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Analytical Methods

Determination of minor carbohydrates in carrot (*Daucus carota* L.) by GC–MS

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ABSTRACT

Minor carbohydrates present in carrot (*Daucus carota* L.) have been studied by GC–MS analysis of their trimethylsilyl derivatives because of their remarkable role in a variety of biological functions. *Scyllo*-inositol and sedoheptulose (D-*altro*-2-heptulose), identified for the first time in this paper were present in all the carrots analysed in concentrations ranging 1.5–5.8 and 1.4–24.6 mg g^{-1} dried weight, respectively. Other minor carbohydrates detected in carrot were *myo*- inositol (2.2–9.8 mg g^{-1}) and mannitol (traces-1.3 mg g^{-1}). Whereas small amounts (close to 2 mg g^{-1}) of *scyllo*-inositol were experimentally determined in other vegetables from the *Apiaceae* family (parsley, coriander and fennel), sedoheptulose was only detected at trace levels.

1. Introduction

Carrot (*Daucus carota* L.) is a vegetable extensively consumed both raw and cooked because of its pleasant flavour and nutritive properties, derived from its high content of provitamin A (especially β-carotene), vitamins, minerals and fibre (Souci, Fachmann, & Kraut, 2000).

Carrot is mainly constituted by water (approximately 90% of fresh weight) and carbohydrates, which account for 5% of carrot edible portion (Souci et al., 2000). In addition to terpenoids, carbohydrates have been reported to be one of the most important sensory indicators for consumer appreciation of this vegetable (Simon, Peterson, & Lindsay, 1980).

As it is known, fructose, glucose and sucrose are the major sugars in carrot and extensive research has been published on their content in carrots of different variety (Alasalvar, Grigor, Zhang, Quantick, & Shahidi, 2001; Svanberg, Nyman, Andersson, & Nilsson, 1997) and/or submitted to different processing and storage conditions (Machewad, Kulkarni, Pawar, & Surve, 2003; Nyman, Svanberg, Andersson, & Nilsson, 2005; Rodríguez-Sevilla, Villanueva-Suárez, & Redondo-Cuenca, 1999; Svanberg et al., 1997). However, to the best of our knowledge, no references have aimed the determination of minor sugars in *D. carota* L.

Inositols (hexahydroxycyclohexanes) are key components of diverse biological molecules with a remarkable variety of functions. Plants adapt to environmental variation by making or accumulating diverse inositol derivatives. Eukaryotes use phosphatidylinositol derivatives for numerous roles in cell signalling and regulation and in protein anchoring at the cell surface. The involvement of inositols in membrane glycerophospholipids is one of their most studied cellular functions (Michell, 2007). Moreover, several inositols have also been described to have positive physiological effects in humans (McLaurin, Golomb, Jurewicz, Antel, & Fraser, 2000; Nestler, Jakubowicz, Reamer, Gunn, & Allan, 1999).

Inositols have been found in different foods of vegetable origin such as grape musts (Versini, dalla Serra, & Margheri, 1984), peanuts (Binder & Haddon, 1984), fresh fruit juices (Sanz, Villamiel, & Martínez-Castro, 2004a), coffee and substitutes (Ruiz-Matute, Montilla, del Castillo, Martínez-Castro, & Sanz, 2007), etc. *Scyllo*- and *myo*-inositol have been described to be stable against storage or processing of orange juice (Villamiel, del Castillo, San Martín, & Corzo, 1998).

The polyalcohol mannitol (2R,3R,4R,5R)-hexane-1,2,3,4,5,6-hexol) serves as a carbon and energy storage compound and as regulator of osmotic potential in a large number of plants such as celery, parsley, etc (Stoop, Williamson, & Pharr, 1996). Mannitol has also been described as a potent scavenger of hydroxyl radicals both *in vitro* and *in vivo* (Shen, Jensen, & Bohnert, 1997a,b). This antioxidant activity might be significant in the context of fungal pathogenesis, because reactive oxygen species play a central role in plant responses to pathogen attack (Alvarez et al., 1998).

Several papers have described the presence of a number of higher-carbon monosaccharides in plant sources (Begbie & Richtmyer, 1966; Johansson & Richtmyer, 1970; Richtmyer, 1970; Tolbert & Zill, 1954). Sedoheptulose (D-*altro*-2-heptulose), was first

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reported to occur in *Sedum spectabile* Bor. by La Forge and Hudson (1917), and it is known to play an important role in the cyclic regeneration of D-ribulose for carbon dioxide fixation in plant photosynthesis (Benson et al., 1951). *Manno*-, *altro*-, *talo*- and *allo*-heptuloses have been determined at different concentrations in several tropical fruits (Ogata, Kawano, Bevenue, & Casarett, 1972; Shaw, Wilson, & Knight, 1980), their concentrations being dependent on the fruit variety, the fruit part analysed and its degree of ripeness.

Among the different chromatographic techniques that can be used for carbohydrate determination, gas chromatography with flame ionization detection (GC–FID) has shown advantages in terms of sensitivity and power resolution in the analysis of complex mixtures of minor carbohydrates (Martínez Montero, Rodríguez Dodero, Guillén Sánchez, & Barroso, 2004). Coupling of selective mass detection to gas chromatography (GC–MS) provide additional information (mass spectrum) of usefulness for structural characterization of unidentified carbohydrates (Sanz, González, de Lorenzo, Sanz, & Martínez-Castro, 2005; Sanz, Sanz, & Martínez-Castro, 2002).

The present paper has been devoted to investigate the presence of minor carbohydrates in raw and dehydrated carrot samples of different varieties and their characterization by gas chromatography–mass spectrometry.

2. Materials and methods

2.1. Standards

Analytical standards of fructose, glucose, sucrose, *scyllo*- and *myo*-inositol and phenyl-β-D-glucoside were obtained from Sigma Chemical Co. (St. Louis, US). Mannitol was purchased from Fluka (Buchs, Switzerland). Mixtures of *n*-alkanes (C10–C20) were employed for linear retention index (*LRI*) calculations.

2.2. Samples

Eight carrot (*D. carota* L.) samples were analysed (see Table 1). Samples A–D were raw carrots purchased at local markets in Madrid (Spain), while samples E–H were commercial dehydrated carrot flakes. Raw carrots were freeze–dried to estimate the dry matter content and all carrot samples were finely ground using a laboratory grinder prior to carbohydrate analysis.

Samples of coriander, parsley, fennel and celery were purchased at different local markets in Madrid (Spain). A *S. spectabile* plant was obtained from a nursery in Madrid.

2.3. Carbohydrate extraction

Carrot carbohydrates were fractionated according to the method described by García-Baños, Olano, and Corzo (2000). 0.3 g of raw vegetables were weighed into a 25-mL volumetric flask and homogenized at ambient temperature with 5 mL of Milli-Q water. The volume was made up to 25 mL with pure ethanol to obtain a final 80% ethanolic solution and extraction was carried out during 20 min under stirring. Then, samples were centrifuged at 9600 g and 10 °C for 10 min. Precipitates were submitted to a second extraction with 25 mL of 80% ethanol to obtain recovery values close to 100%. 1 mL of supernatants was mixed with 0.2 mL of an ethanolic solution of phenyl-β-D-glucoside (1 mg mL^{-1}) used as internal standard. The mixture was evaporated under vacuum at 40 °C. No changes other than the sample amount weighted (0.1 g) and the supernatant volume evaporated (0.5 mL) were introduced in the procedure for carbohydrate extraction from dehydrated carrots.

For isolation of non commercially available carbohydrates, hot water extracts from *Sedum* sp. leaves were prepared. Samples homogenized in water using an Ultra-Turrax® T-25 (ICT, S.L.) were heated at 60 °C for approximately one hour. 1 mL of each of the water extracts, previously filtered through a Whatman 40 filter, was mixed with 0.2 mL of internal standard (phenyl-β-D-glucoside 1 mg mL^{-1}) and evaporated under vacuum prior to carbohydrate derivatization.

Table 1
Carrot samples under analysis.

Code	Variety[a]	Type of product
A	Mokum	Raw carrot
B	Nantes	Raw carrot
C	Lamuyo	Raw carrot
D	Nantes	Raw carrot
E	Nantes	Dehydrated carrot flakes
F	Nadril	Dehydrated carrot flakes
G	Nadril	Dehydrated carrot flakes
H	Nantes	Dehydrated carrot flakes

[a] Variety is shown as provided by the manufacturer.

2.4. Qualitative analysis

2.4.1. GC–MS analysis

Trimethylsilyl oximes (TMS oximes) were prepared according to Sanz et al. (2002). Oximes were obtained by addition of 350 µL of a solution 2.5% hydroxylamine chloride in pyridine after 30 min at 75 °C. They were then silylated with hexamethyldisilazane (350 µL) and trifluoroacetic acid (35 µL) at 45° C for 30 min. After reaction, samples were centrifuged at 10000 rpm for 4 min, and 1 µL of supernatants was injected into the GC injection port. This method opens the hemiacetal link of the sugar and affords two peaks for each reducing sugar, corresponding to *E* and *Z* isomers.

Trimethylsilyl ethers (TMS ethers) were prepared according to Troyano, Olano, Fernández Díaz, Sanz, and Martínez Castro (1991). 100 µL of anhydrous pyridine, 100 µL of trimethylsilylimidazole (TMSI) and 100 µL of trimethylchlorosilane (TMCS) were added to the evaporated samples. Extraction of the TMS ethers was carried out using 100 µL of hexane and 200 µL of Milli-Q water. 1 µL of the hexane upper layer was injected into the GC. This method keeps the sugar ring intact and affords a peak for each tautomer (until five peaks per reducing sugar).

GC–MS analyses were carried out in a Hewlett–Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass detector (both from Agilent, Palo Alto, CA, USA), using He at ~1 mL min^{-1} as carrier gas. A 25 m × 0.2 mm i.d. × 0.33 µm film thickness fused silica column coated with SPB-1 (crosslinked methyl silicone) from Supelco (Bellefonte, PA, USA) was used. Oven temperature was held at 200 °C for 15 min, then programmed to 270 °C at 15 °C min^{-1} and finally programmed to 290 °C at 1 °C min^{-1}. Injector temperature was 300 °C and injections were made in the split mode with a split ratio 1:40. Mass spectrometer was operating in electronic impact (EI) mode at 70 eV, scanning the 35–600 *m/z* range. Interface and source temperature were 280 °C and 230 °C, respectively. Acquisition was done using a HP ChemStation software (Hewlett–Packard, Palo Alto, CA, USA).

Identification of TMS oximes of minor carbohydrates present in carrot samples was carried out by comparison of their linear retention indices (*LRI*) with those of standard compounds (both commercial and extracted from *S. spectabile*) previously derivatized. Mass spectral data reported in the literature were used for further confirmation of polyalcohol identifications (Sanz, Sanz, & Martínez-Castro, 2004b; Sanz et al., 2004a).

2.4.2. Direct-infusion mass spectrometric (DI–MS) analysis

DI–MS analysis was performed using an electrospray ionization (ESI) interface with a quadrupole mass detector (Hewlett–Packard 1100 Series). Ten-microliter samples of carrot extracts (see 2.3. Carbohydrate extraction) were automatically injected and carried through in water/methanol (50:50, v/v, 1% formic acid) at a flow rate of 0.5 mL min^{-1}. Mass spectra were recorded in positive mode and data were collected for 4 min/sample in scan mode (100–1200 uma) at a scan rate of 0.03 min per scan. The ESI conditions used were the following: needle potential (4000 V), fragmentator voltage (30 V), gas temperature (330 °C), drying gas (10 L min^{-1}) and nebuliser pressure (40 psi).

2.5. Quantitative analysis

GC analysis of samples derivatised as TMS oximes was carried out in a gas chromatograph equipped with a FID detector (HP 5890, Palo Alto, CA, USA). Operating conditions other than the carrier gas (nitrogen) and the detector temperature (300 °C) were identical to those previously described for GC–MS analysis. Chromatographic peaks were recorded using a ChromCard 1.20 acquisition system (CE Instruments, Milan, Italy).

Quantitative data were directly obtained from FID peak areas. Standard solutions of fructose, glucose, sucrose, mannitol, *scyllo*- and *myo*-inositol over the expected concentration range in carrot extracts were prepared to calculate the response factor relative to phenyl-β-D-glucoside. Concentration of minor carbohydrates for which standards were not commercially available was estimated assuming a response factor equal to 1.

3. Results and discussion

Fig. 1 shows the typical total ion current (TIC) chromatogram corresponding to TMS oximes of carbohydrates present in carrot extracts. In addition to major sugars fructose (peaks 2a–b), glucose (peaks 3a–b) and sucrose (peak 5), and polyalcohols such as mannitol (MC1) and *myo*-inositol (MC3), all carrot samples analysed presented other minor carbohydrates (MC2–MC4). Peak MC2 with *LRI* on a SPB-1 column of 2082 showed a spectrum compatible with a polyalcohol structure (see Table 2); their identification as *scyllo*-inositol being confirmed by coinjection of the corresponding commercial standard previously derivatized. *Scyllo*-inositol has not been previously reported in carrots.

Mass spectra of MC4a and MC4b eluting at 19.01 and 19.19 min, respectively, were similar and could probably correspond to the *E* and *Z* isomers of a 2-heptulose, as shown by the relative abundance of ions at *m/z* 205, 217 and 319 (Table 2). The peak area ratio MC4a/M4b ≅1, in agreement with that obtained for the two isomers of other derivatised ketoses (Funcke & von Sonntag, 1979), also supported the possibility of peaks MC4a and MC4b being a ketose.

Table 2
GC and MS data for TMS oximes of minor carbohydrates in carrot extracts.

Peak	Linear retention index (*LRI*)	Mass spectrum[a]	Assignation
MC2	2082	318(100), 73(81), 217(77), 305(74), 147(46), 191(43), 204(35)	*Scyllo*-inositol
MC4a	2304	319(100), 73(78), 205(57), 147(44), 217(25), 103(19), 191(6)	Sedoheptulose
MC4b	2320	319(100), 73(65), 205(56), 147(37), 103(21), 217(20), 191(18)	

[a] *m/z* values (relative abundance in brackets).

However, the similarity in the mass spectra and retention times of TMS oximes of 2-heptuloses and those of linear heptitols hindered the characterization of these two peaks and, therefore, three approaches had to be carried out in order to fully confirm their identification. First, analysis of carrot extracts by direct-infusion mass spectrometry (DI–MS) in positive mode showed an ion at *m/z* 249 compatible with a potassium adduct of an heptose (aldoheptose or ketoheptose) with empiric formula $C_7H_{14}O_7$.

Second, in order to determine the ketoheptose isomer present in carrot extracts, the coinjection of a standard was required. Due to the lack of commercial standards for the different ketoheptose isomers, results were compared with those of sedoheptulose from an aqueous extract of *S. spectabile.* As shown in Fig. 2 and Table 2, perfect matching of *LRI* and mass spectral data for sedoheptulose and peaks MC4a and MC4b, allowed to confirm the presence of this minor carbohydrate in carrot.

Third, trimethylsilyl ethers were prepared. Although a high number of peaks can be obtained for reducing sugars (corresponding to the different tautomers present in equilibrium), the characteristic profiles and the quality of mass spectra obtained makes the monosaccharide identification easy (Sweeley, Bentley, Makita, & Wells, 1963; Páez et al., 1987). While the retention times of chromatographic peaks corresponding to mannitol (MC1), *scyllo*- and *myo*-inositol (MC2–MC3) did not show any variation, as expected, peaks MC4a and MC4b disappeared. However, three new peaks with retention times of 13.03, 13.52 and 13.74 and characteristic fragments at *m/z* 191, 204, 217, 359, 539, corresponding to those of a ketose in furanose or pyranose forms, were observed (Table 3). The same profile and mass spectra were obtained for sedoheptulose in the *Sedum* extract, which confirmed the identity of these peaks.

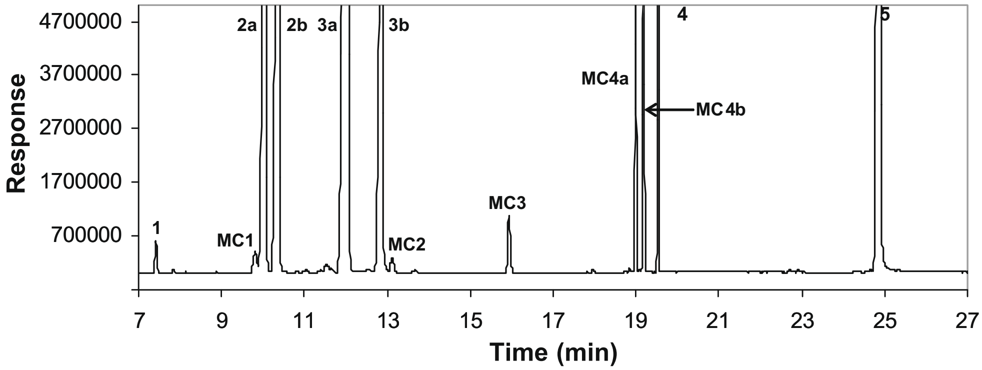

Fig. 1. GC–MS data of TMS oximes of carbohydrates present in carrot sample E. (1) Quinic acid; (2) fructose; (3) glucose; (4) internal standard; (5) sucrose; (MC1) mannitol; (MC2) *scyllo*-inositol; (MC3) *myo*-inositol and (MC4) sedoheptulose.

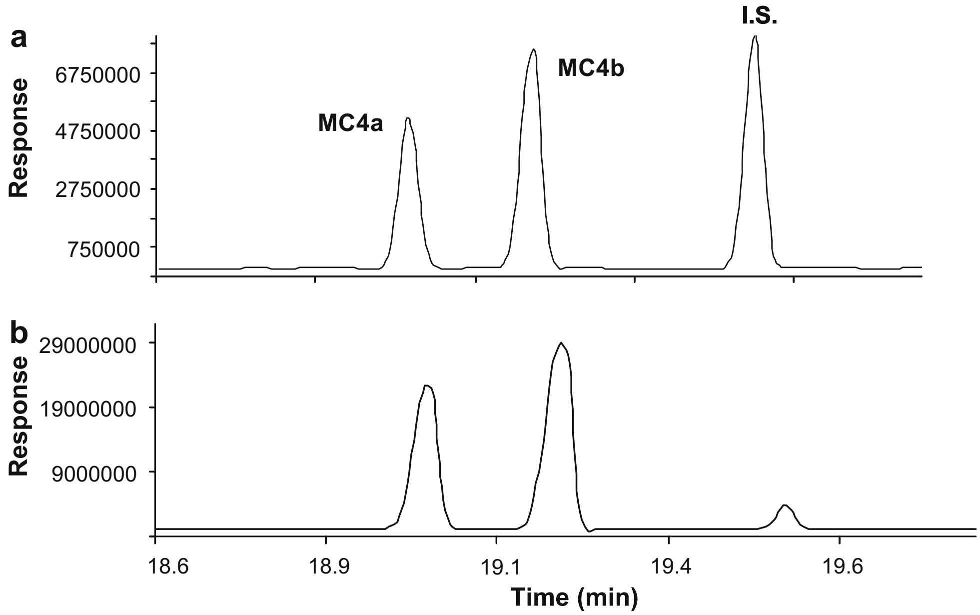

Fig. 2. Profiles obtained by GC–MS analysis of TMS oximes of carbohydrates present in extracts of (a) carrot E and (b) *Sedum* sp. (MC4a–b) sedoheptulose and (I.S.) internal standard.

Table 3
Linear retention indices (*LRI*) and mass spectra of TMS ethers obtained for carrot and *Sedum* sp. extracts.

LRI		Mass spectrum[a]	Assignation
Carrot	Sedum		
2073	2073	204(100), 73(61), 147(33), 217(30), 359(25), 539(25), 191 (12)	Sedoheptulose
2086	2086	217(100), 73(73), 147(46), 539(25), 359(23), 319(16), 191 (13)	
2091	2091	73(100), 217(83), 147(59), 539(49), 359(44), 191 (16)	

[a] *m/z* values (relative abundance in brackets).

All the carbohydrates identified in carrot samples were quantitatively determined by GC–FID using phenyl-β-D-glucoside as internal standard (Table 4). Fructose, glucose, and sucrose content determined on a dried weight basis ranged from 20 to 244, 17 to 245, and 137 to 689 mg g^{-1}, respectively, in raw carrots (A–D); these carbohydrates reaching values of 176, 194, and 407 mg g^{-1} in dehydrated samples (E–H). These results are close to average values reported by Rodríguez-Sevilla et al. (1999), who analysed the effect of processing on soluble sugar content of carrots, among other roots. The high variability observed for some of the samples here analysed is probably due to the carrot variety under analysis and the processing and/or storage conditions employed (Nyman et al., 2005; Svanberg et al., 1997).

The average total content of minor carbohydrates was 17 mg g^{-1} in raw carrots (A–D) and 23 mg g^{-1} in dehydrated carrots (E–H). Mannitol was only present in four of the eight carrot samples analysed, with a content significantly lower than 16 mg g^{-1} reported by Souci et al. (2000). *Myo*-inositol levels (2–10 mg g^{-1}) were in the same range as those reported by Clements and Darnell (1980) in raw, frozen and canned carrots (0.1–0.5 mg g^{-1}), after correction for the average water content of carrots.

Slight variations were observed in the polyalcohol content of the different carrot samples analysed, irrespectively of the carrot variety and type of product considered, whereas a wider range was found for sedoheptulose. In order to verify if the presence of these minor carbohydrates was common to other vegetables of the *Apiacea* family, samples of coriander, parsley, fennel and celery were analysed for sugar content (Table 4). High amounts of mannitol were detected in parsley and celery, in good agreement with

Table 4
Quantitative analysis of carbohydrates in carrot and other vegetables from the *Apiaceae* family. For identification of carrot samples A–H, see Table 1.

Sample code	Concentration (mg g^{-1} dried weight)						
	Fructose	Glucose	Sucrose	Sedoheptulose	*Scyllo*-inositol	*Myo*-inositol	Mannitol
A	244.0	244.8	497.3	14.6	1.6	2.8	1.3
B	204.6	225.8	136.9	5.5	1.5	2.4	1.3
C	122.0	122.8	285.7	17.0	2.3	2.2	––
D	19.9	16.8	688.8	1.4	5.8	9.8	––
E	175.8	194.4	232.9	22.2	2.0	3.4	0.9
F	77.1	52.8	284.0	24.6	2.9	3.6	0.4
G	51.5	43.8	391.5	10.2	2.4	4.2	––
H	24.8	14.9	406.8	7.9	2.7	4.5	––
Coriander	62.3	58.2	41.7	tr[a]	1.6	22.8	1.0
Parsley	11.2	20.6	10.4	tr[a]	1.9	5.4	31.5
Fennel	256.3	230.7	62.6	tr[a]	2.4	5.4	––
Celery	152.4	132.8	24.8	––	––	––	124.3

[a] tr: Traces detected in the ion chromatograms (*m/z* = 319) extracted from TIC profiles.

previous studies (Lewis, 1984; Stoop et al., 1996). Minor amounts of this polyalcohol were also observed in the coriander sample. Neither *scyllo*- nor *myo*-inositol were detected in the celery sample, these polyalcohols being present in the remaining vegetables from the *Apiacea* family under study. When characteristic ion masses of heptuloses were extracted from TIC, only traces of sedoheptulose were found in parsley, coriander and fennel.

4. Conclusions

At the sight of the results found in this work, we can conclude that together with mannitol and *myo*-inositol, two other minor carbohydrates (*scyllo*-inositol and sedoheptulose) were detected and identified in carrot for the first time. Whereas *scyllo*-inositol content was rather similar for the different carrot samples analysed, a wide range of variability in the concentration of sedoheptulose was found. This could probably be attributed to the different carrot variety and/or processing conditions of the samples under study.

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