

Separation, Identification and Quantification of Monomers from Cutin Polymers by High Performance Liquid Chromatography and Evaporative Light Scattering Detection

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A silicic acid high performance liquid chromatographic method which utilizes an evaporative light scattering detector has been developed for the separation of cutin monomers. This binary gradient system was successfully applied to separate underivatized monomers of several fruit cutins including those from apple cvs. Golden Delicious and Red Delicious (*Malus pumila*), tomato (*Lycopersicon esculentum*), grapefruit (*Citrus paradisi*) green pepper (*Capsidum annum*) and pumpkin (*Cucurbita pepo*). All cutin monomers, from the least polar, monohydroxy saturated and unsaturated fatty acids, to the most polar, trihydroxy saturated and unsaturated fatty acids, were separated. Monomer identity was confirmed by gas chromatography–mass spectrometry of collected peaks. The gradient was also able to separate positional isomers of dihydroxy fatty acids, a separation not yet achieved by gas chromatography. The relationship between detector response and the mass of five common cutin monomers was obtained.

INTRODUCTION

The aerial surfaces of fruits and vegetables are covered with an extracellular layer called the cuticle. Because of its key protective role between the plant and the environment, the chemical composition of cutin (polymers of hydroxy, epoxy and keto fatty acids) present in plant cuticles has been studied by many authors (Deas *et al.*, 1974; Holloway, 1977, 1980; Kolattukudy, 1980a, b; Espelie *et al.*, 1983). Cutin structure has been analysed by various chemical methods including hydrogenolysis with LiAlH_4 (Walton and Kolattukudy, 1972) and hydrolysis with alcoholic KOH (Holloway, 1980; Kolattukudy, 1980b) or HCl (Holloway, 1980). The cutin monomers released have been identified by various techniques such as thin layer chromatography (TLC), gas chromatography (GC) and GC-coupled mass spectrometry (GC–MS). These techniques have limitations: TLC analysis allows only a general separation into major lipid classes and is primarily a qualitative method. GC and GC–MS analyses give both qualitative and quantitative information, but require derivatization of free carboxyl and hydroxyl groups.

Until now, no high-performance (pressure) liquid chromatographic (HPLC) method has been reported for the separation of cutin monomers. Our laboratory has developed HPLC methods for the quantitative analysis of plant lipids which utilize a flame ionization detector or an evaporative light-scattering detector

(ELSD) (Moreau *et al.*, 1990; Moreau, 1990). More recently, we reported an HPLC method to separate and quantify hydroxy and epoxy fatty acids on a silicic acid column with an ELSD (Gerard *et al.*, 1992). This paper describes the application of this novel HPLC technique for the separation, identification and quantification of underivatized cutin monomers isolated from various fruit cutins.

EXPERIMENTAL

Chemicals. All HPLC-grade solvents (hexane, chloroform, methanol, isopropanol, dichloromethane) were obtained from Burdick and Jackson (Muskegon, MI, USA). Cellulase (E.C. 3.2.1.4) and pectinase (E.C. 3.2.1.15) from *Aspergillus niger* and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). A methanolic solution of boron trifluoride (14% w/v) and *N,O*-bis-trimethylsilylacetylacetamide (BSA) were obtained from Alltech Associates Inc. (Deerfield, IL, USA).

Cutin preparation. Cutin was prepared from mature fruits obtained from a local supermarket as previously described (Holloway, 1977) with minor modifications. Discs of peel from fresh fruits of apple cvs. Golden Delicious and Red Delicious, tomato, grapefruit, green pepper and pumpkin were removed and boiled in oxalate buffer (oxalic acid 4 g/L : ammonium oxalate 16 g/L). Cutin discs were collected, washed several times with deionized water, dried and ground in a Wiley mill (20 mesh followed by 40 mesh). The powdered cutin was extracted in a Soxhlet apparatus with chloroform for 24 h at 50°C overnight, dried under vacuum, washed with

deionized water and treated with a clarified solution of *A. niger* cellulase (5 g/L) and fungal pectinase (1 g/L) in 0.05 M acetate buffer (pH 4.0) at room temperature for 14 to 16 h. The cutin powder was then washed thoroughly with deionized water and dried in a vacuum oven at 40°C.

Standard hydrolysis and lipid extraction. Alkaline hydrolysis was performed by reacting 30 mg of cutin with 1 mL of 10% KOH (w/v) in methanol at 28°C for 18 h. The resulting solution was acidified with acetic acid and lipids were extracted with chloroform:methanol according to the method of Bligh and Dyer (1959). Organic phases were removed and evaporated under nitrogen. The dry residue was weighed, dissolved in 1 mL of chloroform:methanol (85:15), and filtered through glass wool into autosampler vials.

Chromatographic Procedure. Lipid analyses were performed with a Hewlett-Packard Model 1050 HPLC (gradient programmer, autosampler). All studies were performed with a Chrompack ChromSep 7 µm LICHROSORB Si 60 silica cartridge system (10 cm × 3.0 mm i.d.) with a flow rate of 0.5 mL/min. Underivatized samples were analysed using a linear gradient from isopropanol:acetic acid:hexane (7:2:991) to isopropanol:acetic acid:hexane (200:2:778) in 39 min, followed by a return to isopropanol:acetic acid:hexane (7:2:991) for 8 min as previously described (Gerard *et al.*, 1992). A Varex Universal ELSD was used for the detection of hydroxy fatty acids at a temperature of 40°C and with nitrogen (25 psi) as a nebulizing gas.

GC-MS analysis. The collected fractions were analysed by GC-MS (electron impact) with a Hewlett-Packard Model 5990B instrument fitted with an Ultra 1 (methyl silicone) 12 m capillary column temperature programmed from 150 to 250°C at 4°C/min. Prior to this analysis, hydroxy components were methylated with boron trifluoride in methanol. The methyl esters were then silylated with BSA for 30 min at room temperature.

RESULTS AND DISCUSSION

Hydrolysis of cutins and extraction of monomers

One of the aims of our study was to develop an HPLC technique which could separate and quantify cutin monomers that were released by either chemical or enzymatic hydrolyses. Purified cutin from apple cv. Golden Delicious fruit was hydrolysed by methanolic KOH at 28°C and 70°C. Since identical levels of monomer were released at both temperatures, we chose to use the lower temperature for subsequent analyses. Previous reports (Walton and Kolattukudy, 1972; Holloway, 1973) indicated that approximately 80% of the mass of the polymer could be released by methanolic KOH and extracted as lipid monomers. When we hydrolysed apple cutin cv. Golden Delicious with methanolic KOH, acidified with acetic acid and extracted according to the method of Bligh and Dyer (1959), we were able to recover 78% of the original mass of the polymer. Other extraction solvents including hexane:isopropanol (2:3), methylene chloride, chloroform and diethyl ether were found to be inferior to chloroform:methanol.

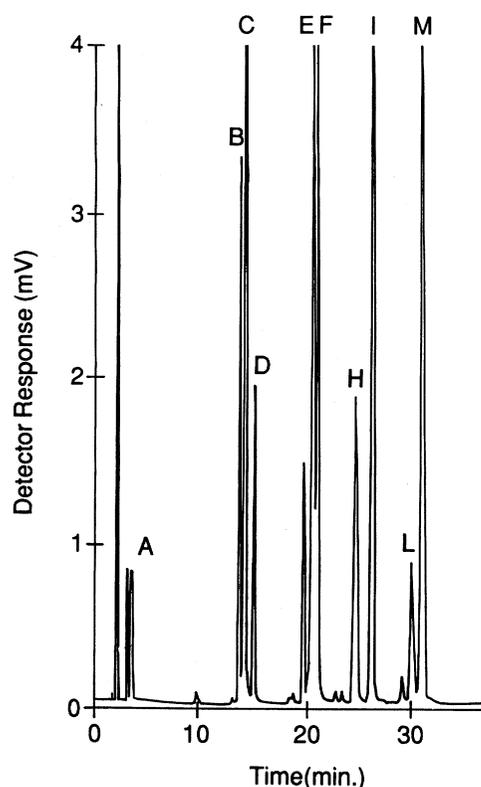


Figure 1. Chromatogram of apple cv. Golden Delicious cutin-free acids. Labels on peaks correspond to those listed in Table 1.

Analysis of apple fruit cutins

Monomers from apple cvs. Golden Delicious and Red Delicious fruit cutin were analysed by HPLC using gradient elution and a Chrompack ChromSep 7 µm LICHROSORB Si 60 silica column as previously described (Gerard *et al.*, 1992). Five major peaks and several minor peaks were observed (Fig. 1). The retention times ranged from 2 to 35 min with the retention times of some of the peaks corresponding to those of hydroxy and epoxy fatty acid standards (Gerard *et al.*, 1992).

The compositions of the apple fruit cutins were similar to those previously reported for the two cultivars (Holloway, 1973, 1980, 1982). The five major components (Fig. 1) were identified as 18-hydroxy-octadeca-9-enoic acid (20.8% for Golden Delicious and 11.7% for Red Delicious), 18-hydroxy-9,10-epoxy-octadecanoic acid (16.0% and 24.2%) and its unsaturated analogue 18-hydroxy-9,10-epoxyoctadeca-12-enoic acid (25.0% and 18.5%), 10,18-dihydroxyoctadecanoic acid (10.9% and 15.9%) and 9,10,18-trihydroxyoctadecanoic acid (15.0% and 19.6%) (Table 1).

The separation achieved allowed all major components to be collected separately and their identities confirmed by GC-MS except for 18-hydroxy-9,10-epoxyoctadecanoic acid and its unsaturated analogue 18-hydroxy-9,10-epoxyoctadeca-12-enoic acid (labelled E and F) which eluted between 20.5 and 21.5 min. Although, the order of elution was not determined, we have labelled the unsaturated epoxyhydroxy fatty acid (peak E) as eluting first, based on our results with other hydroxy fatty acids.

Small peaks eluting at 2.6 min were similar in retention time to the fatty acids hexadecanoic acid and

octadecanoic acid (Table 2). These fatty acids have been previously reported in various cutins (Baker and Holloway, 1970; Eglinton and Hummeman, 1968). Minor peaks eluting between 9 min and 12 min were not identified.

Reaction of the monomer mixture from cv. Golden Delicious with diazomethane (to convert the free acids to methyl esters) appeared to decrease each of the retention times by approximately 2 min (Fig. 3) which indicated that all of the peaks corresponded to carboxylic acids, a result which was confirmed by GC-MS analysis. Although the individual methyl ester peaks were not identified (cf. Fig. 2), their likely identity can be deduced from the corresponding free acid peaks (Fig. 1).

Analyses of other fruit cutin by HPLC

The HPLC technique was applied to the analysis of the monomers of tomato, grapefruit, green pepper and pumpkin fruit cutins. Representative chromatograms are shown in Figs. 3 and 4. The identities of each collected peak were determined by GC-MS and the compositions, based on HPLC analysis, are reported in Table 1.

Tomato. The chromatogram from tomato cutin revealed only two peaks which represented more than 97% of the total monomers. The component comprising the largest peak was identified as 10,16-dihydroxyhexadecanoic acid (labelled K) and represented 93.2% of the total peak area. The earlier eluting peak component was identified as the 9,16-dihydroxyhexadecanoic acid (labelled J) which represented about 4.6% of the total area. These results are consistent with previous reports (Baker *et al.*, 1982).

The HPLC technique allowed us to separate, collect and identify minor peak components. Low levels (0.8%) of 16-hydroxyhexadecanoic acid (labelled D) were present as previously reported (Baker *et al.*, 1982). With this technique, we have also identified 10-oxo, 16-hydroxyhexadecanoic acid (labelled G) as a minor component (1.1%). This hydroxy fatty acid has not been reported previously for tomato cutin. No trihydroxy compounds were detected. It has been

reported that the level of trihydroxy compounds in tomato fruit cutin is dependent on the size of the mature fruit with decreasing amount as the size increases (Baker *et al.*, 1982). The tomatoes we used were particularly large. A small peak eluting at 2.5 min representing 0.2% of total monomers was similar in retention time to hexadecanoic and octadecanoic acids (Table 2).

Green pepper. The major monomer components of green pepper cutin were identified as two isomers of dihydroxy fatty acids eluting between 26.0 and 30.0 min (Fig. 3). They were identified as 9,16-dihydroxy- and 10,16-dihydroxyhexadecanoic acid (40.4% and 41.7%; labelled J, K respectively) (Haas, 1974).

Other components eluting between 14 and 16.5 min were identified as 16-hydroxyhexadecanoic acid and 18-hydroxyoctadeca-9-enoic acid. These two components were not previously reported for green pepper cutin. GC-MS analysis confirmed the presence of both 18-hydroxy-9,10-epoxyoctadecanoic acid and its unsaturated analogue 18-hydroxy-9,10-epoxyoctadeca-12-enoic acid (labelled E and F). The small peak eluting at 2.7 min and representing less than 0.2% of the total monomers corresponded to the retention time of hexadecanoic acid and octadecanoic acid (Table 2). Trace amounts of trihydroxy fatty acids (labelled L and M) were observed.

Grapefruit. One predominant peak eluting at 22.7 min and representing 77.4% of the total monomers was obtained (Fig. 4). The peak component was identified as 10-oxo,16-hydroxyhexadecanoic acid (labelled G). This monomer was previously reported to be the predominant monomer in the fruit cutin of several *Citrus* species including grapefruit (Deas *et al.*, 1974; Baker and Procopiou, 1975; Espelie *et al.*, 1980, 1983).

Grapefruit cutin also contained two dihydroxymonobasic acids identified as 10,18-dihydroxyoctadecanoic acid (labelled I) (14.1% of total area) and 9,18-dihydroxyoctadecanoic (labelled H) (1.8% of total area) (Eglinton and Hummeman, 1968; Holloway, 1982). 16-hydroxyhexadecanoic acid (labelled D) and 18-hydroxyoctadeca-9,12-dienoic acid (labelled B) were also found to be minor components (1.10 and 2.3%, respectively) of grapefruit cutin (Eglinton and

Table 1. Monomer composition of various fruit cutins as determined by HPLC-ELSD

Cutin compounds	Area (%) ^a					
	Golden Delicious apple	Red Delicious apple	Tomato	Grapefruit	Green pepper	Pumpkin
A: Hexadecanoic acid, octadecanoic acid	0.7 ± 0.1	2.5 ± 0.1	0.2 ± 0.1	2.4 ± 0.1	0.1 ± 0.1	12.0 ^b ± 0.1
B: 18-Hydroxyoctadeca-9,12-dienoic acid	3.0 ± 0.1	1.1 ± 0.1	0	2.5 ^b ± 0.1	0	0
C: 18-Hydroxyoctadeca-9-enoic acid	20.8 ± 0.2	11.7 ± 0.1	0	0	0.4 ± 0.1	0
D: 16-Hydroxyhexadecanoic acid	2.9 ± 0.1	1.5 ± 0.1	0.8 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.7 ^b ± 0.1
E: 18-hydroxy-9,10-epoxyoctadecanoic acid	16.0 ± 0.2	24.2 ± 0.3	0	0	7.3 ^b ± 0.1	0
F: 18-Hydroxy-9,10-epoxyoctadeca-12-enoic acid	25.0 ± 0.5	18.5 ± 0.3	0	0	6.5 ^b ± 0.1	0
G: 10-Oxo,16-hydroxyhexadecanoic acid	0	0	1.1 ^b ± 0.1	77.4 ± 0.4	0	0
H: 9,18-Dihydroxyoctadecanoic acid	2.9 ± 0.1	3.5 ± 0.1	0	1.8 ± 0.1	0	84.0 ^b ± 0.5
I: 10,18-Dihydroxyoctadecanoic acid	10.9 ± 0.2	15.9 ± 0.5	0	14.1 ± 0.1	0	12.0 ^b ± 0.1
J: 9,16-Dihydroxyhexadecanoic acid	0	0	4.6 ± 0.1	0	40.4 ± 0.3	0
K: 10,16-Dihydroxyhexadecanoic acid	0	0	93.2 ± 0.6	0	41.7 ± 0.3	0
L: 9,10,18-Trihydroxyoctadeca-12-enoic acid	1.2 ± 0.1	1.1 ± 0.1	0	0	0.1 ^b ± 0.1	0
M: 9,10,18-Trihydroxyoctadecanoic acid	15.0 ± 0.5	19.6 ± 0.5	0	0	0.3 ^b ± 0.1	0

^a Area values are expressed as the mean area % of triplicate determinations ± standard deviation.

^b Cutin monomers not previously reported.

Table 2. Comparative analysis of cutin monomers by HPLC and GC

Compound	Retention time (min)	
	HPLC analysis	GC analysis
A: Hexadecanoic acid	2.5	15.2
A: Octadecanoic acid	2.7	15.9
B: 18-Hydroxyoctadeca-9,12-dienoic acid	14.6	19.7
C: 18-Hydroxyoctadeca-9-enoic acid	15.1	17.2
D: 16-Hydroxyhexadecanoic acid	16.0	16.2
E: 18-hydroxy-9,10-epoxyoctadeca-12-enoic acid	20.5	27.6
F: 18-Hydroxy-9,10-epoxyoctadecanoic acid	21.5	28.7
G: 10-Oxo,16-hydroxyhexadecanoic acid	22.7	20.1
H: 9,18-Dihydroxyoctadecanoic acid	26.9	26.9
I: 10,18-Dihydroxyoctadecanoic acid	28.3	26.9
J: 9,16-Dihydroxyhexadecanoic acid	28.5	24.2
K: 10,16-Dihydroxyhexadecanoic acid	30.3	24.2
L: 9,10,18-Trihydroxyoctadeca-12-enoic acid	33.2	30.2
M: 9,10,18-Trihydroxyoctadecanoic acid	34.2	30.7

Hummeman, 1968; Espelie *et al.*, 1983). A small peak eluting at 2.5 min representing 2.4% of the total monomers had a retention time similar to hexadecanoic acid and octadecanoic acid (Table 2).

Pumpkin. Pumpkin cutin was found to contain one major component eluting at 26.9 min and identified as the 9,18-dihydroxyoctadecanoic acid (84%) (Table 1). Also present were lesser amounts (12.0%) of 10,18-dihydroxyoctadecanoic acid (labelled I) and 16-hydroxyhexadecanoic acid (labelled D). Two peaks eluting between 2.5 and 2.7 min representing 12.0% of total monomers and corresponding to retention times of hexadecanoic acid and octadecanoic acid were also found. To our knowledge this is the first report of pumpkin cutin monomer composition.

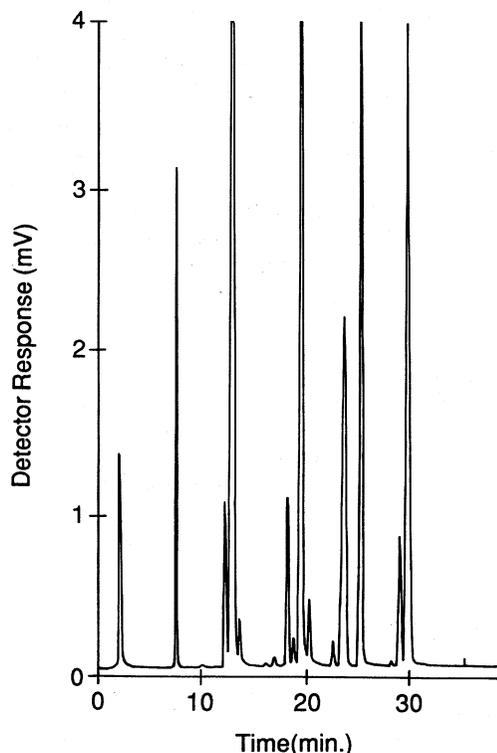


Figure 2. Chromatogram of apple cv. Golden Delicious cutin methyl esters.

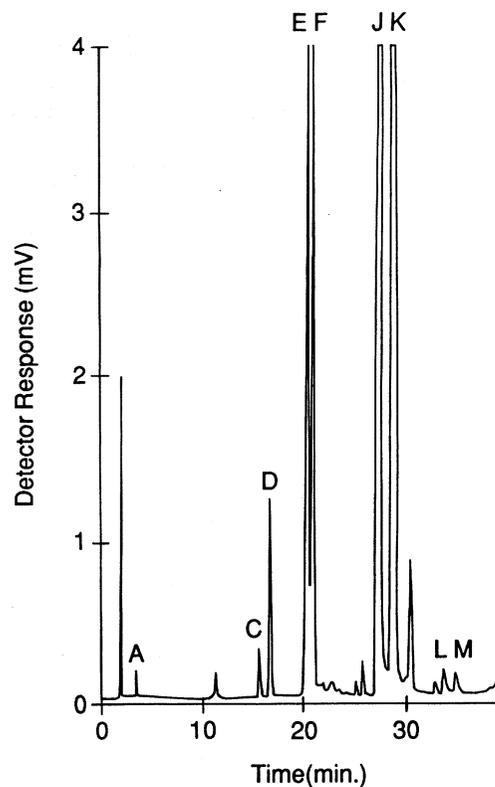


Figure 3. Chromatogram of green pepper cutin-free acids.

Comparison of GC and HPLC

All the major cutin monomers could be separated and identified by the HPLC technique but not by GC (Table 2). In contrast to GC, the HPLC technique was able to discriminate between isomers of dihydroxy fatty acids. To our knowledge no other researchers have

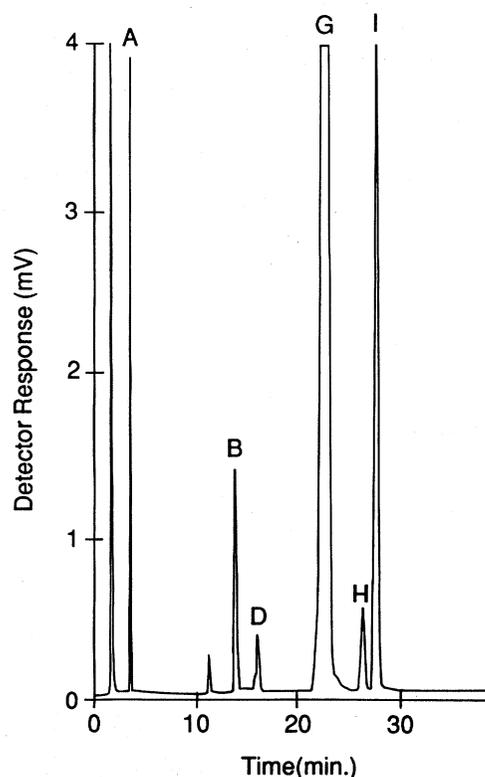


Figure 4. Chromatogram of grapefruit cutin-free acids.

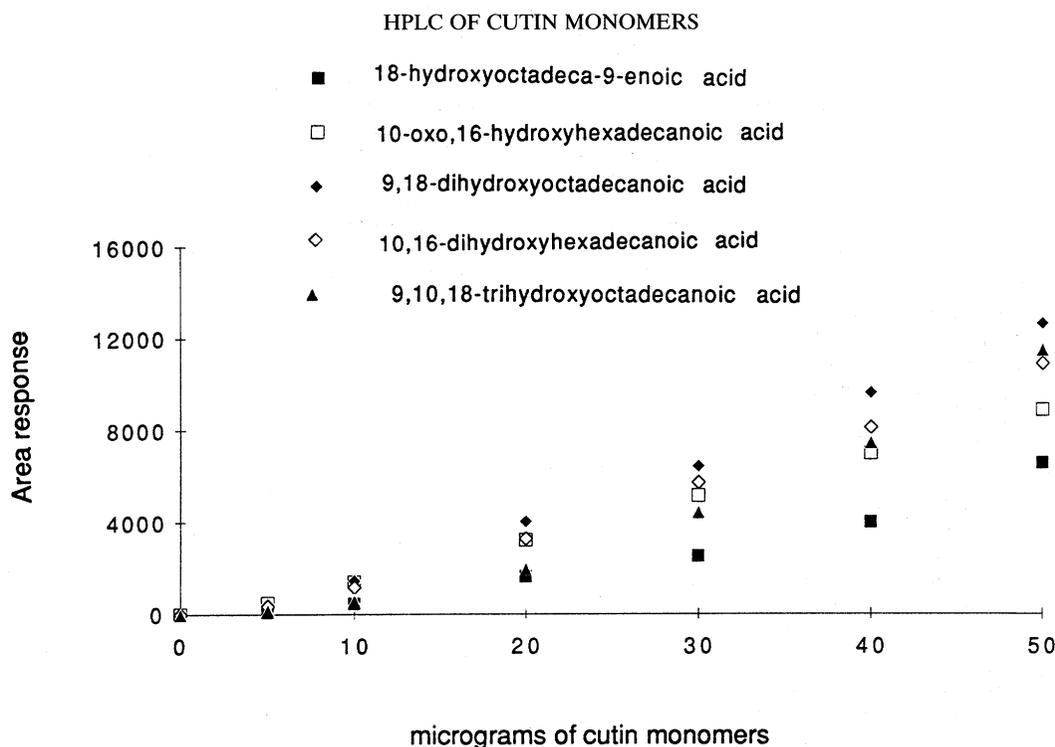


Figure 5. Mass versus detector response for several cutin monomers.

successfully achieved separation of such isomers by GC.

Some or all of the trihydroxy fatty acids analysed by GC-MS as their tri-*O*-silyl fatty acid methyl esters may be artifacts resulting from ring opening of the epoxy compound (analysed as the di-*O*-silylmethoxy fatty acid methyl ester) during base hydrolysis of the cutin. However, when apple fruit cutin was enzymatically hydrolysed (with cutinase from *Fusarium solani* f. sp. *pisi*), the same HPLC profile was observed (data not shown). This indicates that trihydroxy fatty acids are authentic monomers of apple and pepper cutins.

The HPLC method allows for the separation of hydroxy or epoxy fatty acids with different degrees of unsaturation, as well as the resolution of isomers of dihydroxy fatty acids. The combination of HPLC and GC-MS is thus a powerful analytical tool in characterizing cutin monomer composition and is potentially useful in the isolation and characterization of cutin oligomers which should give greater insight into the polymeric structure of cutin.

Quantification of cutin monomers by the HPLC technique

Standard curves (relationship between peak area/mass) were constructed to perform quantitative analyses.

Various cutin monomers were collected manually (10-oxo,16-hydroxyhexadecanoic acid from grapefruit cutin; 9,18-dihydroxyoctadecanoic acid from pumpkin cutin; 10,16-dihydroxyhexadecanoic acid from tomato cutin; 18-hydroxyoctadeca-9-enoic and 9,10,18-trihydroxyoctadecanoic acid from apple cv. Golden Delicious fruit cutin) and analysed in the range 0–50 µg. Although calibration curves for each of the five monomers were linear (Fig. 5), there were differences in detector response, with the highest response being exhibited by dihydroxy compounds, followed by tri-, oxo-, and finally mono-hydroxy compounds. Each data point represented is the mean of three separate injections. These differences may be due to the gradient shape and to the detection system. Although these standard curves can be used to determine the mass of each of the standards shown in Fig. 5, it would be necessary to construct similar curves to quantitatively analyse other cutin monomers.

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