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Extraction and Analysis of Inositols and Other Carbohydrates from Soybean Plant Tissues

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1. Introduction

An outstanding characteristic of soybean plants is their ability to produce large amounts of the carbohydrate pinitol. Pinitol and the closely related inositols are currently undergoing widespread investigation for their biological and nutritional value. These and all the carbohydrates are typically extracted and analyzed together. Therefore, this review includes a general discussion about the extraction and analysis of carbohydrates in plants as well as a more in depth examination of the biosynthesis and use of compounds related to pinitol. The multiple roles of these substances in plants and animals, and their synergism have not been fully realized. This review discusses not only the extraction and analysis, but also the diverse roles of the inositols with an emphasis on inositols from the soybean plant.

2. Carbohydrate production and nitrogen fixation

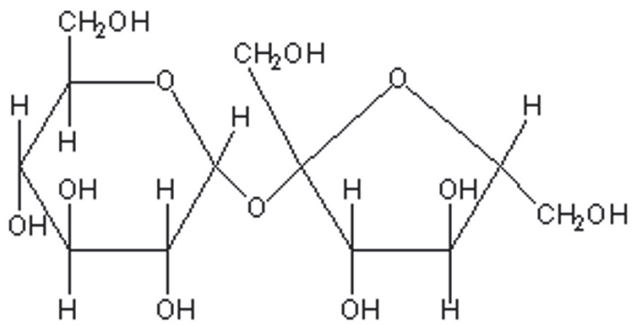
Carbohydrates are produced in plants by photosynthesis. Zhu et al. (2010) reviewed photosynthesis in relation to improving crop yield. Agronomically, there has been little benefit in breeding for increased photosynthesis indicating that the relationship of photosynthesis to yield is still not well understood (Farquhar & Sharkey, 1982; Pessarakli, 2005). The relative growth rate of shoots was shown to be correlated to the soluble carbohydrate level in the plant, but shoot growth was also impacted by plant stress (Masle *et al*, 1990). One commonly studied plant stress in relation to carbohydrate production is drought stress. There is confusion regarding the regulation of carbohydrate synthesis when plants are under drought stress. Drought stress in addition to reducing shoot growth, increases root growth (Sharp & Davies, 1979).

Approximately 70 million tons of fixed nitrogen or about 50 % of the total nitrogen that enters the terrestrial ecosystem comes from biological nitrogen fixation (Brockwell *et al.*, 1995; Tate, 1995). The relationship of carbohydrate availability to photosynthesis, phloem sap supply and N_2 fixation in legumes is complex and knowledge is incomplete (Udvardi & Day, 1997).

Carbohydrates are the main energy source for humans. Carbohydrates are classified according to the number of monomers they contain as monosaccharides (simple sugars), oligosaccharides, or polysaccharides. Carbohydrate metabolism in plants has been reviewed (Colowick & Kaplan, 1951; Ochoa & Stern, 1952; and Horecker & Mehler, 1955). Carbohydrate levels in soybean seed are highest at growth stage R 5.5, or when the seed is half-developed (Wilson, 2004). A significant portion of the carbohydrate produced by photosynthesis is respired in the plant roots. (Lambers *et al*., 1996).

3. Simple sugars

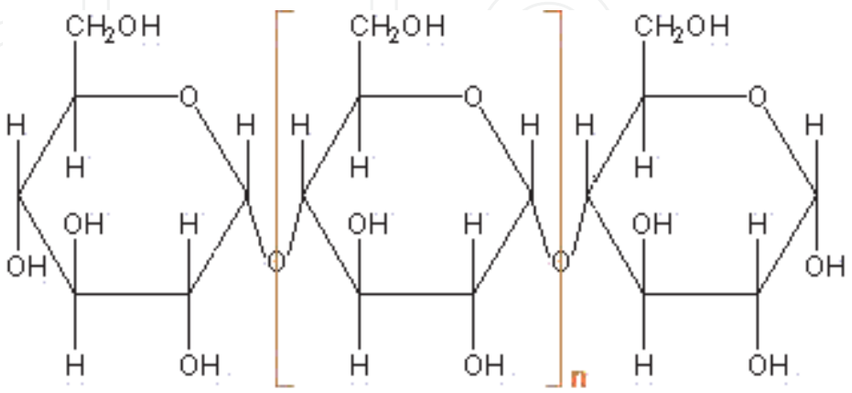
The most common simple sugars are glucose and fructose. Disaccharides consist of two covalently bound sugar molecules. Sucrose, for example, is a disaccharide consisting of glucose and fructose. Sugars have a role in energy, carbon transport molecules, hormone-like signaling factors, and as the source for building proteins, polysaccharides, oils and woody materials (Halford *et al*., 2010). Plant genotype and environment greatly affect the levels found in plants (Halford *et al*., 2010).



Sucrose

4. Complex carbohydrates

Complex carbohydrates (polysaccharides) are polymers of the simple sugars . Starch is the principal polysaccharide used by plants to store glucose.



(n is the number of repeating glucose units and ranges in the 1,000's)

Starch

Zeeman *et al*., 2010 reviewed the role of starches in plants. Starch breakdown commonly occurs when seeds germinate. Starch is also involved in malting (Halford *et al*., 2010). Glycogen, also a polymer of glucose, is the polysaccharide used by animals to store energy.
Another important polysaccharide is cellulose. Cellulose is used as a structural molecule to add support to leaves, stems, and other parts of plants. Although cellulose can't be used as an energy source in most animals, it provides essential fiber in the diet. Cell wall polysaccharides vary with plant groups and can include cellulose, xyloglucan, arabinoxylan, and pectin. In plants they make up the primary biomass and contribute to fiber in the human diet. This area has been reviewed by Scheller & Ulvskov, 2010; Fontes & Gilbert, 2010.

5. Extraction and cleanup

The methods used for isolating carbohydrates depend on the carbohydrate type, matrix, and purpose or type of analysis. However, some extraction procedures are commonly used for isolating carbohydrates from other classes of compounds in plants and foods. As an example, foods are usually dried under vacuum to prevent thermal degradation, ground to a fine powder to enhance extraction efficiency, and then remove the fats using appropriate solvent extraction.
A commonly used method for extracting low molecular weight carbohydrates from foods is to boil a sample with a 70-80% alcohol solution (Hall 2003, Asp 1993, Smith 1973.). Monosaccharides and oligosaccharides are soluble in alcohol solutions; however, most proteins, polysaccharides and dietary fiber are insoluble. The soluble components can then be separated from the insoluble components by filtering, soluble portion passes through the filter and the insoluble part retained by the filter. The two fractions can then be dried using lyophilization or nitrogen blow down techniques. In addition, monosaccharides and oligosaccharides and various other small molecules (e.g. organic acids, amino acids) may be present in the alcoholic extract. It is usually necessary to remove those components prior to carrying out a carbohydrate analysis, for example, with clarifying agents or by elution through one or more ion-exchange resins.
Water extracts of many foods contain substances that are colored or produce turbidity, and may interfere with analyses of carbohydrates; as a result, clarifiers may be needed. The most commonly used clarifying agents are heavy metals (e.g. lead acetate) which form insoluble complexes with interfering substances that can't be removed by either filtration or centrifugation. Ion-exchange is another method for removing interfering components prior to analysis. Many monosaccharides and polysaccharides are polar non-charged molecules and can therefore be separated from charged molecules by passing samples through an ion-exchange column. By using a combination of cationic and anionic resins it may be possible to remove most charged contaminants. Non-polar molecules can be removed by eluting through a column with a non-polar or hydrophobic stationary phase. Proteins, amino acids, organic acids, and hydrophobic compounds can be potentially removed from the carbohydrates in this manner prior to analyses.
Before analysis of the carbohydrates, residual alcohol (or other organic solvents) can be removed, if necessary, from the solution by evaporating under nitrogen or under vacuum using a rotary evaporator. For aqueous solutions, the sample can be concentrated using lyophilization.
Solid phase extraction (SPE) has also been reported for the cleanup and quantification of sugars and organic acids in herbal dry extracts. A three step SPE sequence was used for the

separation of sugars from the other components. A hydrophobic cartridge was used as the first cartridge followed ion and cation exchange cartridges (Schiller et al., 2002).

6. Analysis

Once the carbohydrate fraction has been isolated from other components of the plant, either the total carbohydrate content can be determined, or individual carbohydrates can be isolated, identified and quantified. The analysis of carbohydrates can be performed using any of several different methods. Two of these techniques include gas chromatography (GC) and liquid chromatography (LC). There are also spectral methods available including nuclear magnetic resonance (NMR), infrared (IR) and Raman spectroscopy. In this review, our focus is on the chromatographic and mass spectrometric methods.

7. Derivatization for GC or GC/MS analyses

The most prevalent method used for analyzing carbohydrates is probably GC and GC coupled with mass spectrometry (MS) due to the high resolution of GC and definitive nature of MS. Since carbohydrates are nonvolatile, it is necessary to hydrolyze the sugars and then derivatize them to increase their volatility so they can elute through a GC column for analysis. Methods involving the formation of methylated glycosides, acetates, acetals, trimethylsilyl ethers, and more volatile alditol acetate derivatives of monosaccharides have been widely used (McInnes et al., 1958; Bishop & Cooper 1960; Bishop 1964; Lehrfeld 1981; Blakeney et al., 1983). More recently, trimethylsilyl (TMS) derivatives of carbohydrates have been used principally due to their relative ease of preparation and increased volatility. (Sweeley et al. 1963; Sullivan & Schewe 1977; Honda et al., 1979; Li et al., 1983; Twilley 1984). Different structural forms of carbohydrates can complicate their chromatograms due to the production of several (as many as 5) peaks for each monosaccharide. Formation of the corresponding oxime TMS-derivative reduces the number of potential peaks (Decker & Schweer 1982; Al-Hazmi & Stauffer 1986; Long & Chism 1987). Dmitriev et al. (1971) prepared the aldononitrile acetate derivatives with the oxime intermediate. Churms (1990) found the derivatization process was not affected by the presence of water in the reaction mixture, helping to minimize processing steps. Methods for the separation of neutral sugars in gums have also been reported using similar methods (Al-Hazmi & Stauffer, 1986).

Silylation is a versatile technique to increase the volatility of various analytes, including carbohydrates, making them amenable to GC and GC/MS analyses. There are several practical considerations that should be addressed prior to derivatization of a sample by this method. One major disadvantage of silylation derivatives is that they are susceptible to hydrolytic attack by any moisture present in the sample, resulting in incomplete silylation. However, the trimethylsilylation of aqueous samples of hydroxyl compounds has been achieved using a large excess of derivatizing reagent (Valdez 1985). Evershetd (1993) discussed another problem associated with silylation of carbohydrates, the existence of multiple reaction products, resulting in complicated chromatograms. The multiple products result from the formation of anomers and interconversion between pyranose and furanose rings. Interconversion of the anomers occurs via the open chain form of the sugar, while mutarotation results from the opening and closing of the ring. The interconversions can be minimized by the use of rapid and mild derivatization conditions. If silylation is the method of choice for derivatization, it may be desirable to protect the keto group of the

monosaccharides prior to silylation in order to prevent the formation of enol-TMS ethers. These derivatives are unstable and complicate the analyses by giving rise to multiple products that can't be prepared quantitatively (Halket 1993).

In most instances, the silylating reagent is an adequate solvent. However, sometimes an additional solvent is required in the reaction. The selection of that solvent is critical to the success of the derivatization process. Any active hydrogens, including those present in the solvent, may be silylated. Pyridine has been found to be an ideal solvent for silylation reactions due to the increased solubility of the carbohydrates and their derivatives in that solvent (Evershed 1993). Heating slightly is often utilized to aid in efficient silylation,

One of the earliest reagents used for silylation was hexamethyldisilazane (HMDS). Usually, there is no need for additional solvents when HMDS is used. Recently, Ruiz-Matute et al. (2010) reviewed derivatization techniques of carbohydrates for GC and GC/MS analyses. Included in the discussion were derivatization of common sugars through the formation of ethers and esters, oximes, alditol acetates, aldononitriles, and dithoacetals (Evershed 1993).

Another silylating reagent is trimethylsilylimadazole (TMSI). Garland et al. (2009) analyzed soybean roots for pinitol using GC/MS (see Figs. 1-3). Roots were extracted in methanol and derivatized using TMSI. In this example a DB-5 capillary column was used in the splitless mode. The column eluents were analyzed by a double-focusing, four-sector mass spectrometer in the electron-ionization mode. Accurate mass measurements were also performed to determine the elemental composition of the parent and fragment ions. Under these conditions, a pinitol standard produced a single peak in the total ion chromatogram with a retention time of 9.18 min as shown in Fig. 1. Although several peaks appeared, pinitol's peak at 9.18 min was well-resolved.

The mass spectrum of TMSI-derivatized pinitol in Fig. 2 shows the major ion fragments detected from this, the most common carbohydrate in soybeans (Garland et al, 2009). In this example, the base ion is m/z 260. A comparison of the extracted ion plots of the soybean extract is shown in Fig. 3. A vertical, solid black line was added to each at the retention time of derivatized pinitol as determined from the standard. In the extracted ion plot of the soybean root, Figure 4 shows the total ion chromatogram of a TMSI-derivatized sugar beet extract. In this example no significant peaks appeared at the retention time of pinitol. The sugar beet root extract also showed no substantial peaks with the m/z 260 mass fragment.

The concentration of pinitol in soybean roots was approximately 4% of the soybean root's dry mass using a dry/fresh weight ratio of 54.5 mg DW/g FW (which is similar to 73.6 mg DW/g FW reported for alfalfa by Fougere, *et al.* (1991). The methanol extraction method appears to be effective for removing pinitol from the root tissue of soybean plants. The extent of extraction at the cost of time was encountered as well by Streeter and Strimbu's simultaneous extraction and derivatization method (Streeter & Strimbu 1998). Although they were able to reduce processing time, they were unable to extract as much pinitol from fibrous plant tissues in pyridine in 1 h when compared to complete extraction with ethanol for 24 h before derivatization (Streeter & Strimbu 1998).

Another benefit to using methanol extraction and TMSI derivatization is the relative simplicity of the resulting chromatograms. Eleven peaks were observed in the soybean extract chromatogram in Fig. 1, with pinitol clearly defined near 9.18 min. This compares with only 6 major peaks from sugar beet (Fig. 4) and 10 from snap bean roots (Fig. 5). The simplicity of the chromatograms is an indicator that pinitol and a small amount of other compounds are present in the methanol extract, which reduces the likelihood of coelution or some other interfering matrix effect with pinitol. This also provides support for the possible

use of methanol extraction as a first step in the purification of pinitol from soybean root tissue.

The mass spectrum of the derivatized pinitol shown in Figure 2 is very similar to that reported previously (Savidge & Forster, 2001). Identification of pinitol by mass spectrometry is made exceedingly easy by the presence of a high-intensity m/z 260 fragment ion. The fragment ion at m/z 260 appears to be a unique ion associated with pinitol and the other O-methylinositols compared with the other sugars observed using this analytical procedure. This allows for a high probability of quantitative results even in the event of another analyte coeluting with pinitol. The elemental composition obtained from accurate mass measurements for m/z = 260 was determined to be $C_{11}H_{24}O_3Si_2$, which was matched within 4.3 millimass units (mmu).

We have also extracted roots using 80% ethanol rather than methanol. This led to the extraction of a greater variety of inositols and O-methylinositols from several plant roots (unpublished data).

Permethylation is another derivatizing method for the analysis of carbohydrates. The methods using permethylation initially provided relatively long retention times. Some of the reactions to form permethylated derivatives include the use of methyl iodide/silver oxide (Gee & Walker 1962; Walker et al., 1962; Kircher, 1960) methylsulfinylcarbanion/methyl iodide (Hakomori 1964; Corey & Chaykovsky 1962; Moor & Waight 1975), and potassium/liquid ammonia/methyl iodide (Muskat 1934a; Muskat 1934b). Permethylation has also become very popular in the LC/MS analysis of carbohydrates.

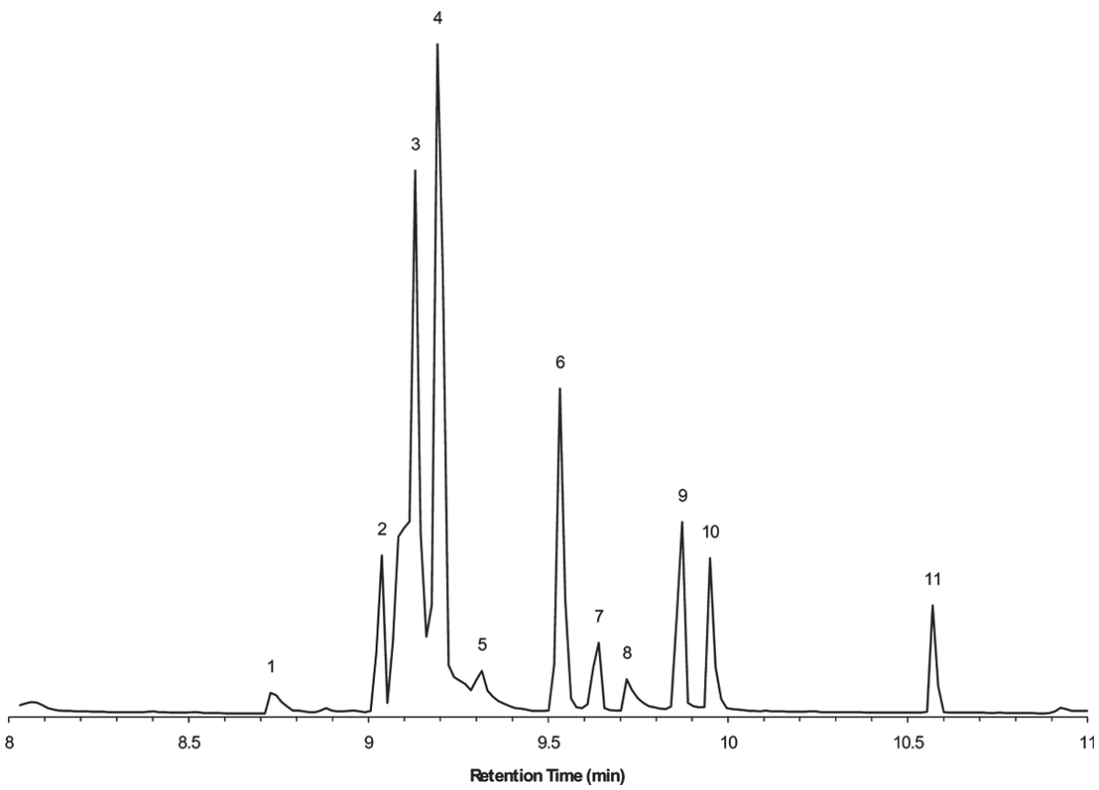


Fig. 1. Total ion chromatogram of an extract of soybean roots. Peak 4 was determined to be pinitol. From Garland, *et al* (2009).

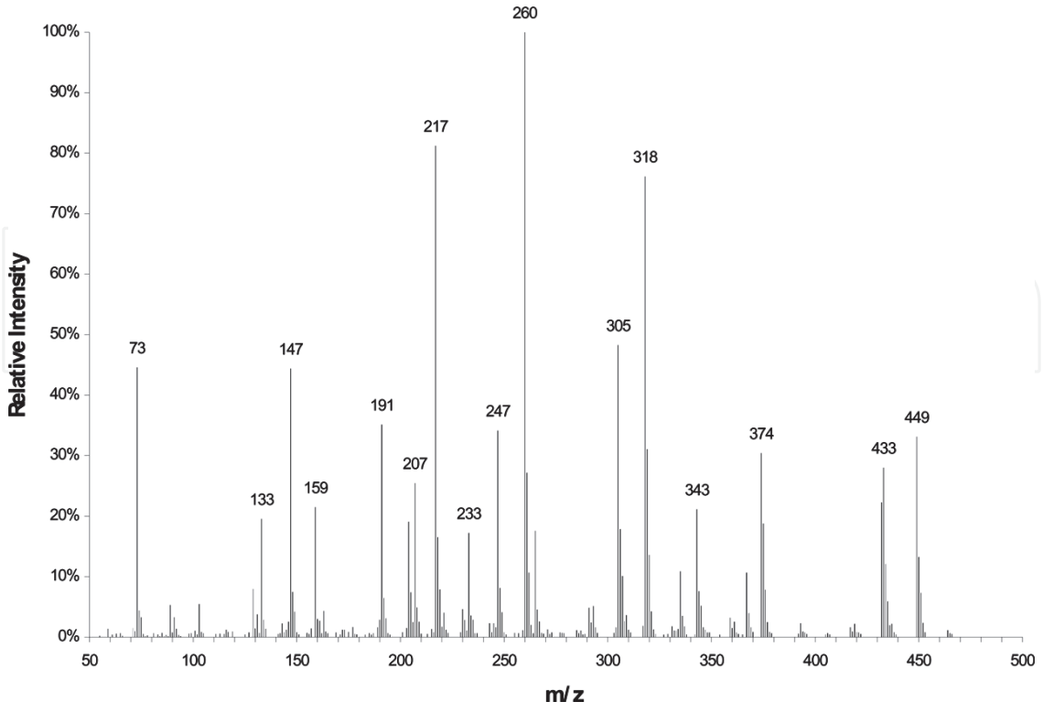


Fig. 2. Mass spectrum of TMSI-derivatized pinitol. From Garland, *et al.* (2009).

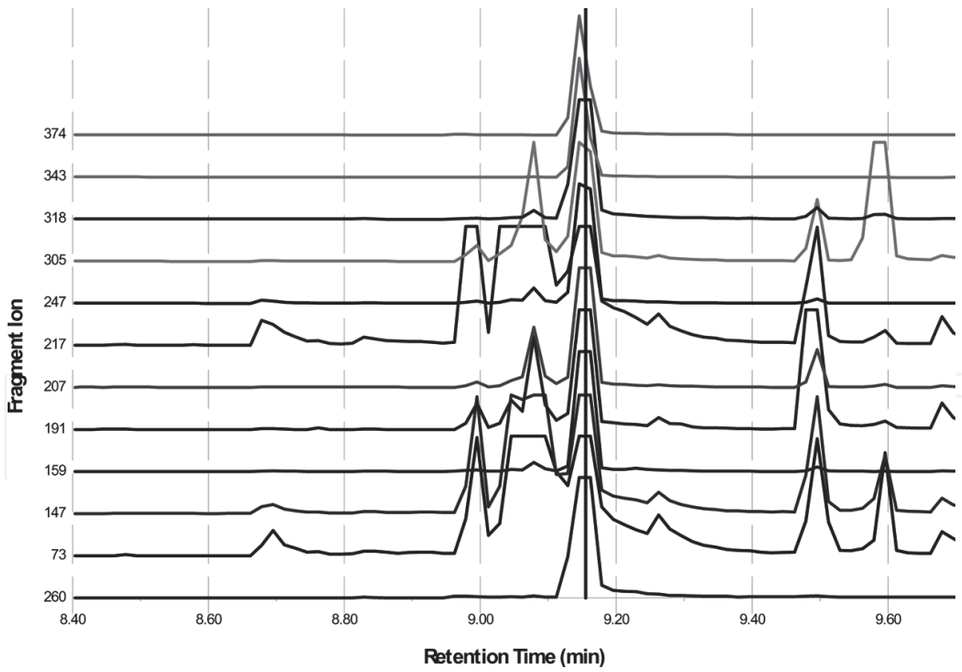


Fig. 3. Extracted ion plot of TMSI-derivatized soybean root extract. The labels on the vertical axis indicate the fragment mass of each extracted ion chromatogram. The chromatograms were spaced for easier representation. All peaks are on the same scale relative to their baselines. A vertical black line was inserted at the retention time of pinitol for reference. From Garland, *et al.* (2009).

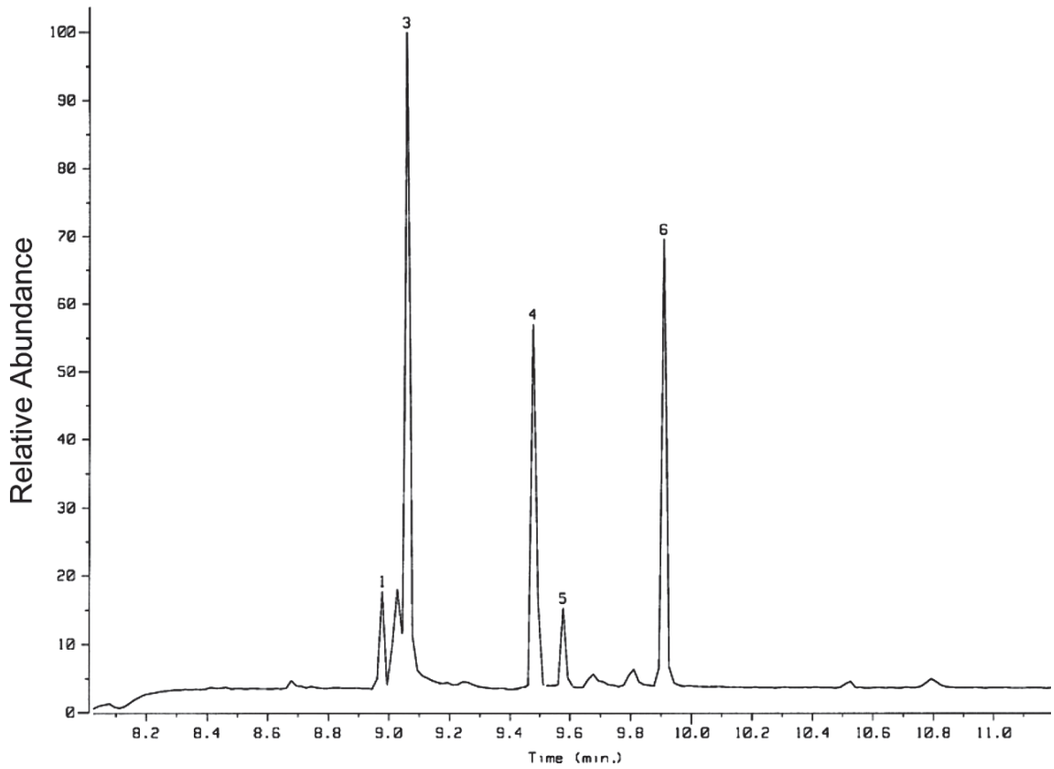


Fig. 4. Total ion chromatogram of derivatized sugar beet extract. Conditions were those of the chromatogram in Figure 1. Pinitol (retention time 9.2 min.) was not detected, as confirmed by MS analysis (Garland, *et al.*, 2009).

8. Other analytical techniques

Another technique for the separation and analysis of carbohydrates is liquid chromatography (LC). The column used in LC to provide the separation depends on whether the carbohydrates have been derivatized or not. Underivatized carbohydrates are commonly separated using ion exchange resins with water as an eluent and refractive index (RI) for detection. Refractive index detectors are, however, typically low in sensitivity, so samples need to be concentrated for quantitative analyses. The concentration of the carbohydrate must be in the percent range, and the RI detector can only be used with isocratic elution (Martens & Frankenberger 1990).

Other alternative detectors including both UV/visible absorbance and fluorescence require either pre-column or pre-detection derivatization of sugars, due to the fact that carbohydrates do not have a chromophore. Evaporative light scattering (ELS) is a detection technique used in high performance chromatography (HPLC) and supercritical fluid chromatography (SFC). It has been used for the analysis of carbohydrates and can act as a qualitative or quantitative detector (Wei & Ding 2000 ; Karlsson et al., 2005). The ELS is limited to solutes of low volatility. With the ELS, the column effluent is passed through a nebulizer and then into a heated drift tube; the solvent is evaporated leaving behind a particulate or aerosol form of the target compound. Light striking the dried particles that exit the drift tube is scattered and the photons are detected by a photodiode or photomultiplier tube at a fixed angle from the incident light. (LaPosse & Herbtreteau 2002).

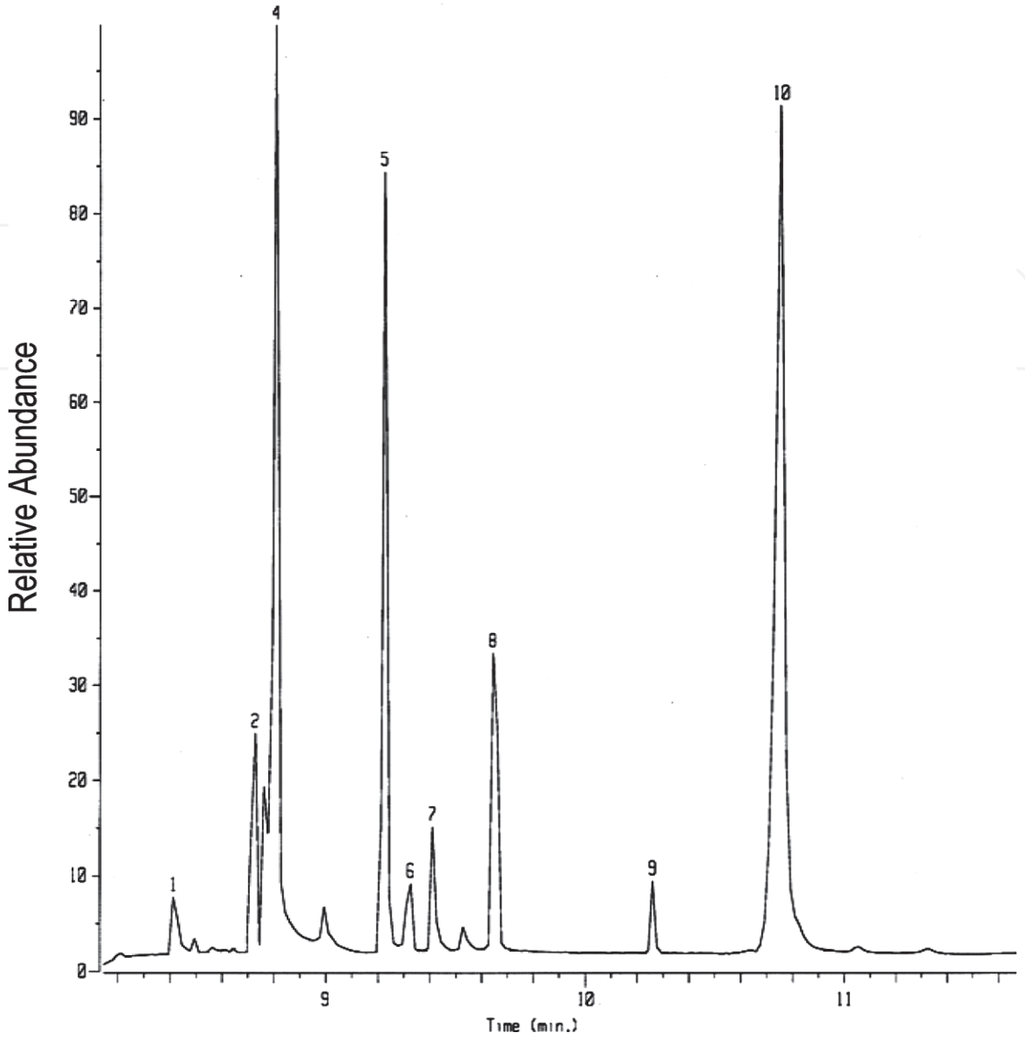


Fig. 5. Total ion chromatogram of derivatized snap bean root extract. Peak 5 was at a similar retention time to that of pinitol in Fig 1 (9.2 min.), but MS analyses were unable to detect pinitol in snap bean root extract (Garland, *et al.,* 2009).

Another detector commonly used is a pulsed amphoteric detector (Lee 1996; Johnson et al., 1993).

One derivatization procedure for carbohydrates to provide a chromophore for LC analysis involves a reaction with p-nitrobenzoyl chloride and pyridine. The reaction replaces the active hydrogens with a nitrobenzoyl group. The method was applicable to mono-, di-, and trisaccharides except fructose (Nachtmann & Budna 1977; Nachtmann 1976). Many of the derivatization reactions for carbohydrates are discussed by Knapp (1979). In addition, other derivatization techniques have been discussed (Meulendijk & Underberg 1990).

Mass spectrometry can also be coupled with LC. Examples are LC/MS and capillary electrophoresis/MS. Many of the LC techniques allow carbohydrates to be analyzed without prior derivatization as is necessary in GC and GC/MS analyses.

It should be noted that there is not one LC column that has been reported to separate every carbohydrate. Togami et al. (1991) discussed the separation of carbohydrates using cation-

exchange columns. Richmond et al. (1991) separated carbohydrates in dairy products. Henderson and Berry (2009) have utilized Zorbax columns for the separation of carbohydrates in Stevia sweetener. Romano (2007) discussed carbohydrate analysis in food products emphasizing column chemistries and detection. Several vendors offer LC columns for carbohydrate separation. Wilcox et al. (2001) also discussed several column types used for carbohydrate separation. Hydrophilic interaction chromatography (HILIC) has also been reported as a method for analyzing ionic or polar compounds, particularly biomolecules and drug metabolites (http://www.laboratoryequipment.com/article-is-hilic-in-your-future-ct92.aspx). Simple carbohydrate separations can also be performed on functionalized silica or resin-based columns (http://www.labnews.co.uk/feature_archive. php/4000/5/just-juice).
The separation of mono- and oligosaccharides are also performed using capillary electrophoresis. Different formats are capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP), and micellar electrokinetic chromatography (MEKC). These techniques are summarized in a review by Thibault and Honda (2003).

9. Liquid chromatography/mass spectrometry (LC/MS) and other MS techniques

Efficient separation methods such as high performance liquid chromatography (HPLC) and capillary electrophoresis combined with detection methods (e.g. mass spectrometry) that supply structural or compositional information is a preferred tool for the analysis of biomolecules, particularly carbohydrates. Liquid chromatography/mass spectrometry with both electrospray (ESI) and atmospheric pressure ionization (APCI) has spurred a major interest in the analysis of carbohydrates.
In ESI , the liquid containing the analyte(s) of interest is dispersed into a fine aerosol. Because the ion formation involves extensive solvent evaporation, the typical solvents for electrospray ionization are prepared by mixing water with volatile organic compounds (e.g. methanol, acetonitrile). To decrease the initial droplet size, compounds that increase the conductivity (e.g. acetic acid) are customarily added to the solution. Large-flow electrosprays can benefit from additional nebulization by an inert gas such as nitrogen. The aerosol is sampled into the first vacuum stage of a mass spectrometer through a capillary, which can be heated to aid further solvent evaporation from the charged droplets. The ions observed by mass spectrometry may be quasimolecular ions created by the addition of a hydrogen ion and denoted $[M + H]^+$, or of another cation such as sodium ion, $[M + Na]^+$, or the removal of a proton, $[M - H]^-$. Multiply-charged ions such as $[M + nH]^{n+}$ are often observed (Gaskell 1997). As examples, Fountain and Grumbach (2009) used negative ion electrospray mass spectrometry for the analysis of fructose, glucose, sucrose, and lactose. Taormina et al. (2007) and Mauri et al. (2002) used flow injection techniques with mass spectrometry. Fugimoto et al. (2005) used rubidium in the mobile phase as a complexing agent for both nuclear magnetic resonance and electrospray mass spectrometry analysis. Taylor et al. (2005) utilized ESI/MS to study fragmentation patterns of carbohydrates. Schlichtherle-Cerny et al. (2003) utilized a HILIC column coupled with ESI/MS for the analysis of amino acids, peptides, glycoconjugates, and organic acids in foods without prior derivatization.
In APCI, typically the mobile phase containing eluting analyte is heated to relatively high temperatures (above 400 C), sprayed with high flow rates of nitrogen and the entire aerosol

cloud is subjected to a corona discharge that creates ions. Often APCI can be performed in a modified ESI source. The ionization occurs in the gas phase, unlike ESI, where the ionization occurs in the liquid phase. A potential advantage of APCI is that it is possible to use a nonpolar solvent as a mobile phase solution, instead of a polar solvent, because the solvent and molecules of interest are converted to a gaseous state before reaching the corona discharge pin. Typically, APCI is a harder ionization technique than ESI, i.e. it generates more fragment ions relative to the parent ion.(Kostianinen et al., 2003). Kumaguai (2001) used atmospheric pressure chemical ionization mass spectrometry for the analysis of sugars and sugar alcohols without derivatization but did use methylene chloride or chloroform that was added post column to increase the sensitivity. The ions detected included $(M+Cl)^-$. Shimadzu application note also used solvent addition post column to improve sensitivity. This application also used APCI in the negative ion mode. Keski-Hynnila et al. (2004) compared APCI, atmospheric pressure photoionization, and electrospray in the analysis of phase II metabolites.

Other types of mass spectrometers used for analysis of carbohydrates include quadrupole time-of-flight (QTOF) mass spectrometers which allow both accurate mass (elemental composition) and MS/MS studies to be performed. Another mass spectrometer very useful for the analysis of carbohydrates is the ion trap (IT) MS. Ion trap technology has been described in (March & Todd 2005a, 2005b), and its major advantage includes the capability of MS^n which can provide additional structural information. Examples of glycoprotein analysis using IT have been described by (Stumpo & Reinhold 2010; Jiao et al., 2010; Reinhold et al., 1990).

Another technique that has been utilized for the analysis of carbohydrates is matrix assisted laser desorption/time-of-flight mass spectrometry (MALDI/TOFMS)(Harvey 1999, 2009)). In MALDI, the sample to be analyzed is mixed with a matrix, which in turns absorbs heat energy from irradiation with a nitrogen laser light. For example, dihydroxybenzoic acid (DHB) or ferulic acid which are commonly used as a matrices have a carboxyl group on a benzene ring. The DHB absorbs the energy and acts as a proton donor (Zenobi & Knochenmuss 1998). Time-of-flight mass spectrometry allows the majority of the ions generated throughout the mass range to be collected by the detector. MALDI has been primarily used to obtain spectra of very large polymers, biomolecules, and a variety of thermally labile materials (Hillenkamp et al., 1991, Nelson et al., 1990. We have also used MALDI/TOF for the analysis of smaller molecules (e.g. <500 amu) (Goheen *et al.*, 1997; Campbell *et al.*, 2001).

10. The inositols

Inositols (Fig. 6) are polyols of cyclohexane with the empirical formula $C_6H_{12}O_6$. There are potentially 9 stereoisomers of inositol but only five are naturally occurring (structure shown below). They are *myo*-inositol, *chiro*-inositol, *scyllo*-inositol, *muco*-inositol, and *neo*-inositol. Of these, *myo*-inositol is the precursor of the other four. *myo*-Inositol is synthesized from glucose.

The synthesis of *myo*-inositol uses the enzyme L-*myo*-inositol 1-phosphate synthase to catalyze the reaction which produces L-*myo*-inositol-1-phosphate from D-glucose 6-phosphate (Hoffmann-Ostenhof and Pittner, 1982). The L-*myo*-inositol-1-phosphate is then dephosphorylated through inositol monophosphate to produce *myo*-inositol (Loewus & Murthy, 2000). The enzyme that catalyzes this step is L-*myo*-inositol 1-phosphate synthase

(Stieglitz et al, 2005). The four other inositol isomers are derived from *myo*-inositol (Loewus and Murthy, 2000). The sequoyitol can then be epimerized to D-pinitol (See Fig. 7) which is demethylated to D-*chiro*-inositol using NADP-specific D-pinitol dehydrogenase (Stieglitz et al, 2005).

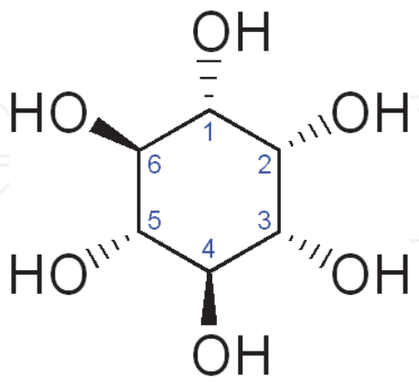


Fig. 6. Inositol.

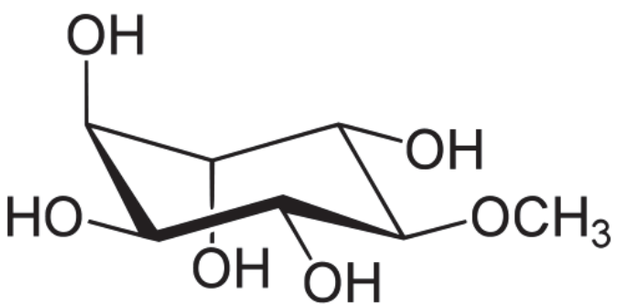


Fig. 7. Pinitol.

In addition to the five stereoisomers of inositol, the *O*-methylinositols can also be synthesized from *myo*-inositol. Of these, ononitol and pinitol are common to soybeans. Ononitol is a precursor to pinitol in soybeans (Loewus and Murthy, 2000; Chiera et al. 2006). Of the *O*-methylinositols, pinitol is most abundant in soybeans.

myo-Inositol is probably the most studied of all the inositols because it is the most commonly available. It has a very important function as it is required in the formation of Lecithin, which protects cells from oxidation and is an important factor in the building of cell membranes. Inositol, also has a metabolic effect in preventing too much fat to be stored in the liver, which is why it is called a lipotropic and is a vital part in maintaining good health.

Inositols have been found in many plants both foodstuff and other plants at varying evolutionary stages (Clements & Darnell, 1980; Chiera *et al*., 2006; Guo & Oosterhuis, 1997; Henry, 1976; Johansen *et al*., 1996; Johnson and Sussex, 1995; Johnson & Wang, 1996; Lind *et al*. 1998; Loewus *et al*., 1984; Manchanda and Garg, 2008; Ogunyemi *et al*., 1978; Phillips, et al 1982; Sheveleva *et al*., 1997; Streeter *et al*., 2001). Different soybean plant parts contain different levels of inositols as do soybean plants in vegetative verses reproductive growth stages (Phillips and Smith, 1974). Comparison of total inositols among plants should be examined carefully because each plant may produce different proportions of the various

inositols (Larson & Raboy, 1999). Research with pinitol in soybean documents that this cyclitol is a major consitutent of soybean (Phillips & Smith, 1974; Streeter, 1980; Phillips, et al. 1982; Dougherty & Smith, 1982). Because pinitol diffuses faster than carbohydrates during imbibition, it is theorized that loss of pinitol from soybean seed encourages the growth of Bradyrhizobium (Rhizobium) species in the soil needed for nitrogen fixation (Nordin, 1984). Accumulation of ononitol and pintol in soybean and other plants under drought conditions has been documented (Streeter *et al*., 2001; Guo & Oosterhuis, 1997; Manchanda & Garg, 2008; Sheveleva, et al, 1997).

Inositols are very important in general plant growth, seed storage, nitrogen fixation and protection of plants during stress. Inositol metabolism and its role in photosynthesis, plant health, and subsequent potential increase in yield is complex but new discoveries in this area may lead to future yield improvements. The role of inositols in nitrogen fixation is also complex and not currently fully understood. Inositols play an important role in phosphorus movement in the environment. Efforts are being made to alter the phytate content of soybean so animals can use the phosphorus and also reduce the amount that is excreted as manure. There are implications here not only for animal health but also for the preservation and sustainability of watersheds.

Phytate, *myo*-inositol hexakisphosphate, is found in almost all plant and animal cells and serves as an important phosphate reserve in plants (Irvine & Schell, 2001). Exposure of soybean cell suspension to *Psuedomonas syringae* pv *glycinea* indicated that whether a virulent or avirulent strain is used, the plant starts defense systems at the expense of housekeeping cell functions (Logemann *et al*., 1995; Shigaki & Bhattacharyya, 2000). Part of this defense reaction involves cellular cyosolic inositol and the IP3 pathway. This pathway is involved in cell division, growth and elongations and there is evidence that this pathway is inhibited when the plant is exposed to pathogens (Perera *et al*., 1999; Shigaki & Bhattacharyya, 2000). Selection of plants with reduced phytate levels raised the question of these plants' response to stress in the form of diseases. Murphy et al., 2008 found that disruption of phytate biosynthesis resulted in increased susceptibility in *Arabidopsis thaliana* to virus (potato virus Y), fungal (*Botrytis cinerea*) and bacterial (*Psueodomonas syringae*) diseases. The role of phytate in basal resistance to plant pathogens was previously unknown. Klink et al. (2009) found 1-phosphatidylinositol phosphodiesterase-related genes expressed when soybean plants are exposed to *Heterodera glycines*, a pathogen of soybean. The findings of inositols in plant defense are important findings and the next step is to determine whether the defense reaction is a general reaction or specific to different types of attacks.

Transgenic plants that release extracellular phytase from their roots have a significantly increased ability to acquire phosphorus from inositol phosphates from growth medium; however, there is less evidence that phosphorus nutrition of plants can be improved in plants grown in soil (George, *et al*., 2004). Phytate and phytic acid represent the major form of phosphorus in animal feed derived from plants. Phosphorus in seeds and tubers is stored primarily as phytate (*myo*-inositol exa*kis*phosphate), which is poorly digested by non-ruminant animals such as swine, poultry and fish (Saghai Maroof *et al*., 2009; Kim *et al*., 2006). The lack of the hydrolytic enzymes necessary for phytate to be utilized by these animals requires supplemental phosphate. Plant breeding efforts involve plant selections for improved phosphorus usage by animals and different feed additives resulting in less environmental pollution.

Inositol is synthesized sparingly in the body but is present in many foods. The inositols are essential nutrients for plants (Loewus and Murthy, 2000) and animals (Holub, 1986). Concentrations of the inositols and their metabolites can be much higher in some plant species than in mammalian tissue. For example, in soybeans, the concentration of pinitol alone approaches 30 mg/g (Streeter & Strimbu 1998; Garland *et al*., 2009) whereas in human blood, the levels of free *myo*-inositol is 3000 times lower (1 mg/100 mL). Levels of pinitol in blood is not widely known, but are anticipated to be orders of magnitude less than *myo*-inositol.

One form of inositol, inositol hexaniacinate, has been used to support circulatory health because it functions like niacin in the body. The major dietary forms of myo-inositol are inositol hexaphosphate or phytic acid, which is widely found in cereals and legumes and associated with dietary fiber, and myo-inositol-containing phospholipids from animal and plant sources.

Inositol is involved in the glucuronic acid and pentose phosphate pathways. Inositol exists as the fiber component phytic acid, which has been investigated for its anti-cancer properties. Inositol is primarily used in the treatment of liver problems, depression, panic disorder, and diabetes (Narayanan, 1987). Used with choline, it also aids in the breakdown of fats, helps in the reduction of blood cholesterol, and helps to prevent thinning hair (Walker, 2010). It promotes the export of fat from the liver. Inositol is required for the proper function of several brain neurotransmitters. Inositol may improve nerve conduction velocities in diabetics with peripheral neuropathy. Inositol may help protect against atherosclerosis and hair loss. There has also been the suggestion that it may help to reverse some nerve damage caused by diabetes (Gregersen et al. 1978; *Ibid*,1983). Inositol has also been tried for other psychological and nerve-related conditions including the treatment of side effects of the medicine lithium. Inositol also has a prominent calming effect on the central nervous system, so it is sometimes helpful to those with insomnia. Inositol may also be involved in depression.

Under pinitol deficiency, detrimental health conditions may exist such as higher blood sugar in diabetics (Geethan and Prince, 2008). Myo-inositol deficiency can lead to depression and other mental disorders (Levine et al, 1995; Benjamin et al, 1995; Fux, et al, 1996). Also, polycystic ovary syndrome (PCOS) has been reported to be related to a deficiency in dietary inositol (Gerli, et al. 2003; Ibid, 2007). Correlations with depression and similar disorders may be related to the abundance of inositol phospholipids in brain and other nervous system tissues. However, the relationship between pinitol and blood sugar levels is more likely correlated with the similarities in structure between the 0-methyl inositol and glucose.

There is no recommended daily allowance for inositol, but the normal human dietary intake is about 1 gram per day. Inositol is available from both plant and animal sources. Natural sources of inositol include soybeans, wheat germ, brewer's yeast, bananas, liver, brown rice, oak flakes, nuts, unrefined molasses, vegetables, and raisins. Most dietary inositol is in the form of phytate, a naturally occurring plant fiber.

Dietary effects of pinitol and ononitol are still in the earlier stages of discovery. It has recently been shown that pinitol lowers blood glucose levels in type II diabetics while significantly decreasing total cholesterol, LDL-cholesterol and the LDL/HDL-cholesterol ratio (Kim et al 2005). The dietary benefits or hazards of the other metabolites of isomers of inositol (other than myo-inositol) are under active investigation.

11. Conclusions and future directions

It is clear from this review that there are many different tools to study the carbohydrates in soybean plants. Results from any of the various analytical methods can be compared as long as they have been tested with adequate standards. The outstanding carbohydrate found in soybeans is pinitol, part of the inositol family. There has been considerable research into the value of the inositols, but most of the emphasis has been on myo-inositol, probably because it is widely available. However, for the soybean industry, it would be valuable to better understand the role of pinitol in health and nutrition. There are good indications that pinitol may have unique nutritional value, and key roles in soybean plant biology. Future directions should include the use of effective analytical methods to perform more research into the roles of pinitol and related inositols in various fields of nutrition, medicine, and plant biology.

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13. List of abbreviations

GC	gas chromatography
LC	liquid chromatography
MS	mass spectrometry
RI	refractive index
UV	ultraviolet
APCI	atmospheric pressure chemical ionization
IT	ion trap
ELS	evaporative light scattering
ESI	electrospray ionization
SPE	solid phase extraction
MS/MS	mass spectrometry/mass spectrometry
LC/MS	liquid chromatography/mass spectrometry
GC/MS	gas chromatography/mass spectrometry
MALDI/TOFMS	matrix assisted laser desorption/time-of-flight mass spectrometry
QTOF	quadrupole time-of-flight
TMS	trimethylsilyl
TMSI	trimethylsilyl imidazole
HMDS	hexamethyldisilazane

14. References

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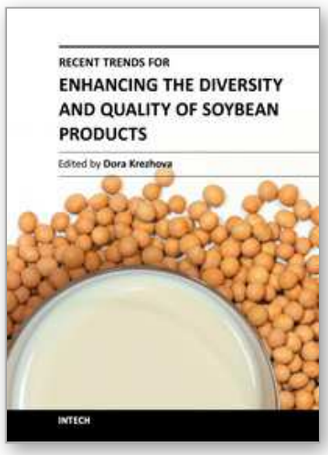

Recent Trends for Enhancing the Diversity and Quality of Soybean Products

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This book presents new aspects and technologies for the applicability of soybean and soybean products in industry (human food, livestock feed, oil and biodiesel production, textile, medicine) as well as for future uses of some soybean sub-products. The contributions are organized in two sections considering soybean in aspects of food, nutrition and health and modern processing technologies. Each of the sections covers a wide range of topics. The authors are from many countries all over the world and this clearly shows that the soybean research and applications are of global significance.

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