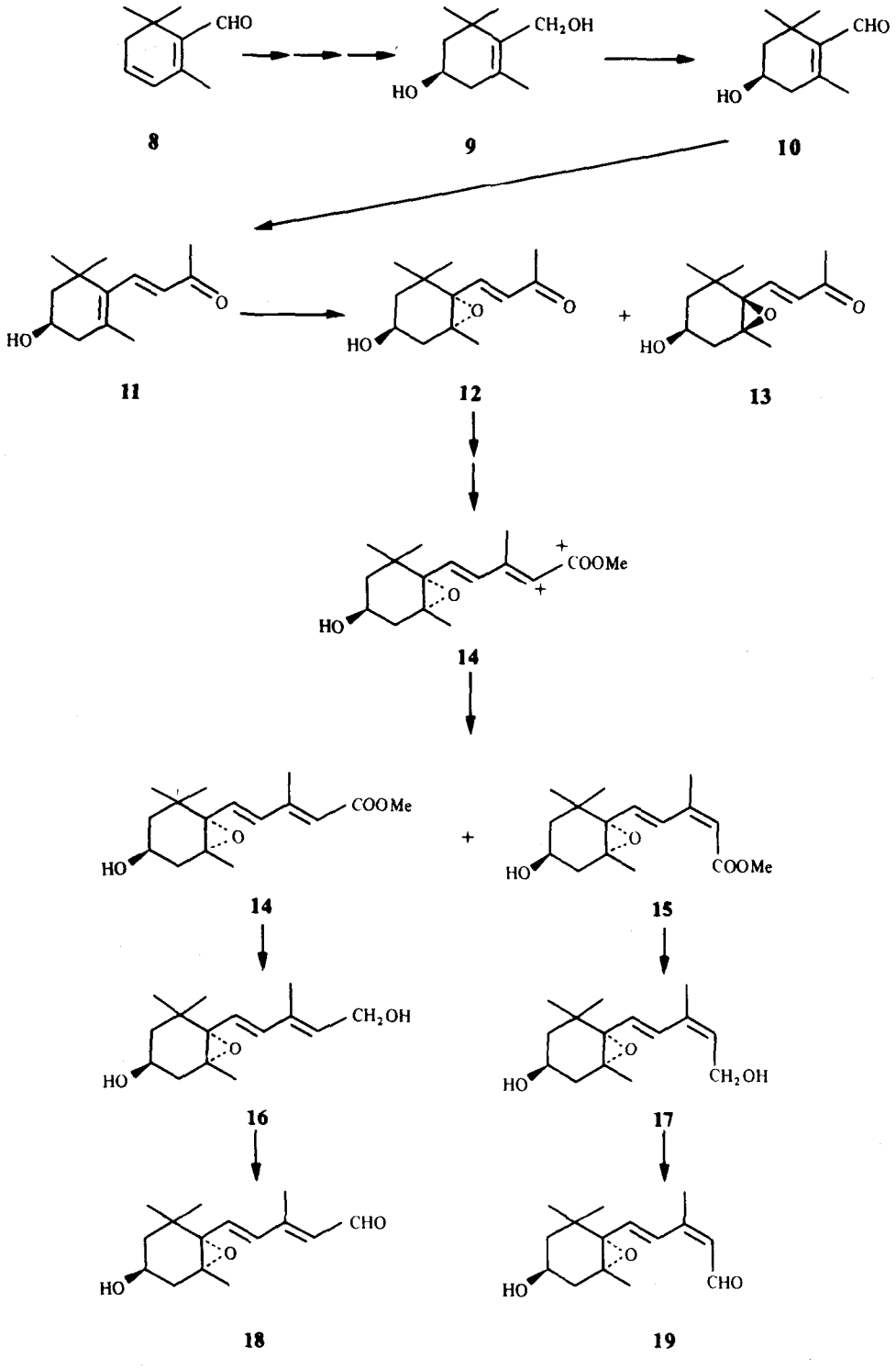

Fig. 1. A simplified scheme for the synthesis of [^{13}C]Xan and *t*-Xan. + = position of ^{13}C label.

stereochemistry of epoxidations of this type and by analogy with the published data on the epoxidation of (2*Z*,4*E*)-5-[(*R*)-4-hydroxy-2,6,6-trimethyl-1-cyclohex-1-enyl]-3-methyl-2,4-pentadienoic acid methyl ester, it was concluded that the major component (75%) was the 5*R*,6*S*-epoxide (**13**) and the minor component (25%) was the required 5*S*,6*R*-epoxide (**12**). This was supported by optical rotation measurements. The putative 5*S*,6*S*-epoxide had $[\alpha]_D^{25}$ +18.6°, whereas the putative 5*S*,6*R*-epoxide had $[\alpha]_D^{25}$ −96.5°.

The 5*S*,6*R*-epoxide was converted into [1,2-^{13}C] (2*E*,4*E*)-5-[(*R*)-4-hydroxy-2,6,6-trimethyl-1-cyclohex-1-

enyl]-3-methyl-2,4-pentadienoic acid methyl ester, ([1,2-^{13}C]2-*trans*-xanthoxin acid methyl ester) (**14**) via a Horner reaction with trimethyl [1,2-^{13}C]phosphonoacetate as described by Taylor and Burden [6]. This compound exhibited a $[\alpha]_D^{25}$ of $-38°$ in chloroform compared to a literature value of $-43.2°$ for xanthoxin acid methyl ester [18]. UV isomerization gave a mixture of xanthoxin acid methyl ester ($[\alpha]_D^{25}$ $-40.5°$) and its 2*E*,4*E* isomer which were separated by PLC. The individual isomers were converted to Xan (**19**) and *t*-Xan (**18**) by standard methods.

The isomerization and breakdown of Xan and *t*-Xan within a plant extract are likely to occur by complex photochemical processes. If reliable measurements of these compounds are to be made then isomerization and breakdown need to be accounted for. The problems associated with the simultaneous determination of Xan and *t*-Xan are exacerbated by the large *t*-Xan:Xan ratio and the low levels of Xan present in plant tissues.

Identical 10 g extracts of tomato leaves were spiked with 100 ng/g [^{13}C] Xan or *t*-Xan. After GC-MS SIM the ratios of endogenous *t*-Xan:Xan were similar in both cases (87:13 and 88:12), but 83% of the added [^{13}C]Xan had isomerized compared to only 7% of the added [^{13}C]*t*-Xan. The endogenous ratio of *t*-Xan:Xan must have been even greater prior to extraction because if the original ratio had been in favour of Xan then a final equilibrium ratio of 3:2 at most would have been obtained. It was apparent that Xan and *t*-Xan could not be quantified independently and simultaneously by the addition of [^{13}C]Xan and [^{13}C]*t*-Xan respectively. A method was developed in which the addition of [^{13}C] *t*-Xan to an extract could be used to calculate the concentration of both endogenous *t*-Xan and Xan.

(i) *Calculating the concentration of* t-*Xan*

Based on preliminary extractions the amount of [^{13}C]*t*-Xan added to an extract would be as similar as possible to the levels of *t*-Xan found endogenously. As the ratio of *t*-Xan:Xan found in all tissues so far examined has been large (10–500:1) [8] and (Table 2), the main assumption was that the direction and rate of isomerization of *t*-Xan to Xan would be the same for the internal standard and endogenous compound. The following calculation could therefore be made:

$$[t\text{-Xan}] = \frac{\text{peak area } m/z \text{ 250 } (t\text{-Xan})}{\text{peak area } m/z \text{ 252 (t-Xan)}} \times \begin{matrix}\text{correction*}\\ \text{factor}\end{matrix} \times \begin{matrix}[^{13}\text{C-}t\text{-Xan}]\\ \text{added}\end{matrix}$$

*from SIM calibration curve.

(ii) *Calculating the concentration of Xan*

The Xan measured by GC-MS SIM represents endogenous Xan and Xan derived from *t*-Xan during extraction. When the [^{13}C]*t*-Xan internal standard is added the percentage of [^{13}C]Xan present is a known small amount (*ca* 2%). It is therefore possible, after GC-MS SIM to calculate the percentage of [^{13}C]*t*-Xan that has isomerized to [^{13}C]Xan during the extraction. Continuing with the above assumption (see i), the percentage of Xan that has arisen via isomerization of *t*-Xan can also be calculated. Thus the corrected *t*-Xan:Xan ratio can be obtained and knowing the levels of *t*-Xan (i) a simple calculation produces the concentration of Xan originally present in the tissue:

$$[\text{Xan}] = \frac{\text{corrected peak area } m/z \text{ 250 (Xan)}}{\text{corrected peak area } m/z \text{ 250 } (t\text{-Xan})} \times [t\text{-Xan}]^*$$

*from (i).

The losses of Xan and *t*-Xan are assumed to be comparable as the isomers are purified and analysed together.

Table 2. Levels of Xan and *t*-Xan in a variety of tissues (ng/g fr. wt)

	Present estimate*		Previous estimate		
Tissue	*t*-Xan	Xan	*t*-Xan	Xan	Ref.
Lycopersicon (leaf)	90.0 ± 2.8	7.8 ± 1.4	18.2	7.8	[29]
Phaseolus (leaf)	192.0 ± 44.0	5.4 ± 2.8	174.0	48.0	[5]
Zea (root tip)	14.3 ± 1.1	n.d.	n.d.	32 ± 10†–20‡	[24]
Helianthus (hypocotyl)					
Control intact	16.0	n.d.	—	10–60	[32, 33]
Control halved	10.1 ± 3.9	1.0 ± 0.7	—	—	
Light half§	11.7 ± 8.7	1.7, n.d.	3.8 ± 1.0	2.4	[14]
Dark half	7.4 ± 1.4	0.1, n.d.	4.3 ± 0.9	0.9	[14]

n.d. = not detected.
*2–4 replicates.
†Root cap.
‡Adjacent 1.5 mm segment.
§Hypocotyls unilaterally illuminated for 2 hr.

The artefactual production of Xan and *t*-Xan from xanthophylls during extraction has been reported to give 100–1000-fold overestimations of the concentrations of Xan that exist in plant tissues [19]. The use of a ^{13}C-labelled internal standard could not account for such Xan generation and so it was important to assess the contribution, if any, xanthophyll breakdown made to the levels of Xan measured during this procedure. Under acidic conditions the 5,6-epoxide groups of xanthophylls such as violaxanthin are converted into 5,8-furanoids [see 20]. These rearranged xanthophylls could not, even if cleaved, generate Xan. De Vit [19] showed that extraction of plant tissue with acidic methanol caused an almost instantaneous conversion of violaxanthin and neoxanthin into their corresponding furanoids whilst minimizing Xan and *t*-Xan breakdown. Yields of Xan from samples extracted in methanol (86.3 ± 2.5 ng/g fr. wt.) were similar to those obtained using acidic methanol ($\equiv 0.1$ M HCl, 8.25 ml conc HCl/l MeOH; 89.5 ± 17.5 ng/g fr. wt), suggesting that Xan is not produced as a result of xanthophyll breakdown during normal methanolic extractions. On extraction into methanol lipoxygenase (LOX) and other enzymes known to cleave carotenoids would be rapidly inactivated but free radical-mediated xanthophyll cleavage could still occur. The antioxidant *tert*-butylatedhydroxyquinoline (TBHQ) completely inhibits both aerobic and anaerobic pigment bleaching by LOX isozymes and their product fatty acid/alkoxy radicals [21–23]. Extractions with the usual antioxidant, sodium diethyldithiocarbonate (DDC), or TBHQ produced similar estimates for the concentrations of both isomers. Interestingly, TBHQ seemed to inhibit isomerization of the internal standard (and presumably endogenous Xan as well) during extraction. This inhibition of [^{13}C]*t*-Xan isomerization was confirmed by further extractions (DDC, $14.2 \pm 2.5\%$ isomerization, $n = 14$; TBHQ, $6.3 \pm 2.2\%$ isomerization, $n = 16$) but the mechanism remained obscure. In subsequent extractions, TBHQ was employed to minimize isomerization and so increase the accuracy of the final measurements.

Positive identification of *t*-Xan, extracted from tomato leaves, was obtained by comparison of the full mass spectra of the endogenous and authentic compounds. Xan was present at very low levels and so identified using SIM of the relevant ions (*m/z* 250 and 149). The ratio of *m/z* 250 to 149 and their GC R_t's were characteristic of authentic Xan. The levels of Xan and *t*-Xan were measured in a variety of tissues and are compared to previously published values in Table 2. Recoveries for total Xan plus *t*-Xan were estimated, following HPLC, at $28 \pm 7\%$ ($n = 17$).

In all tissues examined, the levels of *t*-Xan were much greater than those of Xan, with *t*-Xan:Xan ratios varying from 10 to 70:1. This contrasts with values found in the literature where *t*-Xan:Xan ratios varying from 1.4:1 to a maximum of 4.8:1 are quoted. In fact if all the published data for Xan and *t*-Xan (excepting that of Feldman *et al.* [24]; see below) are analysed, then a value of 3.0 ± 1.0 can be calculated for the ratios of *t*-Xan:Xan ($n = 42$: [4, 5, 25–29]).

This discrepancy between previous estimates of Xan levels and *t*-Xan:Xan ratios and those found during this study has arisen because of an inability of previous techniques to take account of the inevitable isomerization that occurred between Xan and *t*-Xan during extraction. In addition, it is now known that derivatization of Xan, carried out as a part of most previous methods, actually causes isomerization [30]. It is therefore not surprising that past estimates for *t*-Xan:Xan ratios have been consistent and near the equilibrium ratio. Together with an inability to assess accurately losses that occurred during purification the above problem has resulted in an underestimation of *t*-Xan and an overestimation of Xan levels in most tissues.

Feldman *et al.* [24] claimed that *Zea mays* roots contained almost exclusively Xan and little or no *t*-Xan. In contrast, present extractions of *Zea mays* roots revealed modest levels of *t*-Xan and no detectable Xan. The reasons for these conflicting results are unknown but are unlikely to be due to the use of different cultivars.

Bioassay data has previously suggested that Xan might be involved as a growth inhibitor in the phototropic response of sunflower hypocotyls [14, 31, 32]. In this study Xan was found to occur at low levels or be non-detectable in sunflower hypocotyls. Although variable the data did not suggest that a re-distribution of Xan or *t*-Xan occurred following unilateral illumination. Recently, Bruinsma (Department of Plant Physiology, Agricultural University, Wageningen, The Netherlands, personal communication), has shown that the levels of Xan found in sunflower hypocotyls are insufficient to account for the observed phototropic response.

The results of this study illustrate the necessity for a reliable and accurate procedure for the extraction and measurement of Xan and *t*-Xan. It is clear that the results of previous attempts to correlate Xan levels with physiological responses must now be viewed with scepticism, because Xan can be formed artefactually so readily from the more abundant biologically inactive *t*-Xan. Unfortunately, techniques for the determination of Xan are still being described which cannot account for the losses or isomerization that occur during extraction, one such relies on an immunoassay which does not even distinguish between Xan and *t*-Xan [34].

Whilst the development of the technique described in this paper should assist in a reassessment of the possible physiological role of Xan, it is clear from the results presented here that this is probably a very minor one. It appears unlikely, therefore, that Xan functions as a growth inhibitor *per se* because its concentration in plant tissues is extremely small relative to ABA. This would be predicted from the known facile conversion of Xan to ABA [6–8].

EXPERIMENTAL

Plant material. Plants of *Lycopersicon esculentum* Mill. cv 'Ailsa Craig' and *Phaseolus vulgaris* L. cv 'Canadian Wonder' were grown in John Innes No. 2 compost in glasshouses at 20° under natural light supplemented with artificial light (700 W mercury vapour lamps) where necessary to give a 16 hr photoperiod. Leaves were harvested from 10-week-old plants. Seed of *Zea mays* L. cv 'Golden Bantam' were surface sterilized in 7% NaOCl solution for 30 min, washed and imbibed in dist. H_2O for 2 hr, and germinated in the dark at 22° on wet filter paper. After 4 hr the root cap and terminal 3–4 mm of root were excised and frozen in liquid N. Seeds of *Helianthus annuus* L. cv 'Giganteum' were sown directly onto John Innes No. 2 compost and grown at 20° with a 16 hr photoperiod until one-week-old (5–6 cm tall). Hypocotyls were phototropically stimulated via unilateral illumination from a white fluorescent tube (500 μmol/m^2/sec PAR at source). After 2 hr curvature measurements were taken (20.1

$\pm 3.8°$; $n=25$) and hypocotyls cut into light- and dark-facing halves before freezing in liquid nitrogen. Control hypocotyls were treated in a similar manner but illuminated from directly above. All tissue was stored at $-20°$ until required.

(3R)-3-*Hydroxy-β-cyclogeraniol*. Safranal (**8**) was reduced with $LiAlH_4$ to safranol (**9**) which after conversion to the isopropenyl methyl ester was stereospecifically hydroxylated by hydroboration with (+)-diisopinocampheylborane to yield (3)*R* 3-hydroxy-β-cyclogeraniol isopropenylmethyl ether. Removal of the protecting group followed by oxidation with pyridinium chlorochromate gave (3*R*)-3-hydroxy-β-cyclogeraniol (**10**) as a pale yellow oil, in 18% overall yield. $[\alpha]_D^{25}$ $-82°$ ($CHCl_3$) Lit. [17] $[\alpha]_D^{25}$ $-83.5°$ ($CHCl_3$).

(3R)-3-Hydroxy-*β-ionone*. The aldehyde (**10**) was dissolved in excess Me_2CO and a 20% aq. ethanolic solution of NaOMe was added dropwise over 30 min. After stirring overnight the Me_2CO was removed under vacuum and the residue extracted with Et_2O. The Et_2O extract was washed with H_2O and extracted with Et_2O. Removal of the Et_2O gave a brown oil which after purification by flash chromatography gave **11** as a waxy solid $[\alpha]_D^{25}$ $-83°$ (dioxan), lit. $[\alpha]_D^{25}$ $-85°$ (dioxan).

Epoxidation of 3(R)-3-*hydroxy-β-ionone*. Compound **11** was dissolved in CH_2Cl_2 and stirred rapidly in with 0.5 M $NaHCO_3$ while *m*-chloroperbenzoic acid was added over 2 hr. The mixture was stirred at room temp. for an additional 2 hr. After removal of the $NaHCO_3$ layer the solution was washed twice with 0.2 M KOH followed by H_2O and dried over $MgSO_4$. Removal of the CH_2Cl_2 gave a pale yellow oil which showed two peaks, in a 3:1 ratio, by HPLC analysis on system D (eluate monitored at 235 nm). Flash chromatography on silica gel (Keiselgel 60, Merck) using Et_2O as eluant resulted in the isolation of 10% of the sample as the pure minor component $[\alpha]_D^{25}$ $-96.5°$ (dioxane) and 25% as the pure major component $[\alpha]_D^{25}$ $+18.6°$ (dioxane). From the known stereochemistry of the reaction and by analogy with the epoxidation of (2*Z*,4*E*)-5*R*-4-hydroxy-2,6,6-trimethyl-1-cyclohex-1-enyl)-3-methyl-2,4-pentadienoic acid methyl ester [18], it was concluded that the minor component was the 5*S*,6*R*-epoxide (**12**) and the major component the 5R,6S-epoxide (**13**).

(2E,4E)-5-[(1S,2R,4S)-1,2-*Epoxy*-4-*hydroxy*-2,6,6-*trimethyl cyclohexyl*]-3-*methyl*-2,4-*pentadienoic acid methyl ester* (t-*xanthoxin acid methyl ester* (**14**). [1,2-^{13}C]Bromoacetic acid was methylated with CH_2N_2 in Et_2O. The methyl ester was converted into trimethyl [1, 2-^{13}C]phosphonoacetate as described in ref. [35]. The phosphonoacetate was dissolved in dry THF and added to a slurry of NaH in dry THF under N_2. After stirring for 10 min the 5*S*,6*R*-epoxide (**12**) was added in dry THF and the mixture stirred at room temp. for 48 hr. GLC analysis revealed 60% conversion to the 2*E*,4*E*-ester. No detectable quantity of the required 2*Z*,4*E* compound was formed. The reaction mixture was quenched with H_2O and extracted $\times 3$ with EtOAc. After washing with H_2O and drying, the EtOAc was removed under vacuum. The residue was subjected to PLC on silica gel developed in Et_2O. Elution of the major UV absorbing band at R_f 0.36 gave **14** which was pure on GLC analysis. $[\alpha]_D^{25}$ $-38°$ ($CHCl_3$). Proof that this compound was the 2*E*,4*E* isomer was furnished by the ^{1}H NMR spectrum which showed the doublet from H-3 at δ5.8. The PLC purified material was dissolved in EtOAc, purged with N_2 and exposed to sunlight on a windowsill for 2 weeks. After this time GLC analysis revealed the presence of a 1:1 mixture of the 2*Z*,4*E* and 2*E*,4*E* isomers. The isomers were separated by PLC using 4 developments in solvent A. The 2*Z*,4*E* isomer, $[\alpha]_D^{25}$ $-40.5°$ ($CHCl_3$) lit. $[\alpha]_D^{25}$ $-42.2°$ ($CHCl_3$), showed the expected downfield shift of the H-3 doublet to δ7.7 in the ^{1}H NMR spectrum.

[1,2-^{13}C](**14**). EIMS (GC-MS), *m/z* (rel. int.): 282 (40), 250 (32), 222 (20), 208 (20), 181 (35), 153 (22), 149 (35), 136 (30), 123 (100), 120 (40), 106 (19), 94 (32), 69 (19), 43 (41).

[1,2-^{13}C](**15**). EIMS (GC-MS), *m/z* (rel. int.): 282 (22), 250 (23), 222 (18), 209 (20), 181 (38), 163 (25), 149 (35), 136 (30), 123 (100), 120 (40), 106 (19), 94 (32), 42 (51).

[1,2-^{13}C] *Xan and t-Xan*. The individual isomers **15** and **14** were reduced with $LiAlH_4$ to the alcohols **17** and **16** which were oxidized with MnO_2 in $CHCl_3$ to Xan and *t*-Xan. These were purified by HPLC on system B.

[1,2-^{13}C]-Xan (**19**). EIMS (GC-MS), *m/z* (rel. int.): 252 (18), 234 (5), 222 (3), 210 (4), 193 (5), 177 (5), 175 (7), 168 (31), 153 (28), 151 (40), 137 (16), 135 (20), 123 (34), 109 (25), 97 (42), 84 (39), 69 (18), 55 (20), 43 (100).

[1,2-^{13}C]-*t*-Xan (**18**). EIMS (GC-MS), *m/z* (rel. int.): 252 (24), 237 (3), 234 (4), 222 (5), 210 (3), 193 (8), 191 (10), 177 (5), 168 (40), 153 (42), 151 (61), 137 (25), 135 (23), 123 (48), 109 (32), 97 (48), 84 (45), 69 (21), 55 (29), 43 (100).

Extraction and purification of Xan. The basic extraction procedure was as described previously [8]. Frozen tissue samples (5–10 g) were homogenized in MeOH containing one of the antioxidants DDC (200 mg/l) or TBHQ (80 mg/l). During and prior to homogenization the extraction solvent was purged with Ar. A known amount of [1,2-^{13}C]Xan or *t*-Xan was added to the extract which was then filtered. The residue was re-extracted twice, filtered, and the combined filtrates reduced to dryness under vacuum at 30°. The extract was then dissolved in dist. H_2O (pH 7.0) and partitioned with EtOAc (3×25 ml). After drying over $MgSO_4$ and filtering, the EtOAc was reduced to dryness. The extract was dissolved in 1–3 ml of 20% MeOH containing dist. H_2O, filtered and fractionated by reverse phase HPLC using system A. The fraction corresponding to the R_t of Xan and *t*-Xan (19–20 min) was collected and reduced to dryness. Xan and *t*-Xan were further purified by adsorption HPLC using system B. Xan and *t*-Xan eluted between 3.5 and 5 min and were collected together and reduced to dryness. These procedures were performed with the minimum exposure to light and elevated temps. Quantification was carried out by GC-MS using selected ion monitoring (SIM) of the molecular ions of Xan and *t*-Xan (*m/z* 250) and [^{13}C]Xan and *t*-Xan (*m/z* 252). SIM calibration curves, using 5 ng of [^{13}C]Xan or *t*-Xan and varying amounts of [^{12}C]Xan or *t*-Xan, were constructed as described previously for MeABA [15]. A comparison of gradients and intercepts revealed no statistical difference between the curves for Xan and *t*-Xan. This was expected as both isomers were derived from the same synthesis and had identical mass spectra. A single calibration curve for use with both isomers was obtained by combining the original data and this had an equation of: $y=0.08+0.99x$, where y is the peak area ratio and x is the mole ratio injected. Amounts of Xan as low as 1–5 ng could routinely be measured using this procedure.

Chromatography. HPLC systems: A ODS-Spherisorb (150 $\times$ 10 mm i.d.), eluted with a linear gradient of 20–100% MeOH in dist. H_2O over 40 min, 5 ml/min; B Spherisorb (250×4.5 mm i.d.), eluted with $CHCl_3$–MeCN (9:1), 2 ml/min; C Spherisorb (250×4.5 mm i.d.) eluted with $CHCl_3$–MeCN (18:1), 2 ml/min. UV detector set at 280 nm in all cases. D Partisil (250×4.5 mm i.d.) eluted with Et_2O, UV detector set at 235 nm.

PLC: Merck $200 \times 200 \times 2$ mm plates, solvent A CH_2Cl_2:MeCN (10:1.5).

GC-MS. GC: Mega-2 (Carlo Erba Instruments, Crawley, U.K.) OV-bonded-phase column (15 m $\times$ 0.32 mm i.d.; 0.45 μm film thickness) with a temperature programme of 35–150° (ballistic)-210° (at 8°/min), He at 20 ml/min, linked to MS via single stage jet separator at 230°. MS: Resolution >600, ionising voltage 70 eV, source 180°. SIM was performed under computer

control with a dwell time of 140 msec, a settling time of 100 msec and a 50 μsec sample period.

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