Simultaneous Enzyme Catalysis Extraction: A Versatile Technique for the Study of Flavor Precursors

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The identification of glycosidically bound volatiles from apple fruit (*Malus sylvestris* Mill cult. Jonathan) was achieved by isolation of a glycosidic extract by Amberlite XAD-2 adsorption and methanol elution followed by enzymatic hydrolysis with emulsin. Capillary gas chromatography and combined capillary gas chromatography–mass spectrometry revealed for the first time the occurrence of the following aglycons: 1-butanol, 2-methyl-1-butanol, 1-hexanol, 2-methylbutanoic acid, benzyl alcohol, 1-octanol, 2-phenylethanol, 1,3-oct-5(*Z*)-enediol, 1,3-octanediol, 4-vinylguaiacol, eugenol, 4-hydroxyphenylacetic acid, 3-hydroxy-β-damascone, 4-hydroxy-3-methoxyphenylacetic acid, 3-oxo-α-ionol, and vomifoliol. The concentrations of the liberated aglycons were found to change over time when the glycosides were subjected to enzymatic hydrolysis. Thus, the attractive C_{13} norisoprenoids were isolated in later hydrolysis steps in large amounts, although they occurred only in traces in the first hydrolysate. An apparatus was developed allowing continuously the simultaneous enzyme catalysis and extraction (SECE). Mathematical calculations of the enzymatic cleavage of glycosides were performed.

Recent interest in flavor research is increasingly devoted to the analysis of flavor precursors and intermediates (Schreier, 1988). In the last few years attention was focused on the enzymatic formation of plant volatiles including their water-soluble derivatives such as polyols, diphosphates, and glycosides (Braell et al., 1986; Schreier and Winterhalter, 1986; Croteau, 1986; Strauss et al., 1986). The identification of glycosidically bound volatiles is commonly achieved by isolation of a glycosidic extract from the natural material using different methods (Williams et al., 1982) followed by subsequent enzymatic treatment with a glycosidase. In most cases the commercial preparations emulsin (almond β-glucosidase EC 3.2.1.21) or Rohapect C (pectinase) are employed to yield the volatile aglycons.

In this paper it is demonstrated that the concentration ratios of the aglycons liberated from a glycosidic extract changed, after the enzymatic hydrolysis was repeated over a period of time. Thus, some aglycons could be identified in later hydrolysis steps, although these occurred only in trace concentrations in the first hydrolysate. An apparatus was developed allowing continuously the simultaneous enzymatic catalysis and extraction (SECE) of the liberated aglycons. The suitability of the system is discussed by means of glycosidically bound volatiles from apple fruit.

EXPERIMENTAL SECTION

Fruits. Fresh, ripe apple fruits (*Malus sylvestris* Mill cult. Jonathan) were obtained from the local market.

XAD Conditioning. Amberlite XAD-2 (100 g, SERVA particle diameter 1.0–3.0 mm) was successively extracted for 24 h in a Soxhlet apparatus with 250 mL of pentane, 250 mL of ethyl acetate, and 250 mL of methanol. The last slurry was filled into a chromatographic column (100 × 2.5 cm) conditioned by passing 2.0 L of distilled water (20 mL/min).

Isolation of Glycosides by the XAD Method (Gunata et al., 1985). After removing the seeds of 1.2 kg of apples and cutting into small pieces, the fruits were submerged in 1 L of 0.2 M phosphate buffer (pH 7.4; containing 0.2 M glucono-δ-lactone as glycosidase inhibitor and 5.0 mg of phenyl β-D-glucopyranoside as internal standard), homogenized with a Braun blender for 30 s and centrifuged (4000*g*; 0 °C). The supernatant was passed through the conditioned XAD column. After the column was washed with 1.5 L of distilled water, 0.5 L of pentane, and 0.75 L of ethyl acetate, the glycosidic extract was obtained by eluting with 500 mL of methanol.

Cleanup of the Glycosidic Extract. The methanolic extract was concentrated in vacuo followed by dissolving the residue in 100 mL of 0.2 M phosphate buffer (pH 5) and extracting continuously with 100 mL of diethyl ether in a liquid–liquid extractor to separate the volatiles.

Enzymatic Hydrolysis with Emulsin. The purified glycosidic extract was removed from the diethyl ether by decanting the organic layer. Ten milligrams of emulsin (Boehringer) was added to the aqueous phase and the resultant fraction incubated at 40 °C for 24 h. Finally, the aglycons were isolated by continuously extracting the enzyme hydrolysate with 100 mL of diethyl ether. The obtained alcohols were identified in the organic phase by means of HRGC and HRGC–MS. To the remaining aqueous layer of this extraction was added 10 mg of emulsin again; this was incubated for 24 h at 40 °C and extracted. In addition, three enzymatic hydrolysis steps with emulsin were undertaken to identify the aglycons of a glycosidic apple extract by means of HRGC–MS.

Enzymatic Hydrolysis with Hesperidinase. In a fashion analogous to the enzymatic hydrolysis with emulsin, five sequential enzymatic hydrolysis steps of a cleaned glycosidic extract from apples were performed with 1000 mg of hesperidinase (Sigma) in each step. The aglycons were analyzed by means of HRGC and HRGC–MS. The enzymatic hydrolysis with hesperidinase was repeated with the SECE method (cf. Results and Discussion). After the SECE the enzyme activity was measured. It decreased to one-fourth of the activity at the beginning.

Capillary Gas Chromatography (HRGC). A Carlo-Erba Fractovap 4100 gas chromatograph with FID equipped with a J&W fused silica DB5-30W capillary column (30 m, 0.25 mm (i.d.), film thickness = 0.25 μm) was used. Split injection (1:50) was employed. The temperature program was 60–300 °C at 5 °C/min. The flow rates for the carrier gas were 2.0 mL/min He, for the makeup gas 30 mL/min N_2, and for the detector gases 30 mL/min H_2 and 300 mL/min air, respectively. The injector temperature was kept at 250 °C and the detector temperature at 300 °C. Volumes of 1.0 μL were injected.

Capillary Gas Chromatography–Mass Spectrometry (HRGC–MS). A Varian Aerograph 1440 gas chromatograph equipped with a Carlo-Erba water-cooled on-column injection system was coupled by an open-split

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Table I. Aglycons Identified in Apple Fruit by HRGC and HRGC-MS after Enzymatic Treatment (Emulsin) of a Methanolic Eluate Obtained from XAD-Separated Glycosidic Fraction

peak no.[a]	R_t[b]	compound
1	640	1-butanol
2	732	2-methyl-1-butanol
3	867/863	1-hexanol/2-methylbutanoic acid
4	984	phenol (from internal standard)
5	1038	benzyl alcohol
6	1075	1-octanol
7	1119	2-phenylethanol
8	1252	1,3-oct-5(*Z*)-enediol
9	1275	1,3-octanediol
10	1336	4-vinylguaiacol
11	1365	eugenol
12	1545	4-hydroxyphenylacetic acid
13	1624	3-hydroxy-β-damascone
14	1657	4-hydroxy-3-methoxyphenylacetic acid
15	1645	3-oxo-α-ionol
16	1800	vomifoliol

[a] The peak numbers correspond to the numbers in Figure 1. [b] HRGC conditions, see the Experimental Section.

interface to a Finnigan MAT 44 mass spectrometer with SS 200 data system. A J&W DB5-30W fused silica capillary column (30 m, 0.25 mm (i.d.), film thickness = 0.25 μm) connected to a 2-m uncoated piece of fused silica capillary column as the "retention gap" (Grob and Müller, 1982) was used. The conditions were as follows: temperature from 60 to 300 °C at 5 °C/min; carrier gas flow rate, 2.5 mL/min He; temperature of ion source and all connection parts, 200 °C; electron energy, 70 eV; cathodic current, 0.8 mA; injection volumes, 1.0 μL.

Results of qualitative analyses were verified by comparison of HRGC retention (R_t) and mass spectral data with those of authentic reference substances. Quantitative HRGC determinations were carried out on a Hewlett-Packard 3388 A laboratory data system.

RESULTS AND DISCUSSION

A glycosidic extract was isolated by the suggested XAD method (Gunata et al., 1985) at pH 7.4 from apple fruits (*M. sylvestris* Mill cult. Jonathan). The eluate obtained by treatment with methanol was evaporated in vacuo and dissolved in phosphate buffer (pH 5). Incubation with emulsin liberated the aglycons, which were isolated by solvent extraction and analyzed by HRGC and HRGC-MS. The substances listed in Table I (cf. Figure 1a) were identified. This enzymatic hydrolysis was repeated twice under the same conditions as before. The HRGC separations of the aglycons isolated after the second and third hydrolysis steps are shown in Figure 1b,1c. Surprisingly, only in the extracts obtained after the second and third enzymatic hydrolyses are the interesting C_{13} norisoprenoids, such as 3-hydroxy-β-damascone, 3-oxo-α-ionol, and vomifoliol, found in larger amounts (standard: phenol, from phenyl β-D-glucopyranoside).

Apparatus for the SECE. Because of the labourious and time-consuming repetitions of the enzymatic hydrolysis and sample preparation, an apparatus based on a publication of Bednarski et al. (1987) was developed for the continuous simultaneous enzyme catalysis and extraction of the samples. The system consists of a reaction tube (Figure 2, right) for the enzyme catalysis and a modified liquid–liquid extractor (Figure 2, left) for the solvent extraction of the products. For the reaction tube a thermostatically controllable chromatographic column (volume 200 mL, temperature 25 °C) is employed containing a dialysis tubing (SERVA Servapor, 2 × 45 cm; thickness of the membrane, 0.025 mm) filled with the

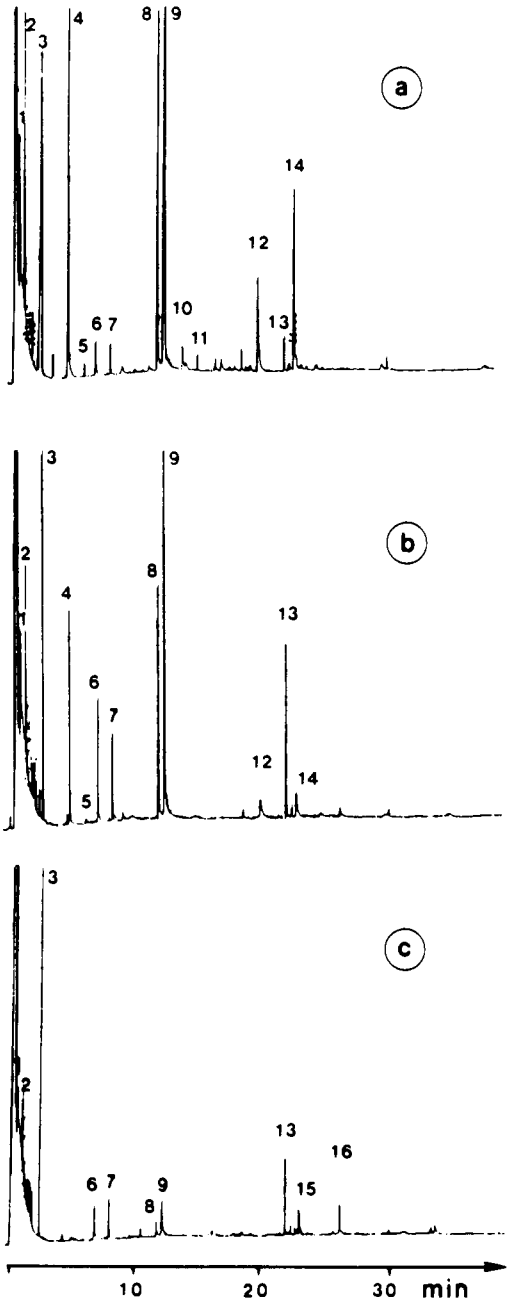

Figure 1. HRGC separations (J&W, 30 m × 0.259 mm (i.d.), fused silica WCOT DB-5 capillary column, df = 0.25 μm) of apple fruit aglycons obtained after repeated enzymatic treatments (emulsin) of a methanolic eluate from XAD-separated glycosidic fraction: a = first, b = second, c = third enzymatic hydrolysis. The peak numbers correspond to the numbers in Table I.

enzyme and the glycosidic extract both dissolved in a buffer optimized for the enzyme. Through the stopcock (Figure 2, right, T1) the buffer is pumped (flow rate 1 mL/min) into the reaction tube by means of a micropump. The substrates and the products of hydrolysis increasingly penetrate through the dialysis membrane into the buffer. The outer buffer runs off through the connection at the upper end of the column (Figure 2, T2). For the extraction of the liberated aglycons a modified liquid–liquid extractor (Figure 2, left) is used. The advantage of the additional two glass tubes connected to the liquid–liquid extractor (Figure 2, G1, G2) is the permanent change of the aqueous solution. Through the Teflon connection (Figure 2, left, T2) the buffer with the substrates and the products flows into the extractor where it immediately contacts the organic phase. The aglycons as the less polar compounds

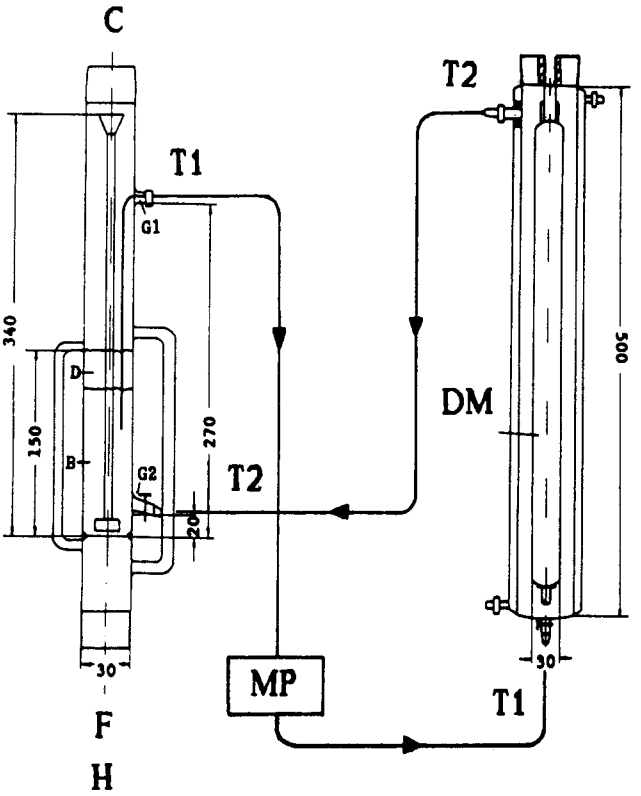

Figure 2. Apparatus for simultaneous enzyme catalysis extraction consisting of a modified liquid–liquid extractor (left) and a reaction tube (right). Key: C = condenser; F = flask; H = heater; T1, T2 = Teflon connections; G1, G2 = additional glass tubes; B = buffer; D = diethyl ether phase; DM = dialysis membrane; MP = micropump.

of the buffer enrich in the organic phase and flow back with the organic layer (Figure 2D) into the flask. The polar substances of the buffer solution (Figure 2B), like the carbohydrate residues and the nonhydrolyzed glycosides, flow back through the Teflon connection 1 (Figure 2, left, T1) into the reaction tube. There the nonhydrolyzed glycosides penetrate again the dialysis membrane and are hydrolyzed.

The essential advantage of this modified assembly is the fact that once the system is started, the flask with organic layer of the modified extractor can be exchanged without interrupting the continuous process. A further advantage is the small amount of enzyme used. For each above-mentioned enzymatic hydrolysis step a new portion of enzyme had to be added. This handicap is eliminated with the developed simultaneous system. Furthermore, the organic layer does not contact the enzyme solution. Emulsions often occurring during the single hydrolysis steps are no longer a problem. The organic solution of the aglycons is cleaner. Additionally, the system saves time, since enzymatic treatment and solvent extraction run simultaneously. Finally, by continuously removing the products from the equilibrium of reaction, the enzyme works more effectively. Thus, a higher amount of aglycons is available for identification and characterization.

Theoretical Mathematical Considerations. For the mathematical calculations of the enzymatic cleavage of glycosides two different glycosides both occurring in the same solution have to be considered. Their enzymatic hydrolysis are represented by the following equations:

$$Z_1\text{-O-}A_1 + H_2O \xrightarrow{K_1} Z_1\text{-OH} + A_1\text{-OH}$$

$$Z_2\text{-O-}A_2 + H_2O \xrightarrow{K_2} Z_2\text{-OH} + A_2\text{-OH}$$

Z_1-O-A_1 and Z_2-O-A_2 represent two glycosides, 1 and 2, that are split into their carbohydrate residues Z_1-OH and Z_2-OH and their aglycons A_1-OH and A_2-OH. K_1 and K_2 (k_1, k_2) are equilibrium constants. If we set Z_1-O-A_1 = Z_1A_1, Z_2-O-A_2 = Z_2A_2, Z_1-OH = Z_1, Z_2-OH = Z_2, A_1-OH = A_1, and A_2-OH = A_2, then the law of mass action of the hydrolysis can be written

$$K_1 = [Z_1][A_1]/[Z_1A_1][H_2O] \quad \text{(I)}$$

where $[H_2O]$ = constant (valid for aqueous solutions).

$$k_1 = [Z_1][A_1]/[Z_1A_1] \quad \text{(II)}$$

Analogously, for the second glycoside

$$k_2 = [Z_2][A_2]/[Z_2A_2] \quad \text{(III)}$$

Suppose glycoside 1 is to be hydrolyzed more effectively than glycoside 2 by a factor x:

$$x = k_1/k_2 \qquad x > 1 \quad \text{(IV)}$$

Additionally, the initial concentrations of the two glycosides are assumed to be equal:

$$[Z_1A_1] = [Z_2A_2] = [ZA] \quad \text{(V)}$$

As a third prerequisite it is supposed that the carbohydrate residues of glycoside 1 and glycoside 2 are identical:

$$[Z_1] = [Z_2] = [Z] \quad \text{(VI)}$$

Considering these assumptions, the concentration ratios of the two aglycons after the first and second hydrolyses are compared. For the first hydrolysis

$$k_1/k_2 = [Z_1][A_1][Z_2A_2]/[Z_1A_1][Z_2][A_2] \quad \text{(VII)}$$

or, with IV–VI

$$x = [Z][A_1][ZA]/[ZA][Z][A_2] \quad \text{(VIII)}$$

We obtain

$$x = [A_1]/[A_2] \quad \text{(IX)}$$

The concentration ratio of the aglycons equals the ratio of the equilibrium constants.

Consider the second hydrolysis now. The initial concentrations of the two glycosides have changed.

$$[Z_1A_1]^1 = [ZA] - [A_1]^1 = [ZA] - x[A_2]^1 \quad \text{(Xa)}$$

$$[Z_2A_2]^1 = [ZA] - [A_2]^1 \quad \text{(Xb)}$$

$[...]^n$ = concentration after n hydrolysis steps. The new equilibrium is expressed by

$$\frac{k_1}{k_2} = \frac{[Z][A_1]^2[Z_2A_2]^1}{[Z_1A_1]^1[Z][A_2]^2} \quad \text{(XI)}$$

With eq IV and X we get

$$x = \frac{[A_1]^2}{[A_2]^2}\,\frac{[ZA] - [A_2]^1}{[ZA] - x[A_2]^1} \quad \text{(XII)}$$

$$[A_1]^2 = x\frac{[ZA] - x[A_2]^1}{[ZA] - [A_2]^1}[A_2]^2 \quad \text{(XIII)}$$

We define

$$x_1 = x\frac{[ZA] - x[A_2]^1}{[ZA] - [A_2]^1} \quad \text{(XIV)}$$

and thus obtain

$$[A_1]^2 = x_1[A_2]^2 \quad \text{(XV)}$$

or

$$x_1 = [A_1]^2/[A_2]^2 \quad \text{(XVI)}$$

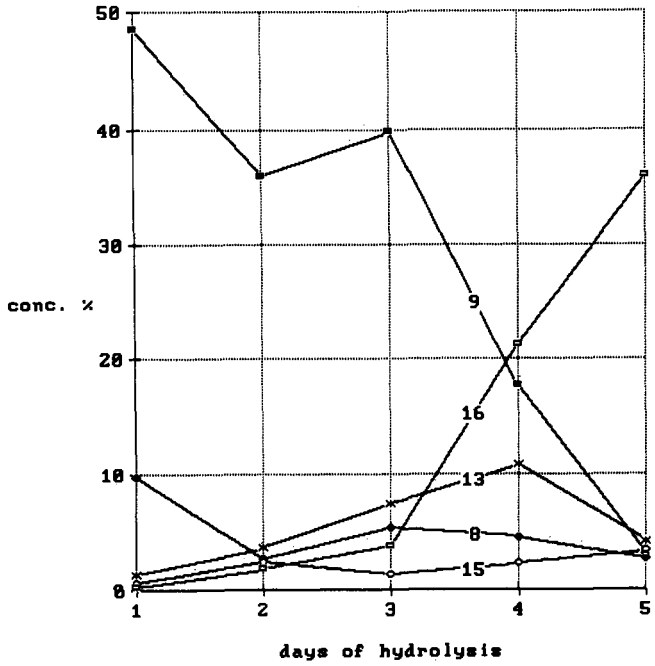

Figure 3. Enzymatic release of C_8 diols **8** and **9** and C_{13} norisoprenoids **13**, **15**, and **16** during SECE with hesperidinase of a glycosidic fraction from apple fruit. Area percentages of HRGC detectable peaks (=100%) are represented.

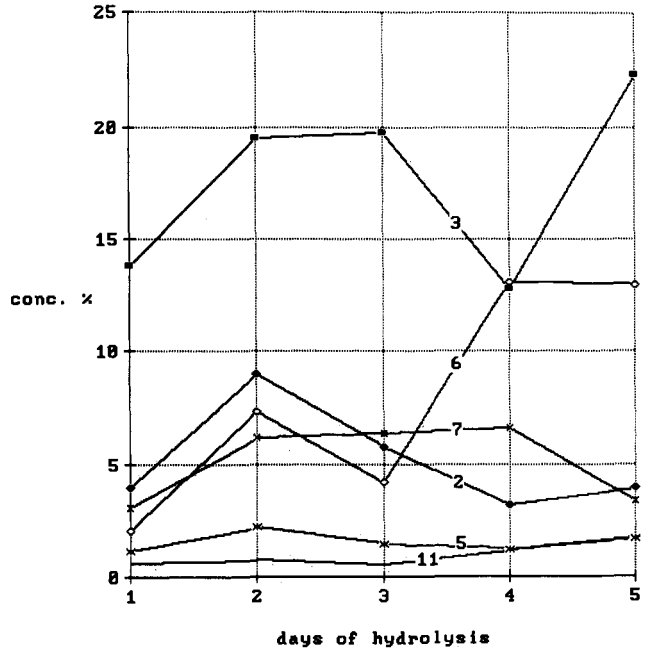

Figure 4. Enzymatic release of aliphatic and aromatic alcohols **2**, **3**, **5**, **6**, **7**, and **11** during SECE with hesperidinase of a glycosidic fraction from apple fruit. Area percentages of HRGC detectable peaks (=100%) are represented.

The result is an expression for the concentration ratio of the aglycons after step 2 and looks similar to eq IX for the hydrolysis step 1. Looking closer at the definition of x_1 (eq XIV) we can compare the two concentration ratios:

$$\frac{x_1}{x} = \frac{[ZA] - x[A_2]^1}{[ZA] - [A_2]^1} \quad \text{(XVII)}$$

$$\frac{[ZA] - x[A_2]^1}{[ZA] - [A_2]^1} < 1 \text{ for } x > 1 \text{ (cf. eq III)} \quad \text{(XVIII)}$$

$$\Rightarrow x > x_1 \quad \text{(XIX)}$$

$$\Rightarrow \frac{[A_1]^1}{[A_2]^1} > \frac{[A_1]^2}{[A_2]^2} \quad \text{(XX)}$$

This means that during step 2 of the hydrolysis the portion of aglyon 2 increases. The above concept applies for all following steps of hydrolysis. Thus, it is possible to obtain detectable amounts of an aglycon that at the start only appears in traces.

SECE of a Glycosidic Apple Extract with Hesperidinase. In this experiment using SECE the glycosides from apple fruit obtained by XAD separation and subsequent methanol elution were dissolved in phosphate buffer (pH 5) and incubated with hesperidinase. Hesperidinase was chosen due to its known effect of releasing vomifoliol from conjugates (Okamura et al., 1981). SECE was carried out at room temperature, and the diethyl ether phase was changed daily. After 5 days, SECE was stopped and the aglycons were examined by HRGC and HRGC–MS. Figures 3–5 represent the quantitative results of the release of aglycons during this enzymatic hydrolysis of apple glycosides. In Figure 3 a decreasing release of both C_8 diols **8** and **9** as well as an increase in the concentrations of C_{13} alcohols **13**, **15**, and **16** with increasing number of hydrolysis steps is shown. The concentrations of the aliphatic and aromatic alcohols **2**, **3**, **5**, **7**, and **11** did not show strong variations and exhibited similar deviations during the single hydrolysis steps (Figure 4). Only the concentration

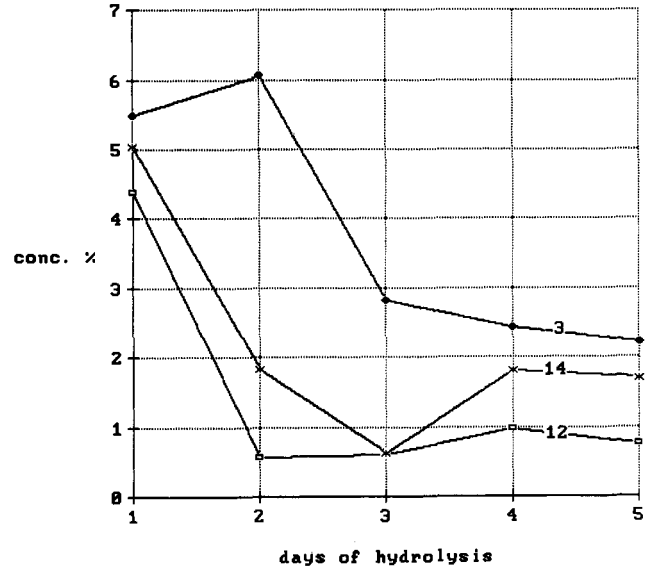

Figure 5. Enzymatic release of aliphatic and aromatic acids **3**, **12**, and **14** during SECE with hesperidinase of a glycosidic fraction from apple fruit. Area percentages of HRGC detectable peaks (=100%) are represented.

of alcohol **6** increased over time. The release of acids was low; parallel curves for the aromatic acids were observed (Figure 5). The curves of concentration of the C_8 diols showed a minimum during the second hydrolysis step, whereby those of the aliphatic alcohols exhibited a maximum (Figures 3 and 4).

The slight increase in the amounts of the C_8 diols during the third hydrolysis step (Figure 3) might be caused by the occurrence of different carbohydrate moieties. In our mathematical and theoretical considerations of the enzymatic hydrolysis it was postulated that the carbohydrate residues of the two glycosides are identical. However, in glycosides aglycons can be combined with carbohydrate residues of various structures comprising not only different monosaccharides but also disaccharides, trisaccharides, etc. The concentrations of the C_8 diols showed a minimum on

the second day because likely the species of C_8 diol glycosides most sensitive to hydrolysis were cleaved on the first day. On the second day, glycosides of other aglycons were preferably hydrolyzed. When their concentration had decreased, now the less sensitive species of C_8 diol glycosides reacted on the third day. In the literature (Bohlmann and Le Van, 1977; Williams et al., 1982) there are examples for the presence of several glycosides of a defined aglycon. The parallel curves of related aglycons shown in Figures 3–5 also indicate the occurrence of different glycosides of defined aglycons in apples.

CONCLUSIONS

The results represented in this paper confirm earlier findings about the specificity of glycosidases toward glycosides with different aglycons. In our previous work carried out on β-glucosidase it was shown that the enzyme preferably catalyzed the liberation of aromatic aglycons, such as, e.g., benzyl alcohol and 2-phenylethanol; more interesting, aglycons like terpenoids exhibited much higher K_m values (Hartmann-Schreier and Schreier, 1987). Thus, repeating the enzymatic hydrolysis several times, as done in the present study, was the logical next step of our previous investigations. The development of a continuous process now opens the way for potential technological applications in which industrially attractive plant volatiles not commonly available will be accessible from conjugates. The system is generally suitable for enzymatic reactions in which nonpolar products were formed from polar substrates (Bednarski et al., 1987).

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Structure–Odor Relationships for Disubstituted Pyrazines

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The odor characteristics, odor thresholds (T), and retention indices on Carbowax 20M ($I^{CW\ 20M}$) and OV-101 columns ($I^{OV\text{-}101}$) were determined for 60 disubstituted pyrazines. Irrespective of the type of substituents and positions of additional ring substituents for the alkylpyrazines, substituted methyl- or ethylpyrazines, in general, have nutty and/or brown notes, while the longer alkyl-substituted pyrazines tend to have green and/or burdock like odors. The ΔI ($=I^{CW\ 20M} - I^{OV\text{-}101}$) values have been applied to quantitative structure–activity relationships in olfaction. The relationship between odor threshold and ($\sum\delta I_R - \Delta\Delta I$), where δI_R is the parameter for a substituent group and $\Delta\Delta I$ ($=\Delta I - \Delta I_P$) is the difference between the ΔI of the disubstituted pyrazine and that of pyrazine (ΔI_P), for the 60 disubstituted pyrazines is as follows: $\log (1/T) = 0.04(\sum\delta I_R - \Delta\Delta I) + 6.2$. The difference between the observed and calculated threshold value is within 1 order of magnitude.

The pyrazines possess roasted or green odors and low threshold values (Masuda and Mihara, 1986). They have been widely used as flavor constituents or perfume components. Takken et al. (1975) and Seifert et al. (1972) mentioned that the most potent odors for alkylmethoxypyrazines were found when an alkyl substituent occupies the position ortho to a methoxy function. One should be careful to drawing conclusions from a comparison of threshold values determined by different groups, even when they have used the same procedure (Maga, 1982; Takken et al., 1975). In order to elucidate the structure–odor relationships for disubstituted pyrazines, we investigated the olfactive properties for some disubstituted pyrazines.

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EXPERIMENTAL SECTION

Instrumentation. The IR, ^1H NMR, ^{13}C NMR, and GC/MS were recorded on a Hitachi 260-10, a JNM-PMX 60, a Bruker AM-400, and a Hitachi M-80B spectrometer, respectively.

GC analyses were carried out on a Hewlett-Packard Model 5710A gas chromatograph equipped with a flame

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