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Biotechnol. Agron. Soc. Environ. 1999 **3** (1), 5–9

Accumulation of glycosidically bound compounds in *Fragaria* × *ananassa* cv. Elsanta fruits at various developmental stages

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Received 12 May 1998, accepted 10 September 1998.

Isolation of glycosidically bound compounds of *Fragaria* × *ananassa* cv. Elsanta was performed by Amberlite XAD-2 adsorption followed by methanol elution. After glycosidic extract hydrolysis with a commercial β-glucosidase and liquid-liquid extraction with diethyl ether, on-line coupled capillary gas chromatographic - mass spectrometric (HRGC-MS) identification of the liberated aglycons was achieved. Five developmental stages of strawberry fruit were investigated during ripening. The occurrence and accumulation of glycosides were pointed out and several aglycons were identified in relation with the investigated developmental stages, with a maximum for the overripe fruit.
Keywords. Strawberry fruit, glycosides, flavour, *Fragaria* × *ananassa*, ripening, capillary gas chromatography, mass spectrometry.

Accumulation des composés liés de façon glycosidique dans les fruits de *Fragaria* × *ananassa* cv. Elsanta à divers stades de maturité. L'isolement des composés liés de façon glycosidique de *Fragaria* × *ananassa* cv. Elsanta a été réalisé par adsorption sur Amberlite XAD-2 suivie d'une élution au méthanol. Après hydrolyse de l'extrait glycosidique à l'aide d'une β-glucosidase commerciale et une extraction liquide-liquide à l'éther diéthylique, l'identification des aglycones libérés a été réalisée par chromatographie gazeuse couplée à la spectrométrie de masse (CG-SM). Cinq stades de développement des fraises ont été étudiés durant la maturation des fruits. La présence et l'accumulation des glycosides ont été mises en évidence et un nombre variable d'aglycones ont été identifiés en relation avec le stade de développement étudié, avec un nombre maximum pour le stade dit "sur-mûr".
Mots-clés. Fraises, glycosides, arôme, *Fragaria* × *ananassa*, maturation, chromatographie gazeuse, spectrométrie de masse.

1. INTRODUCTION

Glycosidically bound compounds have been identified in a vast number of fruits. They have been extensively studied in the cases of grapes and wines (Williams *et al.*, 1980, 1982 a, b; Strauss *et al.*, 1988; Gunata *et al.*, 1990 a, b; Roscher, Winterhalter, 1993; Baumes *et al.*, 1994; Sefton *et al.*, 1994) and for other fruits such as mangoes (Adedeji *et al.*, 1992), peaches (Krammer *et al.*, 1991; Sumitani *et al.*, 1994), pineapples (Wu *et al.*, 1991), grapefruits (Hasegawa *et al.*, 1989), passion fruits (Engel, Tressl, 1983), raspberries (Pabst *et al.*, 1990, 1991 a, b, 1992 a, b, c, d), but the knowledge about the glycosides occurring in strawberries is not so extended, except for the 2,5-dimethyl-4-hydroxy-3(2H) furanone β-D-glucopyranoside (furaneol glucoside) that has been largely studied (Mayerl *et al.*, 1989; Sanz *et al.*, 1994, 1995; Roscher *et al.*, 1996, 1997). Latza *et al.* (1996) identified the 1-O-trans-cinnamoyl β-D-glucopyranose, which is probably the precursor of volatile cinnamates in strawberry flavour. Neuguebauer *et al.* (1994) isolated the 3-hydroxy-5,6-epoxy-β-ionol β-D-glucopyranoside from strawberry leaves. Pelarginidin 3-O-(6-O-malonyl-β-glucoside) (Tamura *et al.*, 1995) and taxifolin 3-arabinoside (Ishimaru *et al.*, 1995), two flavonoid precursors, were also isolated and identified in strawberries. Wintoch *et al.* (1991) studied the glycosidically bound aroma compounds from two species (*Fragaria vesca f. semperflorens* and *Fragaria* × *ananassa* cv. Korona). Considering the importance of glycosidic precursors as flavour potential and the scarce data about the glycosidically bound molecules in strawberry fruits, it is necessary to extend the knowledge about these compounds. Therefore, we have initiated a study directed towards gaining a better understanding of the occurrence of glycosides and the potential role of endogenous glycosidases in the maturation process of

strawberry fruits. The present paper reports on the isolation of a glycosidic extract of *Fragaria* × *ananassa* cv. Elsanta fruits at various stages of development, the identification of some liberated aglycons, and a comparative study of the occurrence and accumulation of these compounds during ripening.

2. MATERIAL AND METHODS

2.1. Reagents

All commercial solvents were obtained from UCB (Union Chimique Belge, Leuven, Belgium) and Alltech (Deerfield, IL, USA) (analytical grade) and were redistilled before use. Amberlite XAD-2, reference compounds and almond β-glucosidase were purchased from Sigma (St. Louis, USA). Other chemicals were obtained from UCB.

2.2. Fruits

Fresh strawberry fruits (*Fragaria* × *ananassa* cv. Elsanta) were obtained from the Agricultural Research Center (Ministry of Agriculture, Gembloux, Belgium) between July and September 1997. Five developmental stages were investigated. They were characterized on the basis of fruit colours as established by Risser and Navatel (1997): green, white, red 1/2, red 4/4 (physiological maturity) and dark red (overripe fruit).

2.3. Isolation of glycosidic extract

Fresh strawberries (100 g) were mixed with 100 mL cold distilled water in a Waring Blendor for 2 min at room temperature. After a maceration of 1 h at 4 °C, the mixture was centrifuged for 15 min at 4 °C (7500 g). The supernatant was collected and filtered with a butter-cloth. A 100 mL fraction of clear strawberry juice was applied to an Amberlite XAD-2 column which has been successively washed with 250 mL of distilled water and 200 mL of a n-pentane/diethyl ether mixture (1/1 v/v). The glycosidic extract has finally been collected by eluting with 200 mL of methanol.

2.4. Enzymatic hydrolysis

A 10 mL fraction of the methanolic extract containing the glycosides was concentrated to dryness under reduced pressure at 30 °C and redissolved in 4 mL of phosphate-citrate buffer (0.2 M, pH 5.0). After addition of 1 mL of an almond β-glucosidase solution (Sigma, EC 3.2.1.2., 5 units·mg^{-1} solid, concentration of 1 unit·mL^{-1} buffer), the mixture was incubated overnight at 37 °C. After addition of 1.75 g of sodium chloride, the liberated aglycons were extracted with 5 mL of redistilled diethyl ether, dried on anhydrous magnesium sulfate, filtered and concentrated under a gentle stream of pure nitrogen. Blanks consisted in tests carried out in the same conditions but without enzyme addition.

2.5. Capillary Gas Chromatography – Mass Spectrometry (HRGC–MS)

A Hewlett Packard 5890 Series II gas chromatograph fitted with split/splitless injector and coupled to a Hewlett Packard 5972A mass spectrometer was used for aglycon analysis. The column used was a HP5-MS (30 m × 0.25 mm, film thickness = 1 µm). The splitless injection was performed at 250 °C with a purge delay time of 2 min. The temperature programme was 40 °C (1 min isothermal) to 180 °C at 6 °C·min^{-1}, then to 290 °C at 15 °C·min^{-1} and final hold at 290 °C for 5 min. The transfer line was maintained at 290 °C. Carrier gas flow rate was of 1.0 mL·min^{-1} He. The GC-MS analyses were performed in the EI mode at 70 eV, the mass range scanned was 30 to 300 amu. The identifications were made by comparison of the recorded mass spectra with data of two libraries: NBS75K.L and WILEY138.L. They were confirmed by co-injection of pure molecules taken as references. Quantitative data were obtained employing external standard curve (area *vs* concentration, linear regression) for each identified compound.

3. RESULTS AND DISCUSSION

The glycosidic extracts obtained after Amberlite XAD-2 adsorption and methanol elution of fresh strawberry juices *(Fragaria* × *ananassa* cv. Elsanta), were subjected to enzymatic hydrolysis (β-glucosidase), followed by liquid-liquid extraction with diethyl ether. A typical HRGC-MS separation of the liberated aglycons at the overripe stage (dark red fruit) is outlined in **figure 1**. The identified aglycons and their range of concentrations are shown in **table 1**. The maximum number of aglycons identified is 11 in the overripe fruit of Elsanta. Occurrence and accumulation of the liberated aglycons during ripening are outlined in **figure 2**.

Some of the eleven aglycons identified in this study have already been reported in the literature for other strawberry cultivars. For example, Furaneol®, was identified in hydrolyzed glycosidic extracts by a lot of authors such as Mayerl *et al.* (1989), Wintoch *et al.* (1991), Sanz *et al.* (1994, 1995), Perez *et al.* (1996) and Roscher *et al.* (1996, 1997). Wintoch *et al.* (1991) and Latza *et al.* (1996) reported the occurrence of E-cinnamic acid.

For all the compounds identified, except for benzyl alcohol, 2,3-dihydrobenzofuran and E-cinnamic acid, the occurrence of aglycons (and by extension, of

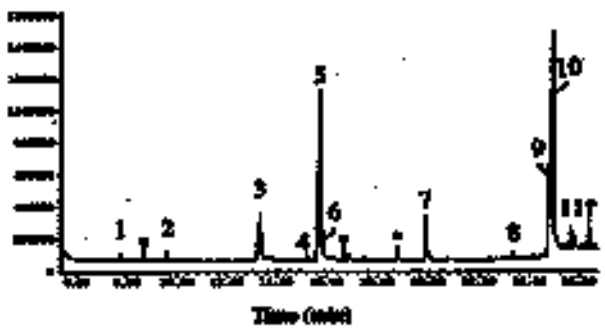

Figure 1. Typical HRGC-MS separation of the aglycons liberated after hydrolysis by β-glucosidase of a methanolic extract (Amberlite XAD-2) obtained from *Fragaria* × *ananassa* cv. Elsanta fruit juice at overripe stage (dark red fruit). · compound not identified. Peak 9 appears as a shoulder of peak 10 — *Séparation classique par CG-SM des aglycones libérés après hydrolyse par une β-glucosidase d'un extrait méthanolique (Amberlite XAD-2) obtenu à partir du jus des fruits de* Fragaria × ananassa *cv. Elsanta au stade de maturité dit "sur-mûr" (fruit rouge foncé). · composé non identifié. Le pic 9 apparaît comme un épaulement du pic 10.*

glycosides) only starts at the ripe stage (red 4/4 fruit) and their contents increase in large amount between red 1/2 and dark red stages, especially for hexanoic acid, Furaneol, (4-hydroxyphenyl) ethanol and E-cinnamic acid. For example, Furaneol was not detected at the red 1/2 stage but was present in overripe strawberries at a concentration of 1296 $\mu g \cdot kg^{-1}$ fresh fruit.

After hydrolysis of the glycosidic extracts, the identified aglycons consisted of acids and alcohols, and the two most important compounds of strawberry flavour, 2,5-dimethyl-4-hydroxy-3(2H) furanone (Furaneol) and 2,5-dimethyl-4-methoxy-3(2H) furanone (mesifurane).

The major aglycons in Elsanta fruits at the ripe and overripe stages are, in decreasing amounts, Furaneol, E-cinnamic acid, mesifurane, hexanoic acid, 2,3-dihydrobenzofuran and (4-hydroxyphenyl) ethanol. In order to verify the origin of the aglycons (hydrolyzed glycosides or, after hydrolysis, degradation of the liberated aglycons), reference compounds corresponding to the eleven identified aglycons were submitted to the same enzymatic hydrolysis procedure than the glycosidic extracts. This was done in order to detect potential alterations which could occur at 37 °C in the presence of β-glucosidase. Since no degradation was pointed out, it seems likely that each identified aglycon results from one specific strawberry glycoside.

Although sugar moiety identifications have not been undertaken within this study, it may be hypothetized that the glycosidically bound compounds

Table 1. Compounds identified in *Fragaria* × *ananassa* cv. Elsanta fruits at five ripening stages by HRGC-MS after hydrolysis of a methanolic extract (Amberlite XAD-2) — *Composés identifiés dans les fruits de* Fragaria × ananassa *cv. Elsanta, à cinq stades de maturité, par CG-SM après hydrolyse d'un extrait méthanolique (Amberlite XAD-2).*

Peak n° (a)	**Compound**	**Amount (mg/kg fresh fruit) at each ripening stage**				
		Green	White	Red 1/2	Red 4/4	Dark red
1	Butanoic acid	nd	nd	nd	nd	11
2	2-Methyl butanoic acid	nd	nd	nd	6	16
3	Hexanoic acid	nd	nd	nd	23	136
4	Benzyl alcohol	nd	nd	<2	5	12
5	2,5-Dimethyl-4-hydroxy-3(2H) furanone (= Furaneol)	nd	nd	nd	494	1296
6	2,5-Dimethyl-4-methoxy-3(2H) furanone (= Mesifurane)	nd	nd	nd	17	56
7	2,3-Dihydrobenzofuran	nd	nd	5	43	81
8	Hydroxylinalool	nd	nd	nd	3	14
9	4-Hydroxybenzeneethanol	nd	nd	nd	nd	125
10	3-Phenyl-2-propenoic acid	nd	nd	21	84	630
11	Derivative of 2(3H) furanone (**b**)	nd	nd	nd	9	35
	TOTAL	-	-	28	684	2412

(**a**) Peak number: see legend of **figure 1.**

(**b**) Tentatively identified as 5-hexyldihydro-2(3H) furanone (gamma-decalactone).

nd = not detected.

Amounts are the mean of five repetitions.

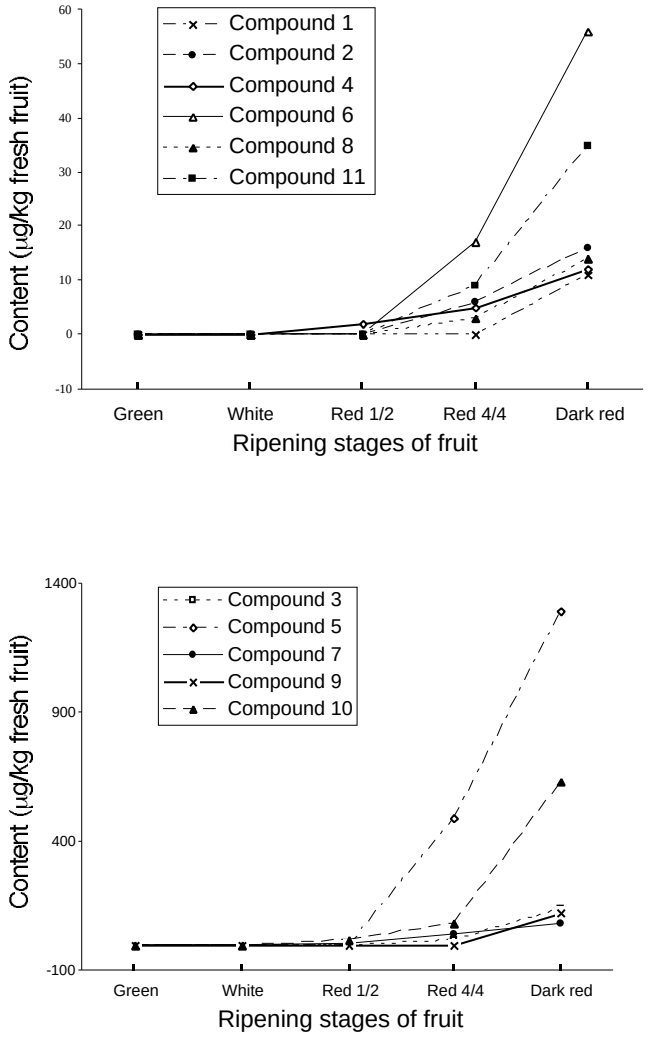

Figure 2. Evolution of the content in aglycons enzymatically liberated from glycosidic extracts (Amberlite XAD-2) of *Fragaria* × *ananassa* cv. Elsanta fruit at various ripening stages — *Évolution du contenu en aglycones libérés de façon enzymatique à partir d'extraits glycosidiques (Amberlite XAD-2) des fruits de* Fragaria × ananassa *cv. Elsanta à divers stades de maturité.*

are β-glucosides. Indeed, the β-glucosidase used for the hydrolysis of glycosidic extracts exhibits a specific action on β-glucosides. The β-D-glucopyranoside structure has already been established for 2,5-dimethyl-4-hydroxy-3(2H) furanone (Furaneol ®) (Mayerl *et al.*, 1989; Wintoch *et al.*, 1991; Sanz *et al.*, 1994 and 1995; Roscher *et al.*, 1996 and 1997), (4-hydroxyphenyl) ethanol (Wintoch *et al.*, 1991), E-cinnamic acid (Latza *et al.*, 1996) and benzyl alcohol (Wintoch *et al.*, 1991). The complete elucidation of the structure of other *Fragaria* × *ananassa* cv. *Elsanta* glycosides is in progress and will be reported later.

Acknowledgment

The authors are grateful to Mr. A. Robbe (Agricultural Research Center, Gembloux, Belgium) for the gift of strawberry fruits (*Fragaria* × *ananassa* cv. Elsanta). This research is supported by University Faculty of Agricultural Sciences (Gembloux, Belgium) funds and by the Unit of General and Organic Chemistry (Prof. M. Marlier).

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