CHAPTER

6

Analytical Methods and Advances to Evaluate Dietary Fiber

M. Garcia-Vaquero

School of Veterinary Medicine, University College Dublin, Dublin, Ireland

OUTLINE

6.1 INTRODUCTION

Dietary fiber is a terminology that encompasses carbohydrates that are not hydrolyzed, digested, or absorbed in the upper gastrointestinal tract. These compounds can be fermented in the lower gastrointestinal tract, providing multiple health benefits when consumed regularly. Thus, it is considered as an essential constituent of a healthy and well-balanced diet (Elleuch et al., 2011; Tobaruela et al., 2018). There is evidence on the role of dietary fiber regulating a normal bowel function and protecting against metabolic disease and cancer. The protective role of fiber against colorectal cancer was recently emphasized by several governmental scientific reports in Denmark (Nordic Council of Ministers, 2014) and the United Kingdom (Scientific Advisory Committee on Nutrition, 2015). Moreover, the evidences of its protective role against cardiovascular diseases and type 2-diabetes have been used to determine the dietary requirements of fiber in the United Kingdom's population (Buyken et al.,

Dietary Fiber: Properties, Recovery, and Applications
https://doi.org/10.1016/B978-0-12-816495-2.00006-X

2018). The recommended dietary intakes of fiber in different countries vary from ≥25 to ≥30 g/day (Buyken et al., 2018) depending on the definition of dietary fiber adopted by those countries as well as the analytical methods used to quantify and identify these carbohydrates.

Recent advances in analytical chemistry, nutrition, and physiology influenced the definition of dietary fiber through the years. The Codex Alimentarius Commission updated the definition of dietary fiber in 2008/2009 setting the basis of new analytical methods, food labeling systems, nutrient reference values, and health claims related to these carbohydrates (Macagnan, Da Silva, & Hecktheuer, 2016). The Codex Alimentarius described dietary fiber as "carbohydrate polymers with 10 or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans" (Codex Alimentarius, 2008, 2009). This definition includes three main groups of compounds: (1) carbohydrates naturally occurring in food; (2) carbohydrates extracted from food raw material by physical, enzymatic, or chemical methods with beneficial health properties; and (3) synthetic polymers showing favorable physiological effects on health. Moreover, the benefits of these compounds for human health have to be demonstrated by generally accepted scientific evidences to the competent authorities (Codex Alimentarius, 2008, 2009).

One of the main controversies of the current definition adopted by the Codex Alimentarius refers to the inclusion of non-digestible oligosaccharides with three to nine monomeric units as dietary fiber. The inclusion of these compounds was left to consideration by the competent national authorities (Codex Alimentarius, 2009). Several countries and institutions updated their official definition of dietary fiber to include these oligosaccharides (see Table 6.1). However, in some cases, these updates in the definition of fiber were not reflected in the analytical

TABLE 6.1 Institutions and Countries That Accepted the Inclusion of Oligosaccharides (3–9 Monomeric Units) in Their Definition of Dietary Fiber

Institutions	Countries
American Association of Cereal Chemists	Brazil
Association Official Analytical Chemists	Canada
Codex Alimentarius Commission	Chile
European Food Safety Authority	China
Food and Drug Administration	Indonesia
Food Standards Australia and New Zealand	Japan
Institute of Medicine	Korea
International Life Science Institute	Malaysia
	Mexico
	Singapore
	Thailand
	Taiwan

(Adapted from Dai, F. J., & Chau, C. F. (2017). Classification and regulatory perspectives of dietary fiber. Journal of Food and Drug Analysis, 25, *37–42.)*

methods of reference used to quantify carbohydrates (Dai & Chau, 2017). For instance, the government of Taiwan defined dietary fiber as edible carbohydrates with a degree of polymerization $\geq$3, including lignin. However, the national reference method for the analysis of dietary fiber in this country remained unaltered, using the traditional method from the Association Official Analytical Chemists (AOAC) 985.29. This situation led to an underestimation of the dietary fiber contents in different products and conflicts in the food labeling system with respect to other countries (Dai & Chau, 2017).

Analytical complications may also derive from the current definition of dietary fiber. The Codex Alimentarius states that carbohydrates have to show health benefits to be considered as dietary fiber (Codex Alimentarius, 2008, 2009). Carbohydrates are normally associated to other bioactive compounds such as phenols, carotenoids, and phytosterols that display beneficial biological activities, i.e., antioxidant activities (Saura-Calixto, 2011). These associated compounds may influence the physicochemical and physiological properties of fiber and thus the health benefits experienced by the population consuming fiber-rich food products (Macagnan et al., 2016). Moreover, when the carbohydrates are extracted from natural matrices, the conditions used during the extraction and purification of these carbohydrates could result in structural modifications of the molecules and affect the biological properties of these compounds (Garcia-Vaquero, Rajauria, O'doherty, & Sweeney, 2017; Garcia-Vaquero, Rajauria, Tiwari, Sweeney, & O'Doherty, 2018).

This chapter reviews the current methodological approaches used to analyze carbohydrates, discussing in detail the official methods to quantify dietary fiber together with recent purification and characterization techniques used to gain a better understanding of the chemical structure and functions of this chemically diverse group of carbohydrates included in the current definition of dietary fiber.

6.2 OFFICIAL METHODS TO ANALYZE DIETARY FIBER

The classical methods to analyze dietary fiber in food before the change in the definition of this concept in 2008 were the AOAC 985.29 and 991.43. Both official methods are only able to quantify high-molecular-weight dietary fiber (HMWDF), expressed as total dietary fiber in the case of AOAC 985.29 or distinguishing between soluble and insoluble fiber to estimate the total dietary fiber in the methodology proposed by the AOAC 991.43 (Macagnan et al., 2016). The new definition of fiber proposed by the Codex Alimentarius Commission in 2008 included additional carbohydrates (i.e., resistant starch, RS) and opened the possibility to include low-molecular-weight oligosaccharides or low-molecular-weight dietary fiber (LMWDF) such as inulin, fructooligosaccharides, galactooligosaccharides, and polydextrose (Westenbrink, Brunt, & van der Kamp, 2013). Following this update in the definition of fiber, the classical methods AOAC 985.29 and 991.43 underestimate the dietary fiber contents of foods and thus, the energy value of food products made of or containing high levels of starch and LMWDF, inducing errors in food labels and composition tables (Macagnan et al., 2016). Furthermore, the inclusion RS as dietary fiber represents an analytical issue as the classical methods are only able to quantify retrograded starch or RS3, neglecting the RS1 (physical inaccessible starch), RS2 (ungelatinized starch granules), and RS4 (chemically modified starch) (Macagnan et al., 2016).

TABLE 6.2 Summary of the Official Methods to Analyze Dietary Fiber, as Described by the Association of Official and Analytical Chemists (AOAC), and the Compounds Measured by Each Method

AOAC Method	Compounds Measured
985.29	Total dietary fiber (high molecular weight)
991.42	Insoluble dietary fiber in foods
991.43	Total dietary fiber (high molecular weight: soluble and insoluble)
993.19	High-molecular-weight soluble dietary fiber in foods
993.21	High-molecular-weight dietary fiber (when >10% fiber and <2% starch)
994.13	High-molecular-weight dietary fiber, provides sugar composition and Klason lignin
995.16	β-Glucan in cereals, feeds, and foods
997.08	Fructans and fructooligosaccharides
999.03	Fructans and fructooligosaccharides (underestimates highly depolymerized compounds)
2000.11	Polydextrose
2001.02	Trans galactooligosaccharides
2001.03	High- and low-molecular-weight dietary fiber (if no resistant starch is present)
2002.02	Resistant starch (2 and 3)
2009.01	Total high- and low-molecular-weight dietary fiber in all foods
2011.25	Insoluble and soluble dietary fiber of high- and low molecular weight in all foods

(Adapted from Mccleary, B. V., Sloane, N., Draga, A., & Lazewska, I. (2013). Measurement of total dietary fiber using AOAC Method 2009.01 (AACC International Approved Method 32–45.01): evaluation and updates. Cereal Chemistry, 90, *396–414.)*

New official methods have been developed to measure specific compounds or fractions of dietary fiber separately, making it difficult to select the most appropriate method to quantify fiber when analyzing unknown food matrices (Westenbrink et al., 2013). The official methods described by the AOAC and the compounds estimated by each method are briefly summarized in Table 6.2.

The application of the classical methods and new AOAC methods combined will result in an overestimation of dietary fiber as several of these analytical determinations overlap (Macagnan et al., 2016; Westenbrink et al., 2013). The classical methods of dietary fiber AOAC 985.29 and 991.43 overlap with the methods to quantify inulin and fructooligosaccharides (AOAC 997.08 and 999.03), RS (AOAC 2002.02), polydextrose (AOAC2000.11), and maltodextrin (AOAC 2001.03). Moreover, the method to quantify maltodextrin (AOAC 2001.03) also overlaps with those of inulin and fructooligosaccharides (AOAC 997.08 and 999.03), polydextrose (AOAC2000.11), and other galactooligosaccharides (AOAC 2001.02). The multiple overlaps between the various AOAC methods are represented in detail in Fig. 6.1.

Recently, novel analytical methods were developed incorporating the use of high-performance liquid chromatography (HPLC), leading to the new official method AOAC 2009.01 and its later modification AOAC 2011.25. The method AOAC 2009.01 is a combination of the sample pretreatments described in AOAC 2002.02, the quantification of HMWDF as in methods AOAC 985.29-991.43, and the deionization and HPLC procedures for the

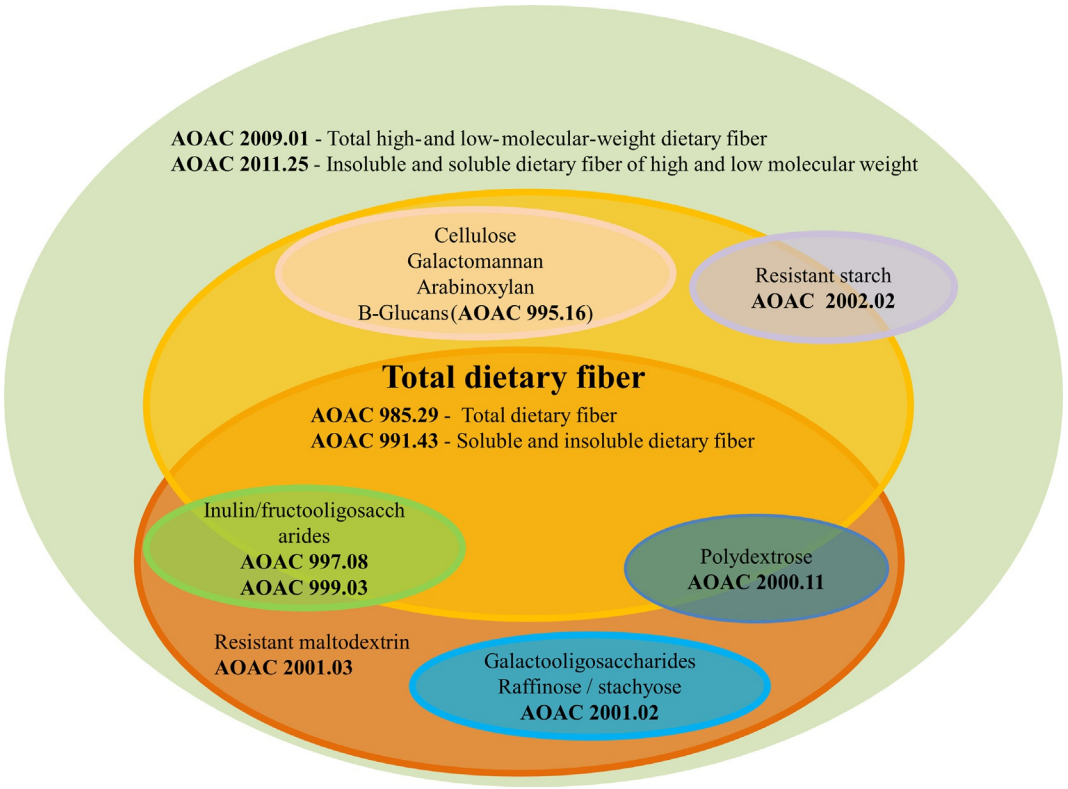

FIG. 6.1 Schematic overview of the official methods to analyze different fractions of dietary fiber and the analytical overlaps appreciated between them. *(Adapted from Westenbrink, S., Brunt, K. & van der Kamp, J. W. 2013. Dietary fibre: challenges in production and use of food composition data. Food Chemistry, 140, 562–567., Macagnan, F. T., Da Silva, L. P. & Hecktheuer, L. H. 2016. Dietary fibre: the scientific search for an ideal definition and methodology of analysis, and its physiological importance as a carrier of bioactive compounds. Food Research International, 85, 144–154. and Devries, J. 2010. Validating official methodology commensurate with dietary fibre research and definitions. Dietary Fibre: New Frontiers for Food and Health, 29–48..)*

quantification of LMWDF of the AOAC 2001.03 method (Mccleary, 2007; Westenbrink et al., 2013). In AOAC 2009.01, the sample is enzymatically hydrolyzed using α-amylase (37°C) followed by a protease treatment (60°C). The HMWDF (both soluble and insoluble HMWDF) is precipitated with 78% ethanol and determined gravimetrically, while the non-digestible oligosaccharides are measured in the ethanol filtrate by HPLC (Mccleary, 2007). The quantification of HMWDF described in AOAC 2009.01 was modified to measure soluble and insoluble fractions of HMWDF separately, leading to the development of the method AOAC 2011.25 (Mccleary et al., 2012).

The methods AOAC 2009.01 and 2011.25 could also result in certain analytical inaccuracies when analyzing starchy food matrices. The starch and maltodextrins may not be hydrolyzed fully by the enzymes, and thus, residual maltooligosaccharides could be erroneously quantified as LMWDF. Several modifications of these methods have been proposed, including additional enzymatic steps with amyloglucosidase (Brunt & Sanders, 2013; Mccleary, 2014) or

modifications of the incubation times of the samples using both α-amylase and amyloglucosidase, simulating the enzymatic treatment of the food passing through the human ileum (Mccleary, Sloane, & Draga, 2015).

6.3 OTHER METHODS TO ANALYZE CARBOHYDRATES

Analyzing dietary fiber is complex as the definition of fiber encompasses a wide variety of molecules of diverse chemical nature, such as polysaccharides (cellulose, β-glucan, hemicelluloses, gums, pectin, inulin, and RS), and oligosaccharides (fructooligosaccharides, oligofructose, polydextrose, galactooligosaccharides, raffinose, and stachyose). Moreover, the polysaccharide chitin and its derived molecule chitosan, obtained mainly from crustaceans' exoskeletons, are also considered as dietary fiber (Elleuch et al., 2011). The chemical nature of these compounds is defined by the number and type of monomeric units constituting the main chain of the carbohydrates, the presence or not of branching, and the type of links between the monomers as summarized in Table 6.3.

TABLE 6.3 Chemical Composition of the Main Carbohydrates Currently Included in the Definition of Dietary Fiber

Carbohydrates	Main Chain (Monosaccharides and Linkages)	Branches (Monosaccharide Composition)
Cellulose	β-(1,4) Glucose	
β-Glucans	β-(1,4) Glucose and β-(1,3) glucose	
Hemicelluloses		
Xylans	β-D-(1,4) xylose	
Arabinoxylans	β-D-(1,4) xylose	Arabinose
Mannans	β-D-(1,4) mannose	
Glucomanns	β-D-(1,4) mannose and β-D-(1,4) glucose	
Galactoglucomannans	β-D-(1,4) mannose, β-D-(1,4) glucose	Galactose
Galactomannans	β-(1,4) mannose	α-D-galactose
Xyloglucans	β-D-(1,4) glucose	α-D-xylose
Inulin	β-(2-1)-D-fructosyl-fructose	
Pectins		
Homogalacturonan	α-(1,4)-D-galacturonic acid	
Rhamnogalacturonan-I	(1,4) Galacturonic acid, (1,2) rhamnose and 1-, 2-, 4-rhamnose	Galactose, arabinose, xylose, rhamnose, galacturonic acid
Rhamnogalacturonan-II	α-(1-4) Galacturonic acid	Unusual sugars (apiose, aceric acid, and fucose)

TABLE 6.3 Chemical Composition of the Main Carbohydrates Currently Included in the Definition of Dietary Fiber—cont'd

Carbohydrates	Main Chain (Monosaccharides and Linkages)	Branches (Monosaccharide Composition)
Arabinanes	α-(1-5)-L-arabinofuranose	α-Arabinose
Galactanes	β-(1-4)-D-galactopyranose	
Arabinogalactanes-I	β-(1-4)-D-galactopyranose	α-Arabinose
Arabinogalactanes-II	β-(1-3)- and β-(1-6)-D-galactopyranose	α-Arabinose
Xylogalacturonan	α-(1-4) Galacturonic acid	Xylose
Gums		
Carrageenan	Sulfato-galactose	
Alginate	β-(1,4)-D-mannuronic acid or α-(1-4)-L-guluronic acid	
Oligofructose	β-(2-1)-D-fructosyl-fructose	
Polydextrose (synthetic)	D-gucose	
Resistant maltodextrins	α-(1-4)-D-glucose	α-(1-6)-D-glucose
Lignin	Polyphenols: syringyl, guaiacyl, and *p*-coumaryl	
Chitosan	β-(1-4)-Linked D-glucosamine and *N*-acetyl-D-glucosamine	

(Modified from Elleuch, M., Bedigian, D., Roiseux, O., Besbes, S., Blecker, C., & Attia, H. (2011). Dietary fibre and fibre-rich by-products of food processing: characterisation, technological functionality and commercial applications: a review. Food Chemistry, 124, *411–421.)*

Moreover, as the definition of dietary fiber includes the demonstration of the health benefits of the carbohydrates to be considered as fiber (Codex Alimentarius, 2008; Codex Alimentarius, 2009), further analytical tools are needed to elucidate the chemical structure of these molecules due to the strong structure-functionality associations described in carbohydrates. β-Glucans and other sulfated polysaccharides extracted from macroalgae showed variable biological activities depending on the chemical structure of the compounds, i.e., molecular weight, monosaccharide composition, sulfate content, and branching (degree and position of the chemical bonds in the molecules) (Garcia-Vaquero et al., 2017). Furthermore, the processes of extraction of carbohydrates from different matrices or food by-products and other experimental conditions used to purify and analyze carbohydrates could influence greatly the chemical structure of these compounds (Garcia-Vaquero et al., 2017). A recent study using ultrasound-assisted extraction to optimize the extraction of glucans for its use as a functional food and nutraceutical emphasizes the need to monitor the biological activities together with the yields of the carbohydrates of interest during the development of new extraction methodologies to obtain molecules with their intact biological benefits (Garcia-Vaquero et al., 2018).

Analytical tools such as chromatographic methods (including liquid and gas chromatography), nonchromatographic separations (field-flow fractionation and capillary electrophoresis), and structural elucidation methods (nuclear magnetic resonance (NMR) spectroscopy and near-infrared spectroscopy) could be used to gain a better understanding of dietary fiber. These techniques could complement the quantification analysis of the official methods and provide relevant information on the chemical composition of carbohydrates (i.e., monosaccharide composition and compounds associated to dietary fiber) together with more advanced chemical structure features (i.e., degree and position of branching, linkage type) that could influence the beneficial effects of these molecules.

The recent chromatographic, nonchromatographic, and other structure elucidation protocols and technological equipment used in recent research are described in detail in this section together with the sample preparation steps (i.e., clean-up, fractionation, and derivatization) needed before analyzing complex carbohydrates.

6.3.1 Sample Preparation

The application of one or more sample pretreatments are usually needed to concentrate or dilute the samples depending on the range of detection of the method and to eliminate other compounds that could interfere in the analysis of the carbohydrates of interest (Garcia-Vaquero & Rajauria, 2018). The preparation of the samples for chromatographic separations or other structural elucidation techniques is a critical process requiring multiple protocols to clean-up (to remove interfering molecules), fractionate (to separate the carbohydrates of interest from others present in the mixture or to increase their concentration for analysis), and generate derivatives of the carbohydrates (conversion of the molecules of interest into a more suitable form for its detection or separation) (Sanz & Martínez-Castro, 2007). The use of one or more of these sample preparation steps will depend on the chemical nature of the analytes (i.e., monosaccharides, oligosaccharides, or polysaccharides), the nature of the samples' matrices (i.e., simple or complex mixtures), and the choice of equipment to analyze the compounds (i.e., type of chromatographic separation and detector of choice). A schematic representation of the sample preparation steps required prior to carbohydrate analysis is presented in Fig. 6.2.

6.3.1.1 Sample Clean-up and Fractionation

A successful clean-up procedure is designed to remove any compound that could interfere with the chromatographic equipment (i.e., insoluble material that could damage the column) and other compounds of the sample with a similar chromatographic behavior to that of the carbohydrates of interest such as carboxylic acids and polyphenols (Garcia-Vaquero & Rajauria, 2018; Sanz & Martínez-Castro, 2007). The most frequently applied clean-up procedures include extraction and filtration techniques.

Extraction procedures include solid-phase extraction (SPE) and liquid-liquid extraction (LLE) used to fractionate carbohydrate mixtures or to eliminate other major residues from the samples before further analysis (Garcia-Vaquero & Rajauria, 2018; Sanz & Martínez-Castro, 2007). SPE uses solid particles of chromatographic material to separate the compounds of a sample based on their different physicochemical characteristics, while in LLE the analytes are transferred from an aqueous sample to a water-immiscible solvent (Garcia-Vaquero & Rajauria, 2018). Recent advances in SPE and LLE extraction procedures

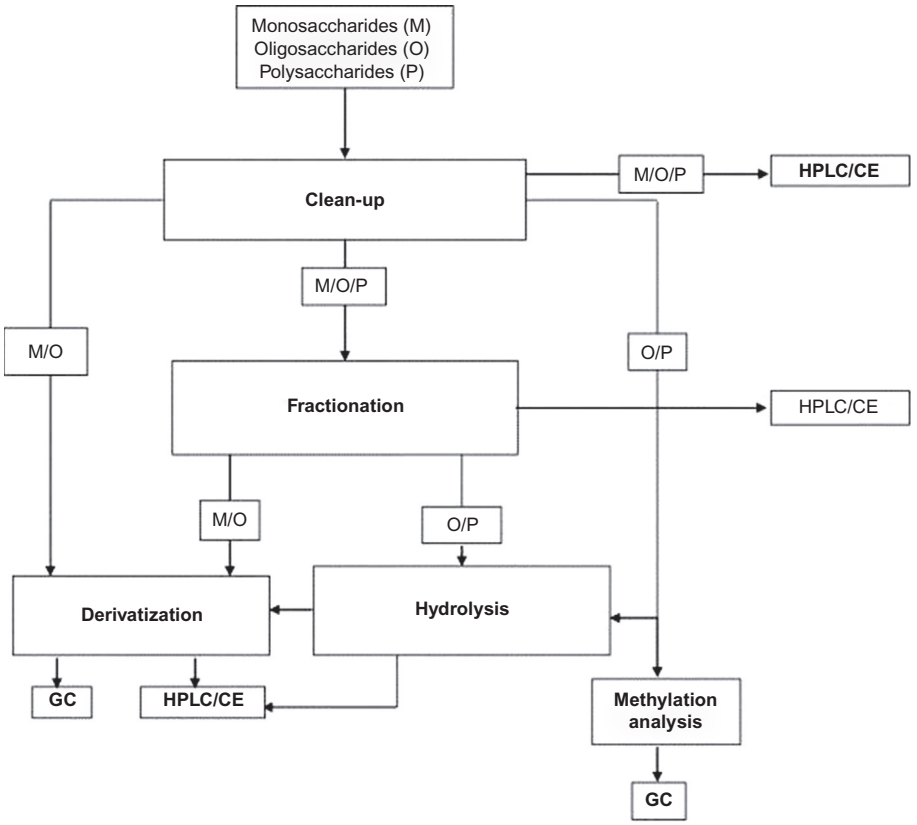

FIG. 6.2 Diagram from Sanz and Martínez-Castro (2007) illustrating the sample preparation steps required for the analyses of carbohydrates using high-performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis (CE).

include the miniaturization of these techniques, resulting in less expensive, time-consuming, and more environmentally friendly alternatives (Garcia-Vaquero & Rajauria, 2018). As an alternative to SPE and LLE, several studies proposed the use of other chemicals, such as Carrez reagents, acetate buffer/acetonitrile, or perchloric acid to precipitate proteins or lipids prior to carbohydrate analysis (Sanz & Martínez-Castro, 2007).

Usually, the sample mixtures are filtrated or centrifuged before their injection into a chromatographic system to avoid the presence of harmful materials that could damage the chromatographic columns (Garcia-Vaquero & Rajauria, 2018). The most widely used filters for this purpose include paper, glass fiber, and membrane filters (Sanz & Martínez-Castro, 2007). Moreover, most carbohydrate isomers only show structural differences in the configuration of their hydroxyl groups or in their molecular weight. The characterization of these molecules usually requires the use of one or several fractionation techniques to enrich the samples, facilitating the performance and interpretation of the analysis. Several filtration techniques have been used to fractionate carbohydrates, including the use of liquid-supported membranes and polymeric materials. The use of membrane technologies, such as ultrafiltration, nanofiltration, or dialysis is a recurrent topic in the recent scientific

literature, and it is explained in detail in recent publications (Galanakis, Tornberg, & Gekas, 2010; Jönsson & Mathiasson, 2001; Patsioura, Galanakis, & Gekas, 2011; Sanz & Martínez-Castro, 2007) and books (Galanakis, 2019).

6.3.1.2 Sample Hydrolysis

The hydrolysis or depolymerization of high molecular weight carbohydrates is normally necessary before performing further chromatographic analysis. For example, the analysis of carbohydrates using gas chromatography (GC) is based on the hydrolysis and later derivatization of the released monosaccharide residues (Ruiz-Matute, Hernández-Hernández, Rodríguez-Sánchez, Sanz, & Martínez-Castro, 2011). The protocols of hydrolysis usually require acidic solutions, i.e., trifluoroacetic, hydrofluoric, sulfuric, or hydrochloric acids, at different concentrations and temperatures. Moreover, the polysaccharides can be hydrolyzed by a methanolysis reaction using hydrochloric acid in methanol (Ruiz-Matute et al., 2011; Sanz & Martínez-Castro, 2007).

The use of enzymes (Sanz & Martínez-Castro, 2007) and other nonconventional methods, such as novel technologies, have been recently studied to hydrolyze carbohydrates. Thereby, microwave and sonication technologies have been successfully applied to reduce the time of hydrolysis and decomposition of the compounds while increasing the yields of depolymerization of the molecules previous to the chromatographic analysis of the samples (Ruiz-Matute et al., 2011). Moreover, there are new protocols combining all these hydrolytic procedures, such as the use of mild acidic solvent (0.2 M trifluoroacetic acid, during 72 h at 80°C of temperature) followed by an enzymatic treatment (Viscozyme L9) to analyze pectins (Garna, Mabon, Wathelet, & Paquot, 2004).

6.3.1.3 Derivatization Techniques

Carbohydrates are normally modified to increase the selectivity or sensitivity of the analysis when using liquid chromatography (LC) or to enhance the volatility of the compounds for analysis using gas chromatography (GC) (Garcia-Vaquero & Rajauria, 2018). The derivatization agents, reactions, and requirements are different if the samples are analyzed by LC or GC although in general all derivatization reactions should ideally be fast, complete, and without the generation of by-products.

In LC, the carbohydrates can be identified using multiple detectors, such as ultraviolet-visible (at low wavelengths), refractive index, and light-scattering detectors. Multiple derivatization methods have been proposed to incorporate chromogenic and fluorogenic groups in carbohydrates and increase the sensitivity and selectivity of the analysis in LC. The derivatization process in LC can be performed prior to pre-column derivatization, during or after the separation technique (post-column derivatization). The derivatizing agents, methodologies, and applications for the analysis of carbohydrates have been previously summarized and reviewed by multiple researchers in the field of analytical chemistry (Honda, 1996; Lamari, Kuhn, & Karamanos, 2003).

In general, when analyzing carbohydrates in GC, the most important features affecting the derivatization process of the molecules are the functional groups containing active hydrogens (-SH, -OH, -NH and -COOH), as these groups may form hydrogen bonds and influence the volatility and thermal stability of the analytes (Garcia-Vaquero & Rajauria, 2018). Classical derivatization methods focused on the replacement of these active hydrogens by other non-polar substituents. Methyl, trifluoroacetyl, trimethylsilyl, and *tert*-butyldimethylsilyl

ethers derivatives have been extensively used in GC to analyze carbohydrates of up to four or five monosaccharides, sugar alcohols, and amino sugars (Sanz & Martínez-Castro, 2007). Derivatization techniques and the applications of these methods to analyze carbohydrates using GC are summarized in literature, specifying the derivatization agents and the chemical reactions between the derivatization agent and the analytes of interest for its later detection (Harvey, 2011; Ruiz-Matute et al., 2011).

6.3.2 Chromatographic Techniques

Chromatographic techniques are based on achieving the separation of multiple compounds by distributing the components of a mixture between two phases: a stationary and a mobile phase (Scott, 2003). The use of chromatographic procedures has recently expanded and evolved due to advances in equipment and column packaging materials. The main chromatographic techniques used to analyze carbohydrates include gas chromatography (GC), liquid chromatography (LC), and thin-layer chromatography (TLC). These techniques could be used to fractionate or purify the carbohydrates of interest from a sample mixture or to characterize in detail the purified polysaccharides depending on the chromatographic separation used. Moreover, the choice of detector associated to the chromatographic separations will depend on the level of detail needed from the analysis. The detectors of choice to fractionate or purify carbohydrates are the ultraviolet-visible (UV) and refractive index (RI) detectors, while mass spectrometry (MS), electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI) detectors are frequently chosen in structural elucidation studies. The monosaccharide composition of carbohydrates and the position of glycosidic linkages between sugar residues of polysaccharides and small oligosaccharides are traditionally analyzed by GC associated with a MS detector (Di Stefano et al., 2012; Ruiz-Matute et al., 2011). LC techniques used to fractionate carbohydrates include high-performance liquid chromatography (HPLC), size exclusion chromatography (SEC), and ion-exchange chromatography (IEC) (Sanz & Martínez-Castro, 2007).

6.3.2.1 Gas Chromatography

Gas chromatography (GC) is based on the separation of analytes using a solid or liquid stationary phase and a gaseous mobile phase (i.e., nitrogen, helium, or hydrogen). The gas transports the analytes along the column, and the separation of the compounds is affected by the interaction of the analytes with the stationary phase, the vapor pressure of the compounds, and other chromatographic conditions used, such as the temperature of the column (Garcia-Vaquero & Rajauria, 2018). GC is an analytical technique with high resolution and sensitivity that could be easily coupled with different detectors including MS, making this analytical tool extremely valuable to analyze complex mixtures of carbohydrates and to provide rather complete structural information of the compounds (Ruiz-Matute et al., 2011).

As previously mentioned, the analysis of carbohydrates by GC requires sample preparation steps such as the derivatization of the compounds to modify the polar groups of the molecules and increase their volatility. The preparation of carbohydrates for GC analysis is complex due to the high number of functional groups in the molecule (mostly hydroxyl, but also carbonyl, carboxyl, amino, and ether groups), but also due to the presence of different

isomeric or tautomeric forms in the same sample solution and the association of carbohydrates to proteins or lipids (glycoproteins or glycolipids) that could generate complex chromatograms and complicate the interpretation of the results (Ruiz-Matute et al., 2011; Sanz & Martínez-Castro, 2007). Moreover, the fragmentation of the analytes provided with MS may be really similar in the case of close isomers, and other techniques will be needed for further elucidation of these chemical structures (Ruiz-Matute et al., 2011).

GC has been recently used by researchers to analyze multiple dietary fiber compounds including β-glucans extracted from mushrooms (Smiderle et al., 2017), pectins from plants, seeds, roots, mushrooms, and other plant by-products (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, & Fernández-Bolaños, 2012; Liang et al., 2012; Liu, Cao, Huang, Cai, & Yao, 2010; Morris, Ralet, Bonnin, Thibault, & Harding, 2010; Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, García, & Fernández-Bolaños, 2015; Wang, Wang, Huang, Liu, & Zhang, 2015) and polysaccharides from multiple sources (Athmouni et al., 2018; Lee et al., 2018; Li et al., 2018; Ma, Adler, Srzednicki, & Arcot, 2017; Xia et al., 2018; Zhang et al., 2018). The sample treatments, chromatographic equipment (i.e., column and detector details) and methodology used to analyze these carbohydrates using GC in the recent literature are summarized in Table 6.4.

Due to the relatively low volatility of carbohydrates, GC analyses are normally used to characterize low-molecular-weight carbohydrates (i.e., mono-, di-, and trisaccharides) (Ruiz-Matute et al., 2011). Complex polysaccharides can also be analyzed in GC by applying a hydrolysis step (normally acidic hydrolysis as seen in Table 6.4) to breakdown the carbohydrates to small saccharides before further derivatization and analysis. However, by using suitable derivatives and adjusting the GC conditions, researchers were able to analyze carbohydrates of up to 12 monosaccharide units (Carlsson, Karlsson, & Sandberg, 1992).

As seen in Table 6.4, the columns used to analyze carbohydrates using GC are mainly capillary columns, characterized by their dimensions, film thickness, and nature of the stationary phase that will influence the separation power or resolution, but also the sample load, speed, and sensitivity of the analysis (Garcia-Vaquero & Rajauria, 2018). The most commonly used detectors coupled to GC to analyze carbohydrates are flame ionization (FID) and mass spectrometry (MS). In FID, the samples are burnt in a flame, generating ions due to the breakdown of C—H bonds that will be detected by electrodes and processed to generate chromatograms (Vitha, 2016). In MS, high-energy electrons cause an ionization and breakdown of the analytes, generating charged fragments that pass through a mass-to-charge analyzer and are later detected as a function of mass-to-time or mass-to-charge ratio (m/z) (Vitha, 2016). The choice of detector coupled to GC will depend on the selectivity, detection limit, and linear ranges needed for the final purpose of each particular analysis (Garcia-Vaquero & Rajauria, 2018).

6.3.2.2 Liquid Chromatography

Liquid chromatography (LC) uses a liquid mobile phase and a column packed with highly porous particles to separate a mixture of compounds due to interactions of the analytes with both the mobile and stationary phases. The analytes that have strong interactions with the stationary phase and little affinity for the mobile phase will be highly retained. Several parameters of the stationary phase, i.e., the diameter of the particles and pore size, will significantly influence the separations in LC (Garcia-Vaquero & Rajauria, 2018). The main LC techniques used to analyze, fractionate, and purify carbohydrates in recent literature include high-performance liquid chromatography (HPLC) used in normal-phase and reverse-phase modes. Other LC methods are also used to fractionate mixtures of carbohydrates depending

TABLE 6.4 Experimental Conditions and Equipment Used to Analyze Carbohydrates Using Gas Chromatography (GC)

Compounds	Samples	Sample Pretreatments	Column	Conditions	Detector	References
Polysaccharides	Plant (*Periploca angustifolia*)	Hydrolysis (2 M trifluoroacetic acid (TFA)) and derivatisation (trimethylsilylation)	HP-5 column (30 m × 0.25 mm × 0.25 μm)	Temperature program: the initial temperature of the column was 150°C (holding this temperature for 1 min) and then it was increased to 180°C at an increase rate of 1°C/min, followed by an increase to 250°C (at 2°C/min) and from 250 to a final temperature of 300°C, holding the maximum temperature for 10 min. Carrier gas: helium at a flow rate of 1 mL/min	Mass spectrometer (MS)	Athmouni et al. (2018)
Polysaccharides	Plant (*Ulmus pumila* L.)	Hydrolysis (4 M TFA) and derivatisation (alditol acetate derivatives)	HP-5 column (30 m × 0.25 mm × 0.25 μm)	Temperature program: the initial temperature of the column was 140°C (holding this temperature for 5 min) and then it was increased at a rate of 4°C/min to a final temperature of 240°C, holding the maximum temperature for 5 min. Carries gas: nitrogen at a flow rate of 1 mL/min	Flame-ionization detector (FID)	Lee et al. (2018)
Polysaccharides	Maca (*Lepidium meyenii*)	Hydrolysis (2 M TFA)	DB-17 capillary column (30 m × 0.25 mm × 0.25 μm)	Temperature program: the initial temperature of the column was 180°C and then it was increased to 210°C at a rate of 5°C/min, followed by an increase from 210°C to 215°C at a rate of 0.3°C/min and a final increase from 215 to the final temperature of 240°C at a rate of 8°C/min	–	Li et al. (2018)

Continued

TABLE 6.4 Experimental Conditions and Equipment Used to Analyze Carbohydrates Using Gas Chromatography (GC)—cont'd

Compounds	Samples	Sample Pretreatments	Column	Conditions	Detector	References
Polysaccharides (aldoses, uronic acids, ketoses and amino sugars)	Mushroom (*Glossy ganoderma*) and plant (*Anemarrhena asphodeloides*)	Hydrolysis (2 M TFA) and derivatisation (trimethylsilylation)	DB-5 silica capillary column (60 m × 0.25 mm × 0.25 μm)	Temperature program: the initial temperature of the column was 80°C and it was increased to 190°C at a rate of 2.5°C/min, from 190°C to 252°C at a rate of 2°C/min, from 252°C to 300°C at 25°C/min and from 300°C to 310°C at a rate of 25°C/min, holding the maximum temperature for 15 min	MS	Xia et al. (2018)
Water-soluble polysaccharides	Mushroom (*Grifola frondosa*)	Hydrolysis (2 M TFA) and derivatisation (alditol acetate derivatives)	HP-5 capillary column (30 m × 0.25 mm × 0.25 μm)	Temperature program: the initial temperature of the column was 120°C (holding this temperature for 1 min) and then it was increased to 240°C at a rate of 10°C/min holding the maximum temperature for 5 min. Carrier gas: nitrogen	FID	Zhang et al. (2018)
Nonstarch polysaccharides (NSP)	Banana pseudostem	Enzymatic removal of starch, acid hydrolysis of the NSP and derivatisation (alditol acetate derivatives)	Supelco SP-2380 (30 m × 0.53 mm × 0.20 μm)	Temperature program: the temperature of the column was fixed at 210°C. Carrier gas: helium at a flow rate of 8 mL/min	FID	Ma et al. (2017)
β-Glucans	Mushroom extracts (*Pleurotus ostreatus* and *Ganoderma lucidum*)	Hydrolysis (2 M TFA) and derivatisation (alditol acetate derivatives)	DB-225 (30 m × 0.25 mm)	Temperature program: the initial temperature of the column was 50°C and it was increased to 210–220°C at an increase rate of 40°C/min. Carrier gas: helium	Mass spectrometer (MS)	Smiderle et al. (2017)

Polysaccharides	Mushroom (*Auricularia auricula*)	Hydrolysis (2 M TFA) and derivatisation	HP-5 capillary column (30 m × 0.32 × 0.5 μm)	Temperature program: the initial temperature of the column was 150°C (holding this temperature for 5 min) and then it was increased to 190°C at a rate of 10°C/min, followed by an increase from 190°C to a final temperature of 210°C at a rate of 2°C/min holding the maximum temperature for 5 min. Carrier gas: helium at flow rate 1.2 mL/min	–	Khaskheli et al. (2015)
Pectins and neutral oligosaccharides	Olive by-products	Hydrolysis (2 M TFA) and derivatisation (alditol acetate derivatives)	Supelco SP-2330 (30 m × 0.25 mm)	Temperature program: the initial temperature of the column was 180°C (holding this temperature for 7 min), followed by an increase to 220°C at a rate of 3°C/min. Carrier gas: helium at a flow rate of 1 mL/min	FID	Lama-Muñoz et al. (2012), Rubio-Senent et al. (2015)
Polysaccharides	Mushroom (*Cynomorium songaricum* Rupr.)	Hydrolysis (4 M TFA) and derivatisation (acetylated derivatives)	HP-5 column (0.25 mm × 30 m × 0.25 μm)	Temperature program: the initial temperature of the column was 160°C (holding this temperature for 3 min) and then it was increased to 210°C at a rate of 2°C/min	MS	Wang et al. (2015)
Pectins	Creeping fig seeds (*Ficus pumila* Linn.)	Hydrolysis (2 M TFA) and derivatisation (acetyl derivatives)	HP-5 column (30 m × 0.25 mm × 0.25 μm)	Temperature program: the initial temperature of the column was 160°C and it was increased to 210°C at a rate of 2°C/min, followed by an increase from 210°C to a final temperature of 240°C at a rate of 5°C/min holding the maximum temperature for 10 min	FID	Liang et al. (2012)

Continued

TABLE 6.4 Experimental Conditions and Equipment Used to Analyze Carbohydrates Using Gas Chromatography (GC)—cont'd

Compounds	Samples	Sample Pretreatments	Column	Conditions	Detector	References
Pectins	Mulberry branch bark	Hydrolysis (0.5 M H_2SO_4) and derivatisation (alditol acetates)	HP-5 column (30 m × 0.25 mm × 0.25 μm)	Temperature program: the initial temperature of the column was 180°C, the temperature increased to 240°C at 3°C/min and then to 260°C at 5°C/min. Carrier gas: nitrogen	MS	Liu et al. (2010)
Pectins	Sugar beet (*Beta vulgaris*)	Hydrolysis (2 M TFA) and derivatisation (alditol acetate derivatives)	DB-225 column (30 m × 0.32 mm)	Temperature of the column was constant at 220°C. Carrier gas: hydrogen	–	Morris et al. (2010)

on certain physicochemical properties of the molecules. For instance, the separation of carbohydrates can be achieved based on the charge of the molecules or on the molecular weight of the compounds by using ion-exchange chromatography (IEC) and size-exclusion chromatography (SEC) respectively.

HPLC methods used in normal-phase involve the use of a polar stationary phase (i.e., silica and alumina) and relatively nonpolar mobile phases (i.e., hexane modified slightly with polar solvents such as ethanol and acetone). Thus, the polar solutes tend to attach to the stationary phase while the nonpolar solutes elute in the mobile phase [44]. Normal-phase chromatography was the first mode developed to achieve chromatographic separations; however, this mode has been recently displaced by the use of reverse-phase HPLC (RP-HPLC) (Vitha, 2016). RP-HPLC uses a non-polar stationary phase and a polar mobile phase to separate the analytes on the basis of their hydrophobicity. RP-HPLC columns are normally packed with silica particles modified with C-18 chains, namely octadecylsilane or ODS columns. As previously mentioned, the detectors associated with RP-HPLC will depend on the information needed from the analyte, being the refractive index (RI), mass spectrometry (MS), and ultraviolet-visible (UV-VIS), the most commonly used detectors in recent studies (see Table 6.5). RP-HPLC was used to evaluate the synthesis of fructooligosaccharides (Ganaie et al., 2017; Ganaie, Rawat, Wani, Gupta, & Kango, 2014) and to analyze the monosaccharide composition of multiple carbohydrates, including polysaccharides, alginates, and pectins after an acidic hydrolysis and derivatization of the compounds depending on the nature of the detectors used (Imbs, Ermakova, Malyarenko, Isakov, & Zvyagintseva, 2016; Rascón-Chu et al., 2009; Wu et al., 2014).

In the case of IEC, the separation or fractionation of different carbohydrates is based on the adsorption of the analytes to the immobilized functional groups of an opposite charge of the column and its elution by changing the concentration or pH of the mobile phase (Garcia-Vaquero et al., 2017). Mixtures of carbohydrates can be fractionated using positively charged resins with affinity for negatively charged analytes (anion-exchange chromatography (AEC)) or negatively charged resins with affinity for positively charged molecules (cation exchange chromatography (CAE)).

Recent advances have been made in CAE resins to fractionate carbohydrates (Sanz & Martínez-Castro, 2007), and several researchers achieved the separation of monosaccharides from mixtures of oligosaccharides using CAE. Resins containing K^+ and Ca^{2+} were effective to purify glucose and fructose respectively (Vente, Bosch, de Haan, & Bussmann, 2005). However, AEC was the most used IEC technique to fractionate and analyze carbohydrates in literature as seen in Table 6.5. AEC techniques associated with pulse amperometric detector (PAD) were commonly used to fractionate oligo- and/or polysaccharide mixtures (Imbs et al., 2016; Lama-Muñoz et al., 2012; Rioux, Turgeon, & Beaulieu, 2007) and to purify multiple carbohydrates such as fucoidan (Garcia-Vaquero et al., 2017), fructans (Fu et al., 2018), and pectins (Morris et al., 2010). Moreover, AEC was used to analyze the monosaccharide composition of carbohydrates from different macroalgal and plant extracts after an acidic or enzymatic treatment of the samples (Lim, Yoo, Ko, & Lee, 2012; Rhein-Knudsen, Ale, Ajalloueian, & Meyer, 2017; Rong et al., 2017; Sousa, Nielsen, Armagan, Larsen, & Sørensen, 2015).

Size-exclusion chromatography (SEC) is one of the most powerful tools for the fractionation of carbohydrates to date. In SEC, a sample solution passes through an inert porous material packed in the column, commonly silica-based porous polymers, and the compounds are separated based on the principle of pore permeation. Unlike IEC, the carbohydrates are

TABLE 6.5 Summary of Liquid Chromatographic (LC) Methods and Conditions Used to Analyze Dietary Fiber, Including Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC), Anion-Exchange Chromatography (AEC), and Size-Exclusion Chromatography (SEC)

LC Method	Compounds	Samples	Sample Pretreatment	Column	Chromatographic Conditions	Detector	References
Reverse-phase high-performance liquid chromatography (RP-HPLC)	Fructooligosaccharides	Synthetic fructooligosaccharides	–	Sugar-pak column	Eluted with distilled H_2O. Flow rate: 0.2 mL/min	RI detector	Ganaie et al. (2014), Ganaie et al. (2017)
	β-Glucans	Seaweed (*Saccharina latissima*)	–	Kinetex C18 column (2.6 μm, 150 × 3 mm)	Eluted with 90% ultrapure H_2O and 10% acetonitrile. Flow rate: 0.2 mL/min	Mass spectrometry (MS)	Graiff, Ruth, Kragl, and Karsten (2016)
	Polysaccharides	Seaweed (*Coccophora langsdorfii*)	Hydrolysis (2 M trifluoroacetic acid (TFA)) and postcolumn derivatization (bicinchoninate reagent)	ISA-07/S2504 column (Shimadzu, Japan)	Eluted with a gradient of sodium borate buffer (from 0 to 0.4 M). Flow rate: 0.5 mL/min	Ultraviolet-visible (UV-VIS) detector	Imbs et al. (2016)
	Alginates	–	Hydrolysis and derivatization (1-phenyl-3-methyl-5-pyrazolone (PMP))	KP C18 column (5 μm, 150 × 4.6 mm)	Eluted with 0.1 M phosphate buffer (pH 7) and acetonitrile at a ratio of 83:17 (v/v). Flow rate: 0.8 mL/min	Variable wavelength detector (VWD)	Wu et al. (2014)
	Pectins	Apples (Golden Delicious)	Hydrolysis (4 N TFA) and filtration (0.45 μm)	Supelcogel Pb column (300 × 7.8 mm)	Eluted with H_2O. Flow rate: 0.6 mL/min	RI detector	Rascón-Chu et al. (2009)
Anion-exchange chromatography (AEC)	Fructans	Extracts from roots (*Codonopsis pilosula*)	Filtration (0.45 μm)	DEAE-Sepharose Fast Flow column (50 mm × 40 cm)	Eluted with H_2O. Flow rate: 2 mL/min	–	Fu et al. (2018)
	Alginates	Brown seaweeds (*Sargassum* and *Padina* spp.)	2 hydrolysis steps (H_2SO_4)	CarboPac PA20 column	Eluted with solvents (doubly deionized H_2O, 200 mM NaOH, and 1 M NaOAc in 200 mM NaOH) used at different gradients	Pulse amperometric detector (PAD)	Rhein-Knudsen et al. (2017)

β-Glucans	Extracts from highland barley	–	CarboPac PA-1 column (4 × 250 mm)	Eluted with 15 mM NaOH. Flow rate: 1 mL/min	PAD	Rong et al. (2017)
Polysaccharides (neutral compounds)	Seaweed (*Coccophora langsdorfii*)	–	DEAE-cellulose column (3 × 14 cm)	Eluted with H_2O and linear gradient of NaCl (from 0 to 2 M)	–	Imbs et al. (2016)
Pectins	Citrus	Viscozyme L multienzyme complex	Carbopac PA-1 column (Dionex Corp., Sunyvale, USA)	Neutral monosaccharides eluted with 100 mM NaOH. Uronic acids were eluted with a gradient (from 0 to 170 mM CH_3COONa ($3H_2O$) and 100 mM NaOH). Flow rate: 1 mL/min	PAD	Sousa et al. (2015)
Oligosaccharides (Neutral and pectic carbohydrates)	Olive by-products	–	Carbopac PA guard column (4 × 50 mm) and Carbopac PA-10 column (10 μm, 4 × 250 mm)	Eluted with combination of solvents 150 mM NaOH (eluent A), 150 mM NaOH and 600 mM CH_3COONa (eluent B) and 18 mM NaOH (eluent C). Flow rate: 1 mL/min	PAD	Lama-Muñoz et al. (2012)
Pectins	Yuza (*Citrus junos*) pomace	–	CarboPac PA1 column (4 × 250 mm)	–	PAD	Lim et al. (2012)
Pectins	Sugar beets (*Beta vulgaris*)	Hydrolysis (pectin A)	DEAE-Sepharose CL 6B column (30 × 2.6 cm)	Eluted with a gradient of NaCl in 0.05 M sodium succinate buffer pH 4.5 (from 0 to 0.4 M). Flow rate: 90 mL/h	–	Morris et al. (2010)

Continued

TABLE 6.5 Summary of Liquid Chromatographic (LC) Methods and Conditions Used to Analyze Dietary Fiber, Including Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC), Anion-Exchange Chromatography (AEC), and Size-Exclusion Chromatography (SEC)—cont'd

LC Method	Compounds	Samples	Sample Pretreatment	Column	Chromatographic Conditions	Detector	References
	Polysaccharides (alginate, β-glucan and fucoidan)	Seaweed (*Ascophyllum nodosum*, *Fucus vesiculosus* and *Saccharina longicruris*)	Filtration (0.45 μm)	Phenomenex Security cartridge (4 × 3) and Phenomenex Rezex RPM monosaccharide (100 × 7.8 mm)	Eluted with HPLC grade H_2O. Flow rate: 0.6 mL/min	Evaporative light scattering detector (ELSD)	Rioux et al. (2007)
Size-exclusion chromatography (SEC)	Alginates	Seaweed (*Sargassum angustifolium* and *Colpomenia peregrina*)	Filtration (cellulose acetate membrane)	TSK G5000 PW (7.5 × 600 mm)	Eluted with 0.15 M $NaNO_3$ and 0.02% NaN_3. Flow rate: 0.4 mL/min	UV, multiangle laser light scattering (MALLS) and RI detector	Borazjani et al. (2017), Rostami et al. (2017)
	β-Glucans	Extracts of malted barley	–	TSK PWXL guard column and 2 columns connected in series (TSKgel G5000PW and TSKgel G4000PW)	Eluted with 0.1 M $NaNO_3$, containing 500 mg/L NaN_3. Flow rate: 1 mL/min	Triple-detector array	Tomasi et al. (2017)
	β-Glucans	Barley	Enzymatic treatment (α-amylase) and filtration.	3 columns connected in series (2 AquaGel PAA-M columns and a PolyAnalytik PAA-203 column)	Eluted with 0.1 M $NaNO_3$ with 0.05% NaN_3 (w/w). Flow rate: 0.6 mL/min	RI detector	Zielke et al. (2017)
	Oligo- and polysaccharides (pectin, inulin, stachyose, raffinose)	Food-grade commercial samples	–	TSK-Gel PWH guard (75 mm × 7.5 mm) and TSK gel G 5000 PW column (300 mm × 7.5 mm)	Eluted with 0.01 M $NH_4CH_3CO_2$. Flow rate: 0.8 mL/min	ELSD	Condezo-Hoyos, Pérez-López, and Rupérez (2015)

Pectins	Citrus	–	4 columns (Biobasic SEC-60, 120, 300, and 1000, Thermo Fisher Scientific, Waltham, MA, USA)	Eluted with 0.3 M CH_3COOLi buffer pH 4.7. Flow rate: 1 mL/min	Light scattering detector (LSD), refractometer and differential viscometer detector (triple detection SEC3)	Sousa et al. (2015)
Neutral and pectic oligosaccharides	Olive by-products	–	2 Superdex Peptide HR 10/30 (30 × 1 cm) columns connected in line	Eluted with 100 mM $NH_4CH_3CO_2$ buffer at pH 5. Flow rate: 0.5 mL/min	Diode array detector (DAD) and RI detector	Lama-Muñoz et al. (2012)
Pectins	Yuza (*Citrus junos*) pomace	–	2 columns (BioSep-SEC-S2000 and S4000) connected in series	–	RI detector	Lim et al. (2012)
Pectins	Sugar beets (*Beta vulgaris*)	–	Shodex OH SB-G guard column (Showa Denko, Tokyo, Japan) and 2 columns (Shodex OH-Pak SB-805 HQ and Shodex OH-Pak SB-804 HQ) connected in series	Eluted with 50 mM $NaNO_3$ buffer containing 0.02 % NaN_3. Flow rate: 42 mL/h	UV, LSD, and RI detectors	Morris et al. (2010)
Polysaccharides (alginate, β-glucan, and fucoidan)	Seaweed (*Ascophyllum nodosum*, *Fucus vesiculosus* and *Saccharina longicruris*)	Filtration (0.45 μm)	TSK-guard column PWXL (6 mm × 40 mm), TSK-G5000 PWXL column (7.5 mm × 300 mm) and TSK-G3000 PWXL column (7.5 mm × 300 mm)	Eluted with 0.1 M NaCl. Flow rate: 0.5 mL/min	MALLS and RI detector	Rioux et al. (2007)

separated according to their molecular size, thus the molecular dimensions of the analytes and the average diameter of the pores of the stationary phase are key factors in this chromatographic separation. In SEC, the compounds do not bind to the stationary phase, and the composition of the buffer does not influence the separation of the analytes (Garcia-Vaquero et al., 2017; Sanz & Martínez-Castro, 2007). Some authors reported difficulties using SEC to analyze cellulose and other polysaccharides due to the formation of crystalline structures that do not allow solvents to penetrate into the molecule and break the intramolecular hydrogen bonds for further analysis (Sanz & Martínez-Castro, 2007). Despite these difficulties, SEC has been used to determine the molecular weight distributions of multiple mixtures of carbohydrates as seen in Table 6.5. SEC techniques using a single column were used to estimate the molecular weight of alginates (Borazjani, Tabarsa, You, & Rezaei, 2017; Rostami, Tabarsa, You, & Rezaei, 2017). However, most authors use SEC columns connected in series to fractionate oligosaccharides of different molecular weights, such as polysaccharides (alginate, β-glucan, and fucoidan) from brown macroalgae (Rioux et al., 2007), β-glucans from barley (Tomasi, Marconi, Sileoni, & Perretti, 2017; Zielke et al., 2017), and pectins from citrus and sugar beet (Lim et al., 2012; Morris et al., 2010; Sousa et al., 2015). Moreover, SEC is a fractionation technique that can be used alone (Borazjani et al., 2017; Rostami et al., 2017; Tomasi et al., 2017; Zielke et al., 2017) or combined with other HPLC methods such as IEC to characterize pectins (Lama-Muñoz et al., 2012; Lim et al., 2012; Morris et al., 2010; Sousa et al., 2015), neutral oligosaccharides (Lama-Muñoz et al., 2012), and polysaccharides, such as alginate, β-glucan, and fucoidan extracted from macroalgae *Ascophyllum nodosum*, *Fucus vesiculosus*, and *Saccharina longicruris* (Rioux et al., 2007).

6.3.2.3 Other Chromatographic Techniques

Thin-layer chromatography (TLC) is a chromatographic technique widely used to separate organic compounds such as amino acids, lipids, carbohydrates, vitamins, and other contaminants in food matrices (Sherma, 2000). TLC uses a solid stationary phase (alumina or silica particles of 12–20 μm) that allows the flow of a mobile phase due to capillary forces, separating the analytes of a sample placed on one end of the stationary phase depending on the degree of adsorption of the different analytes by the stationary phase. The degree of separation depends on the surface area and the particle size of the adsorbent (Touchstone, 1992). TLC was recently instrumentalized to develop high-performance TLC (HPTLC) using a small particle size (5 μm) stationary phase distributed in narrow and thin layers in the plate, increasing the speed of separation and improving the limits of detection of this technique (Sherma, 2000). TLC and HPTLC analyses have certain advantages over other chromatographic methods, such as the relatively easy use of these techniques together with the low cost and high efficiency of these separations, as many samples can be analyzed in one plate using low volumes of solvents (Sherma, 2000).

Initially, inexpensive and fast analyses of carbohydrates were performed using paper chromatography. Traditionally, TLC methods offered certain disadvantages to analyze polar compounds, including carbohydrates, such as the creation of strong interactions between the analytes and the stationary phase and the need to derivatize the carbohydrates to achieve an efficient fractionation (Zhang, Xiao, & Linhardt, 2009). The recent developments in adsorbents, solvents, and derivatization agents used in TLC to analyze carbohydrates (mainly monosaccharides, disaccharides, and oligosaccharides) were summarized by Zhang et al. (2009).

TLC protocols were recently used to evaluate the efficiency of novel enzymes degrading carbohydrates such as β-glucosidase. The carbohydrate mixtures were loaded in a TLC silica

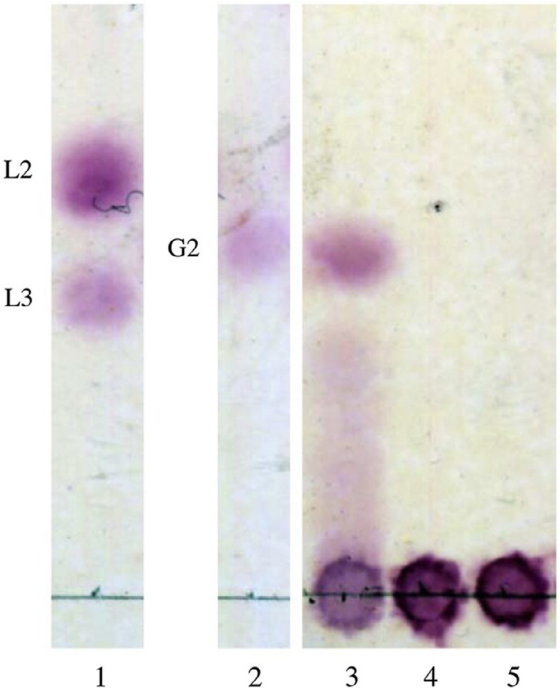

FIG. 6.3 TLC plate provided by Motone et al. (2016) showing the separation of multiple carbohydrate standards (lanes 1 and 2) and the degradation products obtained from laminarin samples (lanes 3 to 5).

gel plate, using as a mobile phase *n*-butanol/acetic acid/water (3:2:2, *v*/*v*/*v*) and visualizing the separation using 10% H_2SO_4 and 0.2% naphthoresorcinol in ethanol (Kim, Kim, Lee, & Kim, 2018). Similarly, the fermentation of β-glucans and changes in carbohydrate profile during the enzymatic process were monitored using TLC (see Fig. 6.3), with acetate/acetic acid/water as mobile phase (3:2:1, *v*/*v*/*v*) and 20% sulfuric acid solution in ethanol containing 0.2% 1,3-naphthalenediol as visualization reagent by heating the plate at 80°C for 5 min (Motone, Takagi, Sasaki, Kuroda, & Ueda, 2016).

HPTLC was the technique of choice to analyze beets and molasses in the sugar industry (Mantovani, Vaccari, Dosi, & Lodi, 1998), and novel HPTLC were developed to analyze carbohydrates in biorefinery streams without applying purification or sample pre-treatments (Oberlerchner, Böhmdorfer, Rosenau, & Potthast, 2018). This method was able to achieve a separation of the main monosaccharides present in the wood lignocellulosic biomass (glucose, xylose, mannose, arabinose, and galactose) without interferences of other compounds present in the original matrix by using acetonitrile/1-pentanol/water (4:1:1, *v*/*v*/*v*) as a mobile phase on a silica stationary phase impregnated with a phosphate buffer (Oberlerchner et al., 2018).

6.3.3 Nonchromatographic Techniques

Other nonchromatographic techniques can also be useful to fractionate carbohydrates, especially when dealing with complex structures that cannot keep their structural integrity intact during the process of analysis using LC techniques. Non-chromatographic analytical tools commonly used to analyze carbohydrates include field-flow fractionation (FFF) and capillary electrophoresis (CE).

6.3.3.1 Field-Flow Fractionation

FFF technique is based on the separation of different compounds of a sample in an opened and unpacked channel. A small volume of sample is injected and pushed through the channel by a longitudinal mobile phase flow while simultaneously applying a perpendicular field (electric, thermal, magnetic, or gravitational forces). The separation of the sample compounds is achieved by the balance of the analytes between the mass transport created by the field acting opposite to the Brownian motion forces inside the channel (Herrero, Cifuentes, Ibáñez, & Castillo, 2011; Sanz & Martínez-Castro, 2007). This method is mainly used to separate large polysaccharides that cannot be fractionated using SEC, such as dextran, pullulan, starch, cellulose derivatives, and heteroglycans (Herrero et al., 2011, Sanz & Martínez-Castro, 2007). FFF coupled with multi-angle light scattering detector was recently used to analyze starch from wheat and barley (Dou, Zhou, Jang, & Lee, 2014) and dextran polymers for their use as an alternative to fossil-derived materials (Faucard et al., 2018; Vuillemin et al., 2018).

6.3.3.2 Capillary Electrophoresis

Carbohydrate mixtures were also fractionated using polyacrylamide slab gels, paper, silylated glass-fiber paper, and capillary tubes. Capillary electrophoresis (CE) is a fast, automated, miniaturized, and highly efficient analytical tool to analyze organic acids, amino acids, or carbohydrates, including monosaccharides and small oligosaccharides, but also complex carbohydrates, such as glycoproteins (Paulus & Klockow, 1996). Unlike HPLC, CE uses capillary tubes to fractionate the analytes using minimum sample preparation procedures as the matrix does not influence the performance of the separation. CE can be associated with quantitative detectors similar to those used in HPLC, being an analytical tool especially suitable to analyze compounds in complex matrices such as food and beverages (Soga & Serwe, 2000).

Carbohydrate analyses using CE normally require derivatization of the compounds for the detection of the molecules using UV as shown in multiple protocols summarized elsewhere (Cortacero-Ramírez, Segura-Carretero, Cruces-Blanco, Hernáinz-Bermúdez de Castro, & Fernández-Gutiérrez, 2004;Soga & Ross, 1999 ; Soga & Serwe, 2000). Several alternatives to derivatization have been developed, including the ionization of carbohydrates by using high-alkaline electrolytes to make them suitable for indirect UV detection. The indirect UV detection of carbohydrates showed promising results analyzing sugars (acidic, neutral, and amino sugars), monosaccharides in glycoproteins, and sugar alcohols in food and beverage samples (Soga & Ross, 1999; Soga & Serwe, 2000).

Unlike HPLC methods, CE offers the possibility to analyze intact complex molecules. The analyses of certain gums such as alginates usually require an enzymatic or acidic depolymerization to release uronic acids detected using colorimetric methods, IEC, and SEC. This indirect quantification is time and cost consuming due to the application of multiple sample pre-treatments, and less accurate if colorimetric methods are used in comparison with other chromatographic techniques (Awad & Aboul-Enein, 2013). A protocol using methanol, HCl, and water to concentrate alginic acid in the samples was developed using fused silica capillary and boric acid buffer to quantify alginates using an UV detector at 200 nm (Moore, Miao, & Benikos, 2004).

6.3.4 Structure Elucidation of Carbohydrates

Isomers are compounds with the same molecular formula and different spatial arrangements of the atoms within the molecule. Large molecules, including carbohydrates, display isomeric forms that could have distinct biological functions within the cells, being involved in structure recognition activities such as substrate-ligand interactions in enzymes and receptors. Different isomeric forms of glycans were linked to cell interaction and signal transduction functions in cells (Furuhashi & Okuda, 2017). Moreover, the cells can produce different isomeric compounds using the same monomeric units. For example, glucose is the monomeric unit of the disaccharides trehalose (1–1 linkage) and laminaribiose (1–3 linkage). However, laminaribiose forms part of laminarin, an energy reserve polysaccharide, while the production of trehalose is induced by abiotic stress in the cells (Furuhashi & Okuda, 2017).

The identification of isomeric forms of carbohydrates requires a meticulous purification of the carbohydrates before using more advanced structural elucidation analyses, such as NMR and Fourier transform infrared (FTIR) spectroscopies.

6.3.4.1 NMR Spectroscopy

NMR spectroscopy is a powerful analytical tool to analyze the structure of carbohydrates, providing information on the type and number of glycosidic linkages and on the presence of α- and β-anomeric configurations in the molecules (Bai & Shi, 2016). NMR is a non-destructive method to obtain structural information of the molecules in a reasonable time. However, NMR equipment is relatively expensive in comparison with other analytical techniques; the samples require labor-intensive and time-consuming pretreatments, and the sensitivity of NMR is lower than that of mass spectrometry (MS). NMR applies a magnetic field and generates a chemical shift or resonance frequency of an atom depending on the chemical environment of the nucleus in a molecule. Most elements have at least one naturally occurring NMR active isotope, i.e., ^{1}H is the most abundant isotope in nature in comparison with ^{13}C and ^{15}N isotopes that will require longer experimental times for their analysis (Jonsson, 2010). For the characterization of carbohydrates, the resonances ^{1}H NMR (4.4–5.5 ppm) and ^{13}C NMR (95–110 ppm) are the most useful spectra to determine the number monosaccharides while ^{1}H NMR (<1 ppm) identifies CH_3-groups and ^{1}H NMR (>2 ppm) are useful to identify *N*-acetyl and *O*-acetyl groups (Jonsson, 2010).

NMR techniques have been recently used to study the chemical structure of multiple carbohydrates such as gums (i.e., carrageenan and alginates) extracted from macroalgae (Youssouf et al., 2017) and polysaccharides extracted from *Ulmus pumila* L. and *Grifola frondosa* (Lee et al., 2018; Zhang et al., 2018). The NMR conditions described in the literature to analyze carbohydrates differ depending on the compounds of interest and the structural information needed in each particular study. ^{1}H NMR and ^{13}C NMR at resonances of 500.13 and 125.75 MHz, respectively, were used to characterize macroalgal polysaccharides (Hmelkov, Zvyagintseva, Shevchenko, Rasin, & Ermakova, 2018), while ^{1}H NMR and ^{13}C NMR at 600 MHz were used to investigate the structure of an inulin-type fructan extracted from *Codonopsis pilosula* (Fu et al., 2018) and ^{1}H NMR spectra obtained at 400 MHz provided relevant structural information of β-glucans extracted from the mushrooms *Pleurotus ostreatus* and *Ganoderma lucidum* (Smiderle et al., 2017).

Samples with overlapping proton resonances that cannot be disentangled by homonuclear NMR require the application of analytical alternatives such as the heteronuclear multiple quantum coherence (HMQC) and heteronuclear single quantum coherence (HSQC) NMR spectroscopies (see an example in Fig. 6.4). The HMQC and HSQC NMR spectroscopies allow the identification of the connections between the ^{1}H and ^{13}C chemical shifts as this methodology spreads the ^{1}H NMR spectrum in the ^{13}C dimension, improving the resolution and effects of strong ^{1}H-couplings. After establishing ^{1}H NMR assignments by homonuclear NMR, the application one-bond heteronuclear ($^{1}H—^{13}C$) correlation spectrum will allow the identification of the multiple monosaccharide units in the sample (Agrawal, 1992).

HSQC NMR technique has been recently used to analyze carbohydrate mixtures from depolymerized chondroitin sulphate extracted from the sea cucumber Holothuria forskali (Brodaczewska, Košťálová, & Uhrín, 2018) and lignin from Ginkgo biloba L. (Jiang, Zhang, Guo, Zhao, & Jin, 2018). HMQC NMR spectrum at 500-MHz has been also applied to characterize water-soluble polysaccharides from the mushroom *Grifola frondosa* (Zhang et al., 2018).

6.3.4.2 FTIR spectroscopy

Infrared (IR) spectroscopy is based on the application of an IR radiation (near-IR (400–10 cm^{-1}), mid-IR (4000–400 cm^{-1}), and far-IR (14,000–4000 cm^{-1})) through a sample. This radiation will be absorbed by the analytes and passed through (transmitted) generating a unique IR spectrum for each chemical structure, with absorption peaks representing the frequencies of vibrations between the bonds of the atoms in the analytes (Dutta, 2017). FTIR spectrometry was developed to measure all IR frequencies simultaneously using an optical device or interferometer that generates a unique signal with all the IR frequencies "encoded," reducing the time of reading per sample. Chemical bonds vibrate at characteristic frequencies absorbing radiation at frequencies that match their vibration modes. The spectrum generated can be used to identify functional groups and compounds as many functional groups have IR absorption at rather specific and narrow frequency ranges independently of the rest of the molecule (Dutta, 2017; Petit & Madejova, 2013). An example of the FTIR spectra used to characterize macroalgal polysaccharides is presented in Fig. 6.5. A few reference values of IR absorption useful to identify functional groups were described in the literature: hydrocarbons showed absorption peaks due to C—H stretching vibrations between 600 and 3300 cm^{-1}, OH or NH groups at 3000–3700 cm^{-1}, and CN triple-bond at 2200–2300 cm^{-1} (Dutta, 2017).

In the case of carbohydrates, absorption ranges of 3200–3500 cm^{-1} were attributed to hydroxyl groups, 2870–2950 cm^{-1} to C—H stretching, 1060–1090 cm^{-1} to C—O—C, and 1024 cm^{-1} to glycosidic linkages when characterizing the structure of carrageenan and alginates (Paula et al., 2015). FTIR has been used alone or in combination with NMR to elucidate the structure of multiple carbohydrates, such as pectins extracted from mulberry branch bark (Liu et al., 2010), pomegranate peels (Pereira et al., 2016), and other complex polysaccharides extracted from mushrooms (Khaskheli et al., 2015; Wang et al., 2015; Zhang et al., 2009), figs (Liang et al., 2012), and macroalgae (Borazjani et al., 2017; Imbs et al., 2016; Rhein-Knudsen et al., 2017).

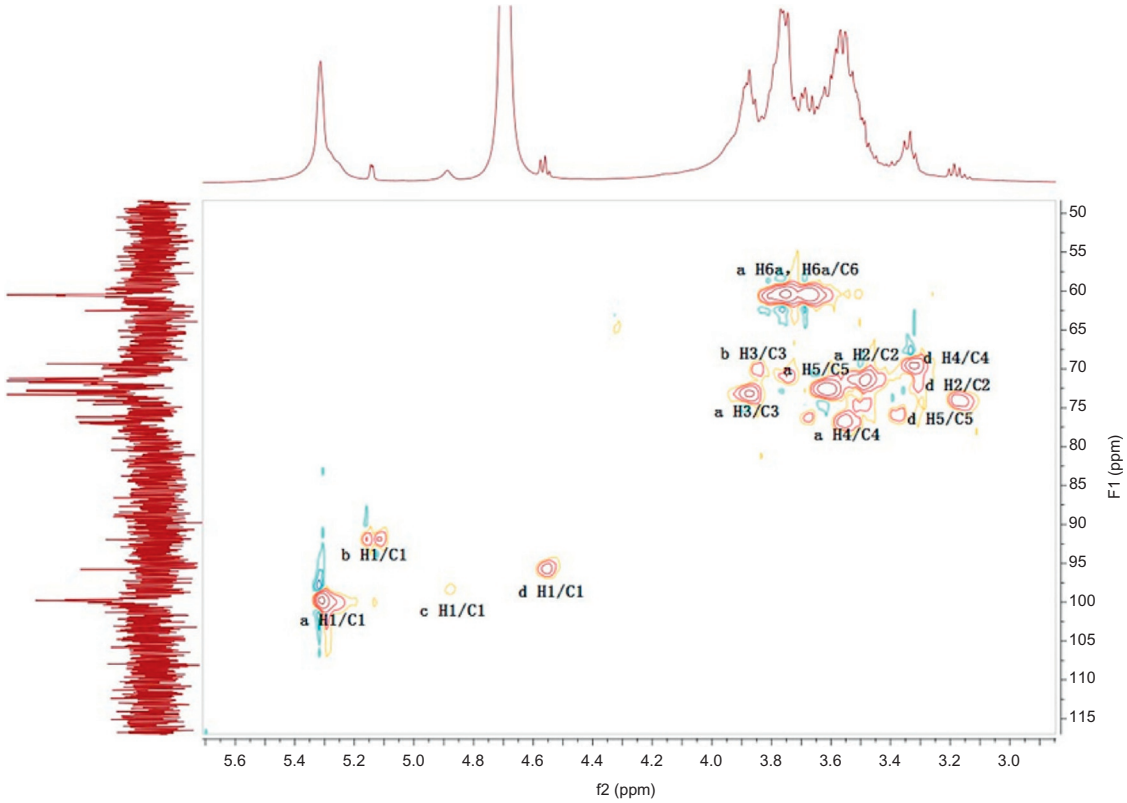

FIG. 6.4 Heteronuclear multiple-quantum coherence (HMQC) NMR spectrum provided by Zhang et al. (2018) when studying polysaccharides extracted from the mushroom Grifola frondosa.

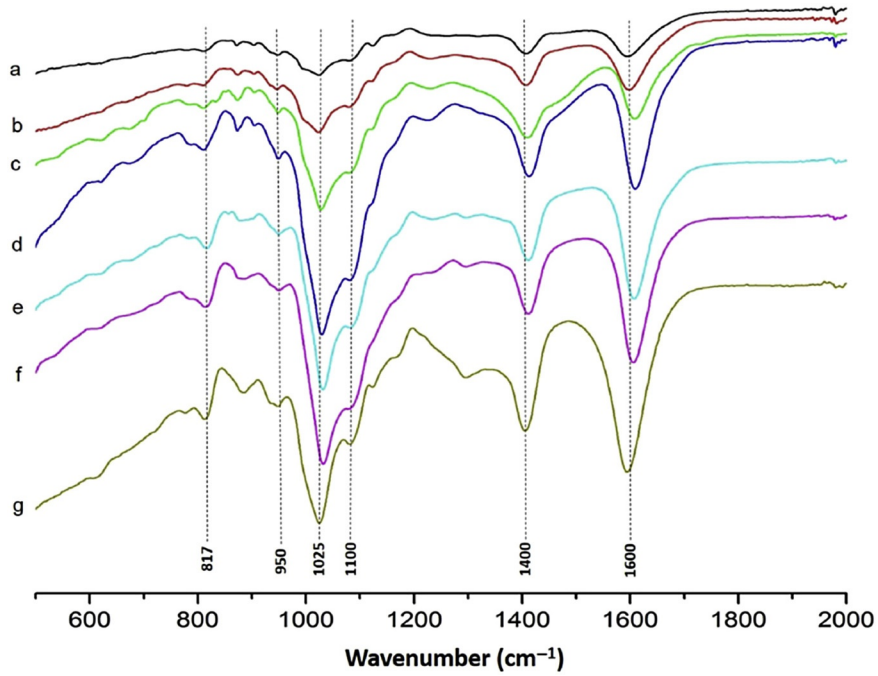

FIG. 6.5 FTIR spectra of alginates extracted from several macroalgae species (A–F) and an alginate commercial standard (G). *(Image taken from Rhein-Knudsen, N., Ale, M. T., Ajalloueian, F. & Meyer, A. S. 2017. Characterization of alginates from Ghanaian brown seaweeds: Sargassum spp. and Padina spp. Food Hydrocolloids, 71, 236–244..)*

6.4 CONCLUSIONS

The concept "dietary fiber" includes carbohydrates of diverse chemical nature, structure, and biological properties. The quantification of fiber or specific fractions of dietary fiber with minimum analytical overlaps have focused the attention of the scientific community, developing comprehensive analytical protocols that were later adopted as official methods by the Association Official Analytical Chemists (AOAC). Due to the changes in the definition of dietary fiber proposed by the Codex Alimentarius, emphasizing the demonstration of the health benefits of carbohydrates to be considered as dietary fiber, new analytical tools are needed to provide a quantitative, but also a qualitative evaluation of fiber. This chapter summarizes key areas for the study of carbohydrates, including the sample preparation steps, analytical tools, and protocols implemented to date. The advances and application of chromatographic methods (liquid, gas, and thin-layer chromatography), nonchromatographic techniques (field flow fractionation and capillary electrophoresis), and other structural elucidation methods (NMR and FTIR) showed promising results, providing detailed structural features of biologically active carbohydrates. As the field of analytical chemistry evolves, new protocols and techniques will be available to advance the knowledge of the complex chemical structures of carbohydrates, gaining a better understanding of the biological health benefits of the multiple compounds classified as dietary fiber.

Acknowledgments

Marco Garcia-Vaquero works within the project "Macroalgal Fibre Initiative" funded by Science Foundation Ireland (SFI) (grant number: 14/IA/2548).

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