Sensitive and Selective Screening for 6′-*O*-Malonylated Glucoconjugates in Plants†

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Using synthesized reference compounds a screening for benzyl, 2-phenylethyl, geranyl, citronellyl, and 2,5-dimethyl-4-hydroxy-3(2H)-furanone 6′-*O*-malonyl β-D-glucopyranosides in various plant tissues was performed by high-performance liquid chromatography−electrospray ionization−tandem mass spectrometry (HPLC−ESI−MS/MS). The results obtained with fruits (guava; raspberry; strawberry), leaves (green tea; vine), and mountain papaya (*Carica pubescens*) peel indicate that malonylation of glycoconjugates is a common pathway in plant secondary metabolism.

Keywords: *Malonylated glycosides; benzyl 6′-O-malonyl β-D-glucopyranoside; 2-phenylethyl 6′-O-malonyl β-D-glucopyranoside; geranyl 6′-O-malonyl β-D-glucopyranoside; citronellyl 6′-O-malonyl β-D-glucopyranoside; 2,5-dimethyl-4-hydroxy-3(2H)-furanone 6′-O-malonyl β-D-glucopyranoside; HPLC−MS/MS, electrospray ionization (ESI)*

INTRODUCTION

In the past, acylated glycoconjugates have been described for flavonoids (Takeda et al., 1986; Saito et al., 1988; Barnes et al., 1994) and a few of glycosidically bound flavor precursors (Schwab and Schreier, 1988; Moon et al., 1994; Roscher et al., 1996a). Malonylated glycoconjugates are also known to occur in the metabolic pathway of pesticides (Suzuki and Casida, 1981; Frear et al., 1985). Recently, a convenient one-pot synthesis has been developed for the regioselective preparation of 6′-*O*-malonylglycosides (Roscher et al., 1996b), which offers an easy access to these polar compounds. At the same time, high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS) has been described as most suitable method for their sensitive and selective identification from complex natural matrices (Roscher et al., 1996a;b). With both appropriate synthetic and analytical methods in hand, the next step was to screen for 6′-*O*-malonylated β-D-glucopyranosides in various plant tissues, in order to get a deeper insight into the natural distribution of these flavor precursors. Benzyl, 2-phenylethyl, geranyl, citronellyl, and 2,5-dimethyl-4-hydroxy-3(2H)-furanone 6′-*O*-malonyl β-D-glucopyranosides (**2a**−**e**, Chart 1) were selected for this screening. The present paper describes the results obtained using a widespread variety of plant tissues, i.e., fruits (guava; raspberry; strawberry), leaves (green tea; vine), and mountain papaya (*Carica pubescens*) peel.

EXPERIMENTAL PROCEDURES

Chemicals. All commercial chemicals used were of analytical grade quality. All solvents employed were of high purity at purchase and were redistilled before use. Water and methanol (each of HPLC gradient grade) and DMSO-d_6 were purchased from Merck (Darmstadt, Germany).

Plant Material. Guava, raspberry and strawberry fruits were available from a local market. Mountain papaya (*C. pubescens*) fruits and fresh green tea leaves were imported from Colombia and South Korea, respectively. Vine leaves cv. Muscat originated from INRA, Montpellier.

Isolation of a Glycosidic Extract. The first step corresponded to the method already described by Gunata et al. (1985). Thus, after mixing of 200 g of fruits (or peel) with 200 mL of 0.2 M citrate−phosphate buffer (pH 7.0) and readjustment to pH 7.0 with 2 N NaOH, a clear extract was obtained by centrifugation (3000*g*, 20 min). To extract leaves the method was modified as follows: 80 g of leaves was mixed with 400 mL of methanol, and after macerization of the mixture (adjusted to pH 7) at ambient temperature overnight, a clear extract was obtained by filtration. Methanol was removed under reduced pressure. The aqueous residue was extracted three times with 100 mL of pentane to remove chlorophyll. The clear extract was then applied to an Amberlite XAD-2 column (3.5 × 35 cm, 10 mL/min). After a rinse with 2000 mL of distilled water and 1000 mL of pentane−diethyl ether (1+1), a glycosidic extract was obtained by eluting with 1000 mL of methanol. The methanol eluate was concentrated under reduced pressure to dryness and redissolved in 10 mL of distilled water (yields ranging from 0.2 to 0.3 g).

The glycosidic extract was applied to a column containing a strong basic anion exchanger (1 × 20 cm; Merck ion exchanger III; 5 mL/min). After a rinse with 1000 mL of distilled water, elution was performed with 1000 mL of 0.75 M Na_2SO_4 solution. The eluate was applied to a XAD-2-column (2.5 × 25 cm; 6 mL/min) to separate the salt. After the column was washed with 1000 mL of distilled water, elution with methanol (1000 mL) yielded the charged glycosidically bound constituents. The methanol solution was concentrated under reduced pressure to dryness and redissolved in 2 mL of distilled water for the analysis of glycoconjugates and aglycons using HPLC−MS/MS and HRGC−MS after enzymatic hydrolysis, respectively.

For the enzymatic hydrolysis an aliquot (corresponding to 100 g of fruits or peel and 40 g of leaves) was used and treated with Rohapect D5L (1 mL; Röhm, Darmstadt, Germany) in 0.2 M citrate−phosphate buffer (pH 5.4). After incubation (24 h at 37 °C) the sample was extracted with diethyl ether, dried over anhydrous sodium sulfate, carefully concentrated by a Vigreux column (45 °C), and analyzed by HRGC−MS.

Reference Compounds. *(a) β-D-Glucopyranosides of Benzyl Alcohol (**1a**), 2-Phenylethanol (**1b**), Geraniol (**1c**), and Citronellol (**1d**).* The synthesis of **1a**−**d** was performed by a modified Koenigs−Knorr method (Paulsen et al., 1985) using the commercially available alcohols (45 mmol) and α-D-acetobromoglucose (12.5 mmol) with Ag_2O (13.5 mmol) as catalyst in dry dichloromethane (25 mL) in the presence of Drierite (4 g). After the mixture was stirred under N_2 for 3

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† Dedicated to Prof. Dr. W. Adam (Institut für Organische Chemie) on the occasion of his 60th birthday.

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Chart 1

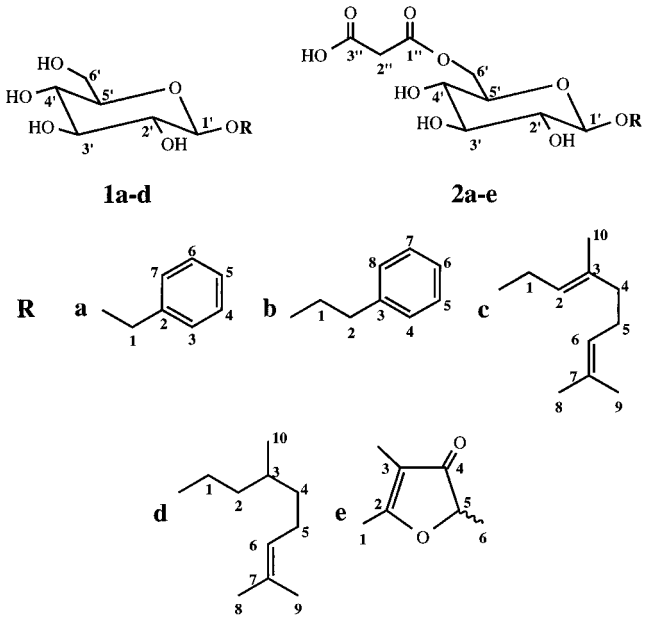

Table 1. ^{1}H NMR Data of Compounds 1a and 2a (400 MHz, DMSO-d_6)[a]

H	1a[b]		2a	
aglycon				
1	4.66	1H, d (12.12)	4.65	1H, d (12.12)
	4.91	1H, d (12.48)	4.85	1H, d (12.12)
3/7	7.47	2H, d (7.00)	7.47	2H, d (7.00)
4/6	7.42	2H, t (6.96)	7.42	2H, t (7.00)
5	7.36	1H, t (7.00)	7.35	1H, t (6.96)
glucose				
1′	4.31	1H, d (7.72)	4.33	1H, d (7.72)
2′	3.09−3.25	m[c]	3.11−3.25	m[c]
3′	3.09−3.25	m[c]	3.11−3.25	m[c]
4′	3.09−3.25	m[c]	3.11−3.25	m[c]
5′	3.09−3.25	m[c]	3.11−3.25	m[c]
6′	3.55	1H, dd (5.88/11.40)	4.20	1H, dd (6.24/11.76)
	3.78	1H, dd (6.24/10.30)	4.27	1H, d (9.92)
malonyl				
2″			3.05	2H, s

[a] Coupling constants in Hz, δ relative to solvent signal of DMSO-d_6. [b] Cf. Schwab and Schreier (1988). [c] Sugar signals overlapped (4H).

days in the dark at room temperature, it was filtered through Celite and purified on silica gel (2 × 60 cm) with pentane−ethyl acetate (3+1) as eluting solvent mixture. After deacetylation with sodium methoxide (0.1 M) the β-D-glucopyranosides were purified on silica gel (2 × 60 cm) by eluting with methanol−ethyl acetate (9+1). ^{1}H and ^{13}C NMR data were identical with published data (Paulsen et al., 1985; Schwab and Schreier, 1988; Yano et al., 1990; Voirin et al., 1990).

*(b) 6′-O-Malonylated β-D-glucopyranosides of Benzyl Alcohol (**2a**), 2-Phenylethanol (**2b**), Geraniol (**2c**), Citronellol (**2d**), and 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (**2e**).* The malonylated glucosides were synthesized as described by Roscher et al. (1996b) using **1a**−**d** (0.35 mmol), malonic acid (1.37 mmol), and *tert*-butylisocyanide (0.67 mmol) in dry CH_3CN (10 mL). Pure **2a**−**d** were obtained by anion exchange chromatography (1 × 20 cm, Merck ion exchanger III; rinse with 1000 mL of distilled water, elution with 1000 mL of 0.75 M Na_2SO_4) and subsequent separation of the salt by a XAD-2-column (2.5 × 25 cm; rinse with 1000 mL of distilled water, elution with 1000 mL of methanol). 2,5-Dimethyl-4-hydroxy-3(2H)-furanone 6′-*O*-malonyl β-D-glucopyranoside (**2e**) was already available (Roscher et al., 1996a).

NMR Data. (**2a**) Cf. Tables 1 and 2. (**2b**) ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.92 (2H, t, J = 7.36 Hz, H-2), 3.05 (2H, s, malonyl CH_2), 3.11−3.25 (4H, m, H-2′−H-5′), 3.73 (1H, t, J = 8.80 Hz, H-1a), 3.93 (1H, q, J = 7.36 Hz, H-1b), 4.15 (1H, d, J = 11.40 Hz, H-6′a), 4.25 (1H, dd, J = 6.20 Hz, 11.76 Hz, H-6′b), 4.30 (1H, d, J = 7.72 Hz, H-1′), 7.28 (1H, m, H-6), 7.34 (4H, m, H-4, H-5, H-7, H-8). ^{13}C NMR (100 MHz, DMSO-d_6): δ 35.8 (C-2), 45.6 (C-2″), 63.6 (C-6′), 69.7 (C-4′), 70.2 (C-1), 73.5 (C-2′), 73.9 (C-5′), 76.5 (C-3′), 103.1 (C-1′), 126.1 (C-6), 128.3 (C-5, C-7), 129.0 (C-4, C-8), 138.9 (C-3), 168.6 (C-1″), 169.6 (C-3″). (**2c**) ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.65 (3H, s, H-8), 1.69 (3H, s, H-10), 1.72 (3H, s, H-9), 2.08 (2H, m, H-5), 2.13 (2H, m, H-4), 2.95−3.08 (4H, m, H-2′ and malonyl CH_2), 3.11−3.27 (3H, m, H-3′, H-4′, H-5′), 4.01 (1H, t, J = 7.00 Hz, H-6′a), 4.08 (1H, d, J = 9.92 Hz, H-6′b), 4.16−4.29 (2H, m, H-1), 4.21 (1H, d, J = 7.36 Hz, H-1′), 5.15 (1H, t, J = 7.00 Hz, H-6), 5.33 (1H, t, J = 6.60 Hz, H-2). ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.0 (C-10), 17.6 (C-9), 25.5 (C-8), 25.9 (C-5), 62.9 (C-6′), 64.2 (C-1), 70.3 (C-4′), 73.4 (C-5′), 73.5 (C-2′), 77.0 (C-3′), 101.3 (C-1′), 120.7 (C-2), 124.0 (C-6), 131.0 (C-7), 139.5 (C-3), 169.8 (C-1″), 170.3 (C-3″) (C-4 and C-2″ overlapped by solvent signals). (**2d**) ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.93 (3H, m, H-10), 1.17 (1H, m, H-3), 1.39 (1H, m, H-4), 1.64 (3H, s, H-8*), 1.69 (3H, s, H-9*), 2.01 (2H, m, H-2), 2.96 (2H, s, malonyl CH_2), 2.99 (1H, t, J = 6.96 Hz, H-2′), 3.11−3.22 (3H, m, H-3′, H-4′, H-5′), 3.63 (1H, m, H-1a), 4.00 (1H, t, J = 6.96 Hz, H-6′a), 4.08 (1H, d, J = 10.28 Hz, H-6′b), 4.10 (1H, m, H-1b), 4.18 (1H, d, J = 7.60 Hz, H-1′), 5.16 (1H, m, H-6) (* signals interchangeable). ^{13}C NMR (100 MHz, DMSO-d_6): δ 17.6 (C-8), 19.4/19.5 (C-10), 25.0 (C-9), 25.4/25.5 (C-5), 28.0/29.0 (C-3), 36.3/36.4 (C-4), 36.7/36.9 (C-2), 63.0 (C-6′), 66.9 (C-1), 70.3 (C-4′), 73.5/73.6 (C-2′), 73.7 (C-5′), 76.7/77.0 (C-3′), 103.0/103.1 (C-1′), 124.8 (C-6), 130.5 (C-7), 170.3 (C-1″), 174.7 (C-3″) (C-2″ overlapped by solvent signals).

Table 2. ^{13}C NMR Data of Compounds 1a and 2a (100 MHz, DMSO-d_6)[a]

C	1a[b]	2a
aglycon		
1	73.6	73.5
2	138.2	138.0
3/7	127.7	127.7
4/6	128.2	128.1
5	127.4	127.4
glucose		
1′	102.2	102.3
2′	70.3	70.2
3′	77.0	76.6
4′	69.6	69.7
5′	76.9	73.8
6′	61.3	63.7
malonyl		
1″		170.3
2″		48.6
3″		171.7

[a] δ relative to solvent signal of DMSO-d_6. [b] Cf. Schwab and Schreier (1988).

Capillary Gas Chromatography (HRGC). A Fisons GC 8160 gas chromatograph (Fisons Instruments, Mainz, Germany) with FID equipped with a J&W fused silica DB-Wax capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm) was used. Split injection (1:20) was employed. The temperature program was 3 min isothermal at 50 °C and then increased from 50 to 240 °C at 4 °C/min. The flow rate of the carrier gas was 2.0 mL/min He and for the make up gas 30 mL/min N_2; for the detector gases the flow rates were 30 mL/min H_2 and 300 mL/min air. Injector and detector temperatures were kept at 220 °C.

Capillary Gas Chromatography−Mass Spectrometry (HRGC−MS). A Fisons gas chromatograph 8060 with split injector (1:30) was combined by direct coupling to a Fisons MD 800 mass spectrometer with MassLab data system (Fisons Instruments, Mainz, Germany). The same type of column and the same temperature program as mentioned above for HRGC analysis were used. Other conditions: temperature of ion

Table 3. HPLC−MS/MS Data of Compounds 2a−e[a]

	2a	2b	2c	2d	2e[b]
molecular weight	356	370	402	404	376
base peak					
$[M + H]^+$					377
$[M + NH_4]^+$	374	388	420	422	
product ions of base peak (CID 1.9 mTorr; −16 eV)					
$[M + H - \text{aglycon}]^+$	**249**[c]	**249**[c]	**249**[c]	**249**[c]	
$[249 - H_2O]^+$	231	231	231	231	231
$[\text{malonic acid} + H]^+$	105	105	105	105	105
$[249 - \text{malonic acid}]^+$	145	145		145	145
$[145 - H_2O]^+$	127	127		127	127
$[\text{aglycon} + H]^+$					**129**[c]
$[\text{aglycon} + H - H_2O]^+$	91		137		
$[M + H - H_2O]^+$	339	353		387	
$[M + H - 2 \times H_2O]^+$	321	335			341

[a] ESI, positive, RP-18; gradient, 5 mM NH_4Ac in H_2O−5 mM NH_4Ac in 90% MeOH. [b] Identical with ESI mass spectral data previously published (Roscher et al., 1996a). [c] Most abundant product ions.

source, 230 °C; temperature of all connection parts, 200 °C; electron energy, 70 eV; cathodic current, 4 mA; mass range, 40−250 g.

LC−MS/MS Analysis. Analysis of 6′-*O*-malonylated glucosides **2a**−**e** was performed on a triple-stage quadrupole TSQ 7000 LC−MS/MS system (Finnigan MAT, Bremen, Germany). Data acquisition and data evaluation were carried out on a Personal DEC station 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT, Bremen, Germany). HPLC separation was carried out on an Eurospher 100-C18 (2 × 100 mm, 5 μm, Knauer, Berlin, Germany) using a linear gradient at a flow rate of 200 μL/min. Electrospray ionization (ESI) in positive mode was used. The temperature of the heated capillary was 200 °C, and the capillary voltage was set to 2.7 kV. Nitrogen served both as sheath (50 psi) and auxiliary gas (10 L/min). The HPLC gradient was as follows: solvent A (5 mM NH_4Ac in water), solvent B (5 mM NH_4Ac in 90% methanol); 0 min, 10% B; 0−10 min, 10−100% B; 10−15 min, 100% B.

The product ion spectra were available by collision-induced dissociation (CID) (1.9 mTorr of argon; −16 eV). From the characteristic fragmentation pattern the most abundant product ion was selected for parent scan mode and selected reaction monitoring (SRM) experiments.

Three different MS/MS experiments were developed for the screening for **2a**−**e** in plants: (i) parent scan mode of *m*/*z* 249.1 (*m*/*z* 300−500); (ii) parent scan mode of *m*/*z* 129.1 (*m*/*z* 300−400); (iii) SRM, time-dependent, 0−7 min, *m*/*z* 377.2/129.1, 374.2/249.1, 388.1/249.1; 7−15 min, *m*/*z* 420.5/249.1, 422.4/249.1; scan-time, 0.5 s.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were recorded on a Bruker WM 400 spectrometer. The spectra were measured in DMSO-d_6 as solvent and referenced to the solvent signal, respectively.

RESULTS AND DISCUSSION

In order to screen for 6′-*O*-malonyl β-D-glucopyranosides in plants, four common β-D-glucopyranosides (**1a**−**d**) were selected. From the synthesized compounds **1a**−**d** the malonylated derivatives **2a**−**d** were prepared. In addition, the already available 2,5-dimethyl-4-hydroxy-3(2H)-furanone 6′-*O*-malonyl β-D-glucopyranoside (**2e**) (Roscher et al., 1996a) was used.

The 6′-*O*-malonylated glucosides were characterized by NMR spectroscopy (cf. Tables 1 and 2 and Experimental Procedures). The additional signal at about 3 ppm in the ^1H NMR spectra of compounds **2a**−**d** in comparison to compounds **1a**−**d** (cf. data for **1a** and **2a** in Table 1) were assigned to the malonyl CH_2 (Horowitz and Asen, 1989). The corresponding ^{13}C NMR signal at 48.6 (**2a**) and 45.63 ppm (**2b**) was overlapped by the solvent signals in the case of compounds **2c** and **2d** (Horowitz and Asen, 1989; Moon et al., 1994). The two signals at about 170 ppm observed in the ^{13}C NMR spectra of 6′-*O*-malonylated glycosides indicated the occurrence of a carboxyl and an ester group. The esterification of OH-6′ of the sugar moiety with the malonic acid was confirmed by a distinct downfield shift of H-6′a and H-6′b in the ^1H NMR spectra of compounds **2a**−**d** in comparison to compounds **1a**−**d**, respectively (cf. data for **1a** and **2a** in Table 1). In addition, the ^{13}C NMR data also showed a downfield shift of C-6′ and an upfield shift of the C-5′ from the corresponding signals of the β-D-glucopyranosides (Kasai et al., 1981).

Using LC−ESI−MS/MS analysis in the positive mode with ammonium acetate in water and methanol, respectively, an abundant pseudomolecular ion $[M + NH_4]^+$ was observed for each malonylated glucoside except for **2e**, whose base peak found to be $[M + H]^+$.

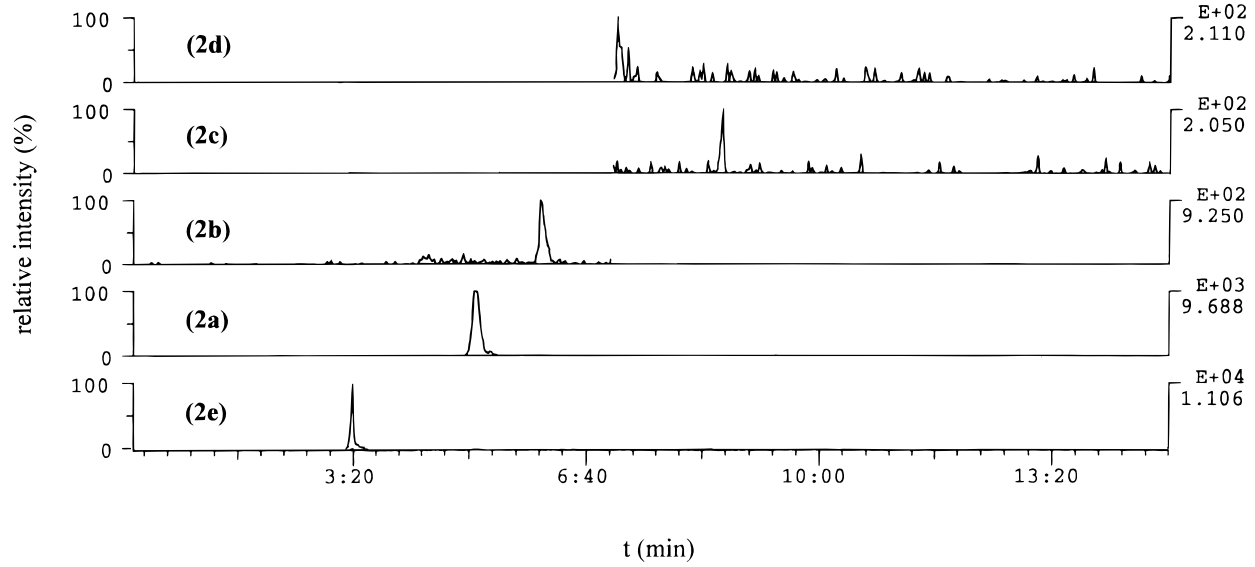

Figure 1. Time-dependent SRM experiment for compounds **2a**−**e** in strawberry extract. The chromatogram shows the product ion traces of **2a**−**e**. The characteristic ion pairs are as follows: *m*/*z* 374.2/249.1 (**2a**); *m*/*z* 388.1/249.1 (**2b**); *m*/*z* 420.5/249.1 (**2c**); *m*/*z* 422.4/249.1 (**2d**); and *m*/*z* 377.2/129.1 (**2e**). Compound **2d** was not detectable in the sample. (ESI, positive mode, RP-18; gradient, 5 mM NH_4Ac in H_2O−5 mM NH_4Ac in 90% MeOH).

Table 4. Screening Results for Compounds 2a−e in Various Plant Tissues

	2a	2b	2c	2d	2e
raspberry	♦	♦	♦		♦
strawberry	♦	♦	♦		♦
mountain papaya peel	♦	♦	♦		
guava	♦	♦			
green tea	♦	♦			
vine leaf (cv. Muscat)	♦	♦	♦		

MS/MS experiments of these "parent ions" produced characteristic product ion spectra.

For all compounds under study nearly the same fragmentation pattern was detectable (Table 3). While the aglycon of **2e** was easily ionized (Roscher et al., 1996a) presumably due to the heteroatoms in the moiety, the aglycons of **2a**−**d** could hardly be ionized. Consequently, ions of the aglycon moiety were less intensive, except for **2e**, whose LC−MS/MS spectrum was dominated by the aglycon fragment.

With a test-mixture of **2a**−**e** a screening method for these compounds in plants was developed. The chosen HPLC gradient with ammonium acetate in water and methanol, respectively, was suitable to separate the synthesized reference compounds **2a**−**e** from each other [relative retention times: (**2a**) 5.0 min; (**2b**) 6.0 min; (**2c**) 8.4 min; (**2d**) 9.1 min; (**2e**) 3.2 min]. Three different MS/MS experiments were performed to identify malonylated glucoconjugates in plants: (i) detection of the *m*/*z* generating the specific product ion *m*/*z* 249 by CID; *m*/*z* 249 was the most abundant product ion of **2a**−**d** and was assigned to be $[M + H - \text{aglycon}]^+$; (ii) detection of the *m*/*z* forming the specific product ion *m*/*z* 129 by CID; due to the different fragmentation pattern of **2e**, *m*/*z* 249 was not suitable for its detection, so *m*/*z* 129 $[\text{aglycon} + H]^+$ was used for the screening as most intensive fragment; and (iii) time-dependent SRM. SRM increased the sensitivity and was highly selective because of excluding matrix effects by filtration of the "parent ion" ($[M + NH_4]^+$ and $[M + H]^+$, respectively) and its specific product ion (*m*/*z* 249 and 129, respectively).

With this method, a widespread variety of plant tissues was screened for compounds **2a**−**e**. Glycosidic extracts from fruits (guava; raspberry; strawberry), leaves (green tea; vine), and mountain papaya (*C. pubescens*) peel were obtained by XAD-2 solid phase extraction and further purified by anion exchange chromatography to separate charged glycoconjugates. A representative example taken from our study on strawberry is outlined in Figure 1. The results of the screening are summarized in Table 4. While the aromatic 6′-*O*-malonyl β-D-glucopyranosides were found in all plants under study, the terpene derivatives were less widely distributed; **2d** was not present in any of the samples. In addition to the already known occurrence of **2e** in several fruits, i.e., strawberry, mango, pineapple, and tomato (Roscher et al., 1996a), it was also detectable in raspberry.

These results were confirmed by enzymic hydrolysis of the extracts with Rohapect D5L and subsequent analysis of the liberated aglycons by HRGC−MS. It has to be stressed, however, that for **2a**−**e** about 10-fold higher enzyme concentration was required than usually employed for the hydrolysis of glycoconjugates. As recently demonstrated, malonylated glycoconjugates are not susceptible to β-glucosidase but to esterase activity the latter constituting a minor activity in Rohapect D5L (Roscher et al., 1996c).

In summary, we have shown that HPLC−ESI−MS/MS offers a highly selective method for the screening for 6′-*O*-malonylated glucoconjugates in plants. These first screening results indicate that 6′-*O*-malonyl β-D-glucopyranosides occur nearly ubiquitous and are common in the composition of bound flavor precursors in foodstuffs. Due to the already known instability observed under traditional sample preparation conditions (Koester et al., 1984; Horowitz and Asen, 1989), these compounds can easily be overlooked as flavor precursors.

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