Food Chemistry 17 (1985) 143–154

Studies on Flavonol Degradation by Peroxidase (Donor: H_2O_2-oxidoreductase, EC 1.11.1.7): Part 1—Kaempferol

Ernst Miller & Peter Schreier

Lehrstuhl für Lebensmittelchemie, Universität Würzburg,
Am Hubland, D-8700 Würzburg, West Germany

(Received: 11 December, 1984)

ABSTRACT

The peroxidatic degradation of kaempferol was studied at pH 5·5 under UV-spectrophotometric control. The reaction products obtained after incubation of kaempferol (dissolved in ethylene glycol monomethyl ether) with peroxidase (Boehringer, Mannheim)/H_2O_2 were fractionated in preparative scale by liquid chromatography on Sephadex LH-20. About twenty compounds were detected by analytical HPLC, from which four substances could be characterized by means of their spectroscopic data: 2,4,6-trihydroxybenzoic acid, 4-hydroxybenzoic acid, 2,2-dihydroxy-1(2,4,6-trihydroxyphenyl)-3(4-hydroxyphenyl)-1,3-propandione and 2-(4-methoxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one (acacetin).

INTRODUCTION

In the past, the importance of the enzyme peroxidase (donor: H_2O_2-oxidoreductase, EC 1.11.1.7) for the enzymic metabolism of various food constituents has been often noticed (Burnette, 1977). Thus, the enzyme is regarded as involved in biochemical pathways, playing a rôle in the course of so-called 'fermentations', e.g. during the processing of tea, tobacco, coffee or cocoa.

Due to the complexity of possible peroxidatic reactions in a heterogeneous matrix, such as food, the study of the metabolism of

Food Chemistry 0308-8146/85/$03·30

individual components and the identification of their enzymic reaction products is not possible. Therefore, model systems with defined substrates and purified enzymes are usually preferred in order to obtain a first insight of the reaction mechanisms which might occur in such complex systems. We have studied the peroxidatic degradation of flavonols in model systems. In this paper the results obtained with kaempferol as substrate are reported.

MATERIAL AND METHODS

Materials

Kaempferol was from Roth, Karlsruhe, and peroxidase (purity grade II) from Boehringer (Mannheim).

Standard incubations

UV-spectrophotometric assay
Fifty microlitres of kaempferol solution (2 mM/litre in ethylene glycol monomethyl ether) and 50 μl of aqueous H_2O_2 solution (4 mM/litre) were added to 1·85 ml 0·1 M citrate–phosphate buffer (pH 5·5). The reaction was started using 50 μl of peroxidase solution (1 mg/ml in citrate–phosphate buffer, pH 5·5). The reaction was photometrically followed (220–400 nm; 20 °C) for at least 20 min. Reference measurements were performed without kaempferol addition.

Large-scale incubations
Twenty-five millilitres of an aqueous H_2O_2 solution (100 mM/litre) and 10 ml of a peroxidase solution (0·2 mg/ml) were added to 500 ml 0·1 M citrate–phosphate buffer (pH 5·5). The reaction was started with 50 ml of kaempferol solution (20 mM/litre in ethylene glycol monomethyl ether) at 20 °C. After 3 min the reaction was stopped by the addition of 100 mg NaN_3. After acidification with HCl (5%) to pH 2·0 the mixture was immediately extracted with diethyl ether (5 × 100 ml). The extracts were combined, dried over Na_2SO_4, and carefully concentrated using a Vigreux column.

TABLE 1
Non-Linear Gradient' Used for Analytical HPLC Separation of Reaction Products from Peroxidatic Kaempferol Degradation

Time (min)	*Methanol (Per cent in the eluent)*
0	0
1	40
10	50
13	55
16	62
18	67
21	73
23	80
25	90
27	100

Liquid chromatographic separations

Analytical HPLC
A Kontron LC 720 apparatus equipped with a 5 × 100 mm Spheri-5 column (RP-18, 5 μ) and an Uvikon 720 detector (Kontron) were used. Measurements were carried out at 295 nm employing a non-linear gradient of acetic acid (5%)/methanol (Table 1). The flow rate was 1 ml/min and 20 μl injections were used.

Liquid chromatography on Sephadex LH-20 (cf. *Fig. 3*)
The concentrated diethyl ether extract (0·2 ml) of reaction products of peroxidatic kaempferol degradation was added onto a Sephadex LH-20 column (1·5 × 60 cm) and, in the first step, fractionated into 41 fractions (1 ml) eluting with methanol (flow rate, 30 ml/h). Rechromatography of combined (and concentrated) fractions 13–18 and 19–29 resulted in the separation of K1, K2 and K3, respectively. From combined and concentrated fractions 21–28 eluates 42–55 were isolated by re-chromatography which were again separated on Sephadex LH-20, yielding K4. As shown in Fig. 3, additional compounds (K5-K8) were isolated, but could not be sufficiently characterized. At each step, the individual eluates were checked by analytical HPLC.

Identification of reaction products

UV-spectroscopy
UV-spectrophotometric measurements were carried out using a Zeiss PMQ II spectrophotometer and different solvents, as well as shift reagents, as described by Markham (1982).

IR-spectroscopy
IR spectroscopic measurements were performed in KBr, employing a Beckman IR 4240 IR spectrophotometer (400–4000 cm^{-1}).

Mass spectrometry
EI mass spectra were recorded using a Finnigan-MAT 44 mass spectrometer at 70 eV. The temperature of the ion source was 250 °C.

^{1}H-NMR spectroscopy
400 MHz ^{1}H-NMR spectra were measured at 20 °C using a Bruker Cryospec WM 400 apparatus, acetone-d_6 as solvent and TMS as internal standard.

^{13}C-NMR spectroscopy
The measurements of ^{13}C-NMR spectra were performed at 20 °C using a Bruker Cryospec WM 400 apparatus, acetone-d_6 as solvent and TMS as internal standard.

Analytical data of isolated reaction products

K1: 2,4,6-Trihydroxybenzoic acid. $C_7H_6O_5$ (170). EI-MS (43-68-125-52-51-69-126-123).

K2: 4-Hydroxybenzoic acid. $C_7H_6O_3$(138). EI-MS (121-138-54-93-63-62-122).

K3: 2,2-Dihydroxy-1-(2,4,6-trihydroxyphenyl)-3-(4-hydroxyphenyl)-1,3-propandione. UV (CH_3OH): λ_{max}, 295 nm (log $\varepsilon = 3{\cdot}85$). IR (KBr): 3500-3000, 1700, 1640, 1600 cm^{-1}. ^{1}H-NMR (400 MHz, acetone-d_6, TMS): $\delta = 6{\cdot}12$ (s, H-6/H-8); 6·95 ($J = 9{\cdot}0$ Hz; H-3′/H-5′); 8·03 ($J = 9{\cdot}0$ Hz; H-2′/H-6′) ppm. ^{13}C-NMR (acetone-d_6, TMS): $\delta = 190$ (C-2); 92 (C-3); 192 (C-4); 159 (C-5); 98 (C-6); 170 (C-7); 98 (C-8); 159 (C-9); 103 (C-10); 126 (C-1′); 133 (C-2′); 116 (C-3′); 163 (C-4′); 116 (C-5′); 133 (C-6′) ppm. EI-MS (121-138-120-122-137-152). $C_{15}H_{12}O_8$ (320). Determined: C, 56·2; H, 3·75; O, 40·0. Found: C, 55·5; H, 4·1; 0, 40·8.

K4: 2-(4-Methoxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one (acacetin). UV (CH_3OH): λ_{max} 270 nm (log $\varepsilon = 4{\cdot}22$), 305 nm (log $\varepsilon = 4{\cdot}20$), 330 nm (log $\varepsilon = 4{\cdot}12$). IR (Mabry *et al.*, 1970). ^{1}H-NMR (400 MHz, acetone-d_6, TMS): $\delta = 6{\cdot}36$ (H-3); 6·27 ($J = 2{\cdot}5$ Hz; H-6); 6·56 ($J = 2{\cdot}5$ Hz; H-8); 8·05 ($J = 9{\cdot}0$ Hz; H-2′/H-6′); 7·15 ($J = 9{\cdot}0$ Hz; H-3′/H-5′); 3·92 (s, —OCH_3) ppm (Geissman & Irwin, 1972). EI-MS (283-284-132-240-241). $C_{16}H_{12}O_5$ (284).

RESULTS

UV-spectrophotometric control of peroxidatic reaction

After incubation of kaempferol with H_2O_2 and peroxidase at pH 5·5 the flavonol degradation was measured by the decrease of UV absorption. The spectral changes observed are outlined in Fig. 1. The characteristic absorptions of kaempferol at 260 and 370 nm rapidly decreased and a pronounced new UV maximum at 300 nm was detected.

Additional UV-spectrophotometric assays showed that the peroxidatic reaction occurred either under aerobic or anaerobic conditions. Furthermore, quantitative iodometric determinations revealed that molar amounts of H_2O_2 were consumed during the reaction.

Preparative isolation of reaction products

Preparative experiments were carried out with kaempferol/H_2O_2/peroxidase (dissolved in ethylene glycol monomethyl ether) at pH 5·5.

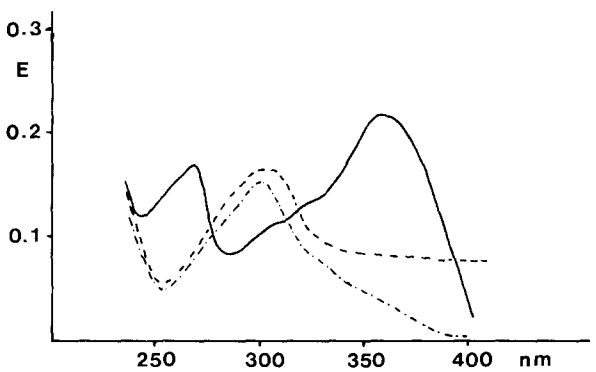

Fig. 1. UV spectrum of kaempferol (——) and newly formed UV maximum after peroxidatic degradation (– – –, after 3 min; – · –, after 60 min.).

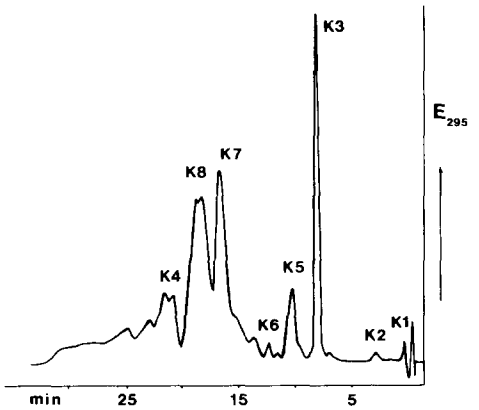

Fig. 2. HPLC separation (Spheri-5 column, 5 μ) of reaction products (K1-K8) extracted by diethyl ether from kaempferol/H_2O_2/peroxidase. Kontron LC 720 with Uvikon 720. Non-linear gradient (acetic acid (5%)/methanol, *cf.* Table 1). Flow rate, 1 ml/min. Detection, 295 nm. Injection, 20 μl.

Quantitative reaction within 3 min was achieved up to a substrate concentration of 3 mM/litre. Thus, several batches had to be used in order to obtain sufficient amounts of reaction products for their subsequent spectroscopic characterization.

First of all, the reaction products extracted by diethyl ether were analyzed by analytical HPLC using RP-18 (Pryde & Gilbert, 1979). With this technique, about twenty reaction products could be detected (Fig. 2).

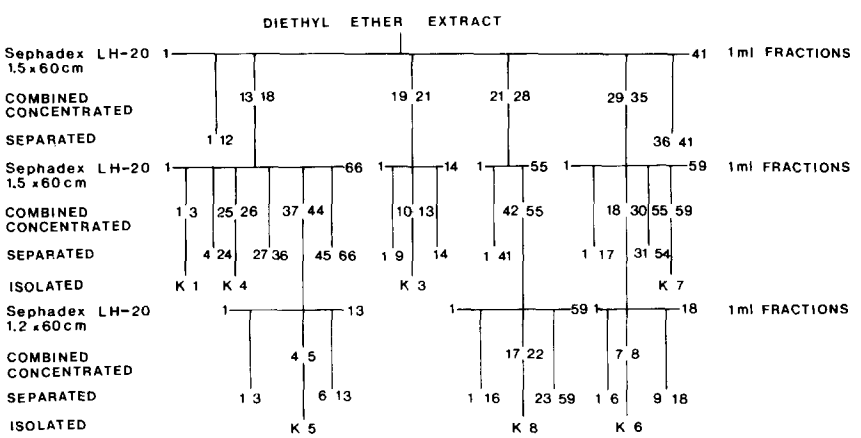

Fig. 3. Scheme of LC separation steps on Sephadex-LH 20 used for isolation of preparative amounts of reaction products from kaempferol/H_2O_2/peroxidase.

No flavonol degradation was observed in parallel assays carried out without peroxidase.

Attempts made (in the follow up) to use HPLC on a preparative scale failed due to strong loss of resolution. Therefore, another technique was sought in order to fractionate the mixture of reaction products. While first studies with cellulose and kieselgel as adsorbents were not successful, a separation of eight components (K1-K8) could be achieved by liquid chromatography on Sephadex LH-20. The separation scheme developed is outlined in Fig. 3. The purity of K1-K8 was checked by analytical HPLC; in each case, only one peak was detected. From these components, four substances could be identified by means of their spectroscopic data.

Characterization of reaction products

K1: About 0·5 mg of K1 was obtained. It was identified as 2,4,6-trihydroxybenzoic acid comparing its gas chromatographic retention (as methyl ester) and mass spectral data with those of an authentic reference compound.

K2: Only small amounts (< 0·5 mg) of K2 were isolated and it was identified as 4-hydroxybenzoic acid by comparing its gas chromatographic retention (as methyl ester) and mass spectral data with those of an authentic reference sample.

K3: This was one of the major components among the reaction products (Fig. 2); about 20 mg of pure K3 could be isolated. The distinct UV maximum at 295 nm indicated the lack of conjugation between the rings. Identical values found in the UV spectrum measured in methanol and after addition of H_3BO_3/NaOAc (Table 2) excluded an *o*-dihydroxyl structure in the A- or B-ring (Markham, 1982). The '$AlCl_3$' spectra recorded in acidic and neutral medium (Table 2) indicated a stable

TABLE 2
Changes of UV Maxima (nm) of K3 after Addition of Shift Reagents (Markham, 1982)

	CH_3OH	*$NaOCH_3$*	*NaOAc*	*H_3BO_3*	*$AlCl_3$*	*$AlCl_3$/HCl*
K3	295	335	335	295	320 380	315 380

TABLE 3
^{1}H-NMR Shifts (ppm; TMS as Internal Standard) of B-Ring Protons of Several C-4′-Hydroxylated Flavonoids (Mabry *et al.*, 1970) Compared with Those Recorded from K3

	H-3′/H-5′	*H-2′/H-6′*
Kaempferol	6·85	7·95
Apigenin	6·8	7·7
Dihydrokaempferol	6·8	7·25
Naringenin	6·7	7·2
K3	6·95	8·05

complex, i.e. the occurrence of a free C-5-OH— and unchanged carbonyl function in position C-4 (Markham, 1982).

The ^{1}H-NMR spectrum (Fig. 4, top) indicated a high-symmetric 5,7,9-trihydroxy structure of the A-ring (6·12 s; H-6/H-8). The B-ring protons appeared as two *ortho*-doublets at 6·95 ppm ($J = 9·0$ Hz; H-3′/H-5′) and 8·05 ppm ($J = 9·0$ Hz; H-2′/H-6′). From the H-NMR spectrum measured after the addition of D_2O (Fig. 4, bottom) it was obvious that the multiplets at 9·5, 3·9 and 3·0 ppm corresponded to free OH-protons (three of them at C-5, C-7 and C-4′). From the downfield position of B-ring protons, in particular, H-2′/H-6′, the remaining OH-functions were assumed to be attached at C-3. The data summarized in Table 3 demonstrate that the olefinic C-2/C-3 double bond (*cf.* apigenin) resonates at 7·7 ppm (H-2′/H-6′) and that, after the introduction of an OH-function at C-3, 7·95 ppm results for H-2′/H-6′. Considering these results an enediol or carbonyl function at C-2 might be suggested.

The occurrence of a carbonyl group at C-2 was confirmed by ^{13}C-NMR analysis. In Table 4 the ^{13}C-NMR shifts of several C-4′-hydroxylated flavonoids (Harborne & Mabry, 1982) are compared with those measured from K3.

In the EI-MS spectrum (70 eV) a M^+-peak could not be detected; *m/e* 121 and 138 were found with highest intensities indicating well-known fragments of A- and B-rings (Markham, 1982).

From the above-mentioned spectroscopic data the structure of K3 was assigned as 2,2-dihydroxy-1(2,4,6-trihydroxyphenyl)-3(4-hydroxyphenyl)-1,3-propandione.

K4: Two milligrams of this product could be isolated. It was identified

TABLE 4

^{13}C-NMR Shifts (ppm; TMS as Internal Standard) of Several C-4′-Hydroxylated Flavonoids (Harborne & Mabry, 1982) Compared with Those Recorded from K3

	C-2	*C-3*	*C-4*	*C-5*	*C-6*	*C-7*	*C-8*	*C-9*	*C-10*	*C-1′*	*C-2′*	*C-3′*	*C-4′*	*C-5′*	*C-6′*
Kaempferol	146·8	135·6	175·9	160·7	96·2	163·9	93·5	156·2	103·0	121·7	129·5	115·4	159·2	115·4	129·4
Apigenin	164·1	102·8	181·8	161·5	98·8	162·7	94·0	157·3	103·7	121·3	128·4	116·0	161·1	116·0	128.4
Naringenin	78·4	42·0	196·2	163·6	95·9	166·7	95·0	162·9	101·8	128·9	128·2	115·2	157·8	115·2	128·2
K3	190	92	192	159	98	170	98	159	103	126	133	116	163	116	133

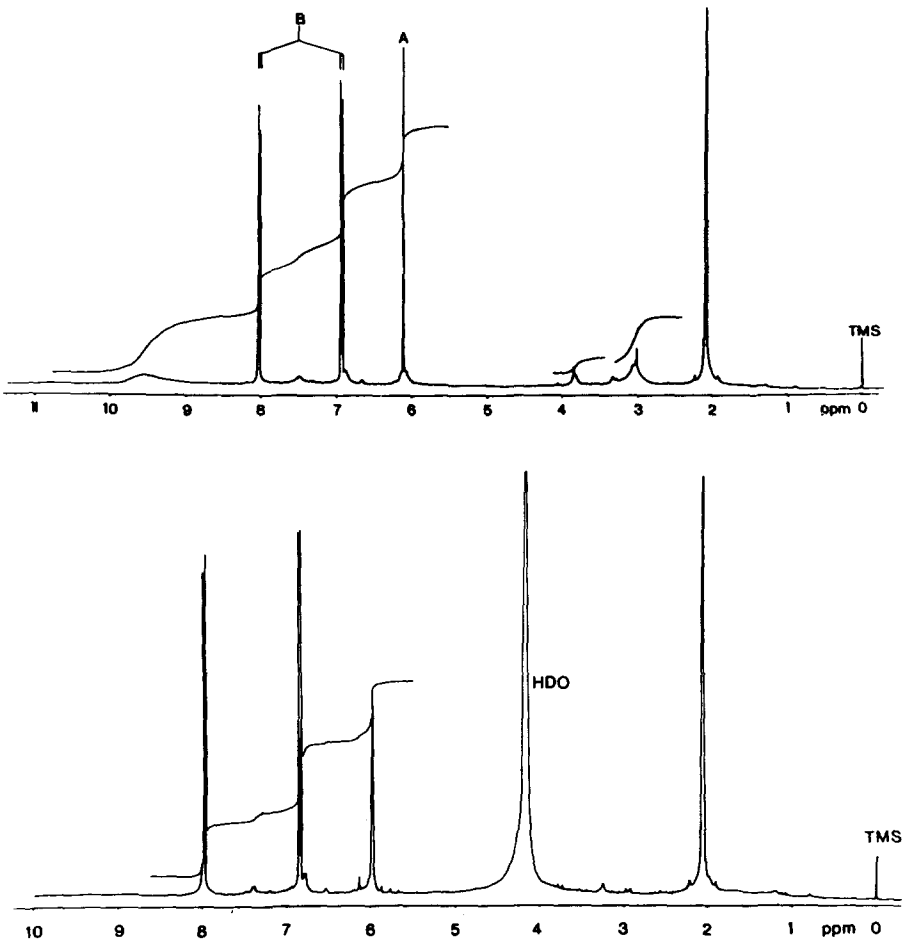

Fig. 4. Top: 400 MHz ^{1}H-NMR spectrum (acetone-d_6, TMS) of K3. Bottom: after addition of D_2O.

as 2(4-methoxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one (acacetin) comparing its spectroscopic data (UV, IR, ^{1}H-NMR, MS) with values taken from the literature (Mabry *et al.*, 1970; Geissman & Irwin, 1972).

DISCUSSION

Studies in several laboratories have demonstrated that phenolic acids (Berlin *et al.*, 1971), flavonols, chalcones, flavanones and aurones (Barz,

1975; 1981) are metabolically active compounds which can be subject to peroxidatic degradation (and polymerization). Using flavonols with free hydroxyl functions at C-3- and C-4′-positions, Frey-Schröder & Barz (1979) observed a peroxidatic degradation of these substances to 2,3-dihydroxy-flavanones, already regarded as primary reaction products of peroxidatic flavonol degradation (Hösel *et al.*, 1972; 1975). Feeding experiments with flavonols and other flavonoids in plant cell cultures revealed that the B-rings became liberated as substituted benzoic acids (Hösel *et al.*, 1972; Berlin *et al.*, 1974). Similar investigations with [ring-A-^{14}C] flavanones further demonstrated that resorcinol and phloroglucinol rings were efficiently degraded, as indicated by $^{14}CO_2$ formation (Berlin *et al.*, 1974). Extensive studies of the peroxidatic degradation of flavanones by Patzlaff & Barz (1978) resulted in the formulation of main degradation pathways; (i) cleavage reactions of the heterocyclic ring yielded phenolic catabolites from ring A and (ii) oxidative destruction of ring A led to C-6/C-3, C-6/C-2 and C-6/C-1 units from ring B. Among the latter substances, 5,7-dihydroxychromanone, 4-hydroxycinnamoic acid, 4-hydroxyphenyl-2-hydroxypropanoic acid and eriodyctiol were identified as major metabolites.

From these results it is obvious that cleavage of the C-ring is one of the most important steps in the peroxidatic degradation of flavonoids. The results obtained in our study with kaempferol as substrate confirm the formation of substituted benzoic acids and enlarge our knowledge characterizing the structure of a newly described intermediate, K3. A similarly structured hydroxypropantrione derivative has been identified previously in studies on HIO_4 oxidation of unsubstituted flavonols (Smith, 1963; Smith *et al.*, 1965). Considering the stable hydrate of 1,3-diphenylpropantrione, the authors pointed out the stability of the substance.

As to acacetin; first of all, the occurrence of a methoxy function may be striking, but, during our study, we remarked that the addition of ethylene glycol monomethyl ether (necessary due to the low solubility of kaempferol) led to a series of side reactions. Thus, diethylenedi- and tetraethylene glycol dimethylether were identified among the products of peroxidatic reaction. This oligomerization of ethylene glycol monomethyl ether did not occur in experiments without kaempferol addition. Methoxylation caused by methanol used during sample preparation could be excluded; before and after the addition of methanol the same analytical HPLC separations were observed.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft. We thank Dr D. Scheutzow, Institut für Organische Chemie, for recording the 400 MHz NMR spectra and Priv. Doz. Dr A. Mosandl for helpful interpretations of the spectra.

REFERENCES

Barz, W. (1975). Abbau von Flavonoiden und Isoflavonoiden—ein Überblick. *Ber. Dtsch. Bot. Ges.* **88**, 71–81.

Barz, W. (1981). Degradation of flavanoids and isoflavanoids. In: *Pigmentation of plants*. (2nd edn) (Czygan, F. (Ed)), Springer, Berlin, Heidelberg, New York, 210–23.

Berlin, J., Barz, W., Harms, H. & Haider, K. (1971). Degradation of phenolic compounds in plant cell cultures. *FEBS Lett.* **16**, 141–6.

Berlin, J., Kiss, P., Müller-Enoch, D., Gierse, D., Barz, W. & Janistyn, B. (1974). Über den Abbau von Chalkonen und Isoflavonen in pflanzlichen Zellsuspensionskulturen. *Z. Naturforsch.* **29c**, 374–83.

Burnette, F. S. (1977). Peroxidase and its relationship to food flavour and quality: A review. *J. Food Sci.* **42**, 1–7.

Frey-Schröder, G. & Barz, W. (1979). Isolation and characterization of flavonol converting enzymes from *Mentha piperita* plants and from *Mentha arvensis* cell suspension cultures. *Z. Naturforsch.* **34c**, 200–9.

Geissman, T. A. & Irwin, M. A. (1972). Methoxylated flavonoids from *Artemisia*. *Phytochemistry* **11**, 3509–11.

Harborne, J. B. & Mabry, T. J. (1982). *The flavonoids. Advances in research*. Chapman & Hall, London, New York.

Hösel, W., Shaw, P. & Barz, W. (1972). Über den Abbau von Flavonoiden in pflanzlichen Zellsuspensionskulturen. *Z. Naturforsch.* **27b**, 946–54.

Hösel, W., Frey, G. & Barz, W. (1975). Über den Abbau von Flavonolen durch pflanzliche Peroxidasen. *Phytochemistry* **14**, 417–22.

Mabry, T. J., Markham, K. R. & Thomas, M. B. (1970). *The systematic identification of flavonoids*, Springer, Berlin, Heidelberg, New York.

Markham, K. R. (1982). *Techniques of flavonoid identification*, Academic Press, London, New York.

Patzlaff, M. & Barz, W. (1978). Peroxidatic degradation of flavanones. *Z. Naturforsch.* **33c**, 675–84.

Pryde, A. & Gilbert, M. T. (1979). *Applications of high performance liquid chromatography*, Chapman & Hall, London.

Smith, M. A. (1963). The oxidation of flavonols by periodic acid. *J. Org. Chem.* **28**, 933–7.

Smith, M. A., Webb, B. A. & Cline, L. J. (1965). The oxidation of flavonols by periodic acid in methanol. *J. Org. Chem.* **30**, 995–7.