

Food Safety and Food Quality Control Beat your own record!

Dirty sample analysis even faster with monolithic Columns
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Noteworthy info about food regulation.
Various application notes, e.g. for constituents and additives.
2 robustness tests on Chromolith® columns.

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Introduction

With the current focus on food issues there is an increasing need for new analytical methods able to cope with large number of analytes in complex matrices. These new analytical assays must provide sensitivity, robustness and high resolution within an acceptable analysis time. Many modern approaches in HPLC analysis enable the reduction of the analysis time without compromise on resolution and/or separation efficiency: the use of monolith columns, liquid chromatography at high temperatures, and ultrahigh pressure liquid chromatography (UHPLC) either using small particle packed columns or superficially porous particle columns. A viable approach to meet these demands is to develop the HPLC assays using Chromolith® columns since monolithic columns are more matrix tolerant than particles packed columns.

Chromolith® columns are made of a single rod of high purity porous silica with defined bimodal pore structure where the macro and meso pores are in the micro and nanometer range. The high permeability and porosity of the rigid silica skeleton result in low column backpressure and more flexibility in flow rates compared to particulate columns. Column equilibration after gradient elution is also much faster on monolithic columns than similar dimension particle packed columns. Overall this offers a possibility to perform high throughput analysis without losing separation efficiency or peak capacity.

Chromolith® columns offer:

- superior matrix tolerance
- extended column lifetime
- a rigid monolithic structure for a longer lifetime with no risk for bed compression
- high throughput at high flow rates, yet with low column backpressure
- an option to perform flow gradients
- a possibility to couple two or more columns in series to enhance peak capacity

Monolith Chromatography Columns

– Ideal Tools for Dirty Sample Analysis

Are monolithic columns less versatile than particle packed columns for routine HPLC assays? Objections such as “there is a lack of sufficient commercially available modifications for my separation needs”, and “this column does not allow me to down/up-scale my methods – It is not available in all the dimensions I need and that prevent the overall applicability in my lab” are sometime raised.

Monolith columns are just as versatile and, in addition, provide several advantages over packed columns especially for “dirty” sample analysis. Especially the much higher tolerance for sample matrix make monolith columns the preferred choice for “dirty” sample analysis. To meet customer demands, Chromolith® columns with cyano (CN), diol (OH) and phenyl stationary phases are being launched in 2013, adding to the already existing silica (Si), amino (NH₂), C₈, and C₁₈ modifications. Chromolith® columns can be used with any HPLC instrument and detection technique, i.e. UV, fluorescence (FL), refractive index (RI), electrochemical (EC), evaporative light scattering (ELSD), and mass spectrometric (MS) detection. The choice of column dimension ultimately depend on the type of instrument used, the sample matrix, sensitivity and separation efficiency (peak capacity) needs. Chromolith® columns are available with 25, 10, 4.6, 3.0 and 2.0 mm I.D. down to 50 micrometer, all with intention to ease scaling of methods.

In the past only few scientific papers were published using monolith columns and MS detection because of incompatibility reasons (need for post-column flow splitter due to high flow rate). Modern MS detectors, however, tolerate flow rates up to 1.0 mL/min without sensitivity loss, where a 2.0 mm I.D. Chromolith® column provide excellent performance over a particle packed column and without loss of separation efficiency or having issues with backpressure. Rapid LC-MS methods are frequently being requested especially in the area of food analytics where large amounts of “dirty and complex samples” are being analysed. It can therefore be expected that more LC-MS methods will be developed using monolith columns where the need is focused at fast, sensitive and selective.

This application compilation contain several methods, with workflows; including calibration curves, recovery calculations, and method robustness overview. Beverages and foodstuff samples have been analyzed with Chromolith® columns having either amino (NH₂) or C₁₈ modifications. LC-UV, LC-FL and LC-MS instruments have been used from various vendors.

As you will experience, Merck Millipore offers virtually everything but the instrument to successfully implement these methods in your laboratory.

Regulations in Food Testing

Chemical analysis of food is a pre-requisite for safeguarding correct labeling of food and protection of consumers against adulteration and misbranding of food. Of course, such tasks can only be achieved together with suitable food legislation, increased controls by food authorities, continuous studies by food safety agencies and universities to improve knowledge about food (processing), and by enhanced responsibility of the food industry. The latter is supported by food quality management systems like HACCP to prevent and / or control chemical, microbiological and physical hazards within the food supply chain. Such quality assurance procedures require chemical analysis throughout the whole food processing chain, starting from the raw materials and up to the final, labeled food product [1]. For ensuring that food complies to a certain minimum standard very often obligatory quality standards are applied in defining the ingredients and what the food must contain at minimum, including the nutritional composition [2, 3, 4]. Sometimes those standards also include the analytical procedures to be used. Examples for such encompassing quality standards are the US standards of identity or the Codex Alimentarius standards [2, 3].

Nutritional data on packaged food are necessary for helping consumers to choose food in accordance to their individual dietary needs and for reducing diet-related diseases [6, 7, 8]. Accordingly, labeling regulations describe in detail the requirements for nutritional labeling (nutrients, amounts and caloric values) on food packages [5, 6]. To ensure a consistent nutrient declaration, the food manufacturer needs to perform additional testing for nutrients like sugars, organic acids, sugar alcohols, fat and fatty acids, protein and sodium as well as for vitamins and minerals [5].

All of these above described measures for ensuring food safety, provision of wholesome food, and consumer protection against adulteration and misbranding require reliable data obtained by chemical analysis of food. Reliable analytical results are also essential to facilitate international food trade.

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Use of Quality Management Systems in Laboratories and Lab Accreditation

The reliability of chemical data depends significantly on how these data have been measured. The implementation of a quality management system for laboratories is an approved measure for safeguarding that the technical equipment and analytical methods are fit for purpose and reproducible [9] and that the staff is suitably qualified and experienced for their tasks.

Consequently, lab accreditation according to an international recognized system is a pre-condition for elaboration of reliable and internationally accepted analytical data [9].

The most important standard for laboratories is the international DIN ISO standard 17025 'General requirements for the competence of testing and calibration laboratories' [9, 10] which is also one of the most important standards for the worldwide globalization of trade. It addresses the technical competence of laboratories to carry out specific tests and is used worldwide by laboratory accreditation bodies as core requirement [9, 10]. The DIN ISO 17025 Standard comprises five elements that are 'Scope', 'Normative References', 'Terms and Definitions', 'Management Requirements' and 'Technical Requirements' [9]. The section 'Technical requirements' includes factors which determines the correctness and reliability of the tests and calibrations performed in laboratory like human factors, accommodation and environmental conditions, test and calibration methods, method validation, equipment, measurement traceability, sampling the handling of test and calibration items [9].

EU – Regulation 882/2004 – Consequences for food testing lab Accreditation

Within the European Union the official laboratories (i.e. those laboratories carrying out the analysis of samples taken during official controls by the competent food authorities of each member state) must be accredited according to DIN ISO 17025 as requested by EU Regulation 882/2004 [11]. The accreditation, however, may relate to individual tests or a group of tests.

Contract or third party laboratories need to be accredited according to this standard only if the results obtained by those laboratories are legally defensible data i.e. that these data are used or are intended to be used in the event of a dispute between the competent food authorities and a food operator / manufacturer. However, as an indirect consequence of this regulation the European food market no longer accepts contract labs for food testing which are not accredited [10].

Accreditation in the USA as requested by the Food Safety Modernization Act

In 2011, the USA enacted the Food Safety Modernization Act (FSMA) [12]. It is an enormous reform program to improve food safety and to avoid intentional as well as non-intentional food adulteration within the whole supply chain also involving food testing labs. Certain provisions of the FSMA are already in effect, others will take effect later in 2013 and 2014 [13]. The FSMA is divided in four main parts. Part 2 of the FSMA ('Improving capacity to detect food safety problems') deals with the detection of food safety problems throughout the food supply chain. Section 202 includes rules to ensure that food testing laboratories (encompassing independent private laboratories, foreign laboratories, and laboratories operated by Federal, State, and local government agencies) meet prescribed standards for quality. The United States Food and Drug Administration (FDA) has not yet decided if in-house labs of food manufacturers may also be eligible for this accreditation.

Until June 2013, the FDA must establish criteria for laboratory accreditation and develop standards that laboratories must meet to be accredited. Correspondingly, accreditation bodies for lab accreditation need to be established by the FDA [12]. Publicly available registry of accreditation bodies shall also be built up by the FDA [12]. According to Section 202 of the FSMA the FDA standards that must be met by accredited labs will include among others that

- appropriate sampling techniques are used,
- analytical procedures must be fit for purpose,
- reports of analyses must be certified,
- an internal quality system must be established and maintained,
- complaint evaluation and response procedures must exist, and
- technicians are qualified by training and experience.

These requirements are more or less the requests described by the DIN ISO Standard 17025 for lab accreditation and therefore already quite common for many labs throughout the world. It is planned that by June 2013, the FDA-accredited labs must be used by food manufacturers/operators at least for

- any specific legal or regulatory testing requirement when addressing an identified or suspected food safety problem;
- any testing required by FDA to address an identified or suspected food safety problem;
- any testing to support admission of an imported food; and
- any testing under an import alert that requires successful consecutive tests.

A key requirement of section 202 is that accredited labs must provide the results of such tests directly to the FDA [12]. Reasons for this requirement may be that due to the other provisions of the FSMA, the number of necessary food samples will increase significantly. On the other hand the FDA does not have the necessary resources to deal with such a sudden rise of food samples to be tested. The FDA, therefore, makes the food manufacturer/operator responsible that the samples are tested and 'outsourced' to third parties, such as contract testing laboratories [14].

The FDA has created a FSMA website giving updated information on FDA's progress [13], the full text of the law and a list of dockets open for comments.

The use of official methods in food analysis

In food analysis, it is especially the complexity of the food matrix which has the largest impact on the performance and reliability of the analytical methods and procedures used [5]. The food matrix consists mainly of chemical compounds like protein, carbohydrate and lipids affecting significantly the performance of analytical methods. For example, high-fat or high-sugar foods can cause different types of interferences compared to low-fat or low-sugar food [5].

The application of extraction steps and digestion procedures, although, being a pre-condition for getting accurate analytical results, is often not only time-consuming, but sometimes also bears the risk of artifact formation [15]. Therefore, analytical methods for food analysis always need to take into account the characteristics and composition of the specific food matrix.

Several non-profit (scientific) organizations, such as the Association of Analytical Communities (AOAC), develop, standardize, and endorse official methods for food analysis. Such official methods play an important role in the analysis of foodstuffs, to ensure that food meets the legal requirements. As a consequence there are e.g. in the USA legal provisions requesting the use of a specific analytical method [16]. Furthermore, such official methods allow for comparability of results between different laboratories that follow the same procedure and for evaluating results obtained using new or more rapid procedures [5].

Official methods by WHO/FAO and Codex Alimentarius

The Codex Alimentarius Commission established in 1962 by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) develops international standards and safety practice for foods and agricultural products (the so-called Codex Alimentarius).

The Codex Alimentarius includes general requirements, code of practices and standards [17]. The Codex Commodity standards include methods for analysis of the respective commodity. Codex Volume 13 includes a list of official methods for analysis and sampling. The methods are sorted according to the specific commodity for which they can be applied (e.g. cereals, fats and oils, infant formulas etc.). There are also some methods which can be used for all kind of food exemplified by the method for detecting and quantification of the sweetener Cyclamate [18]. Codex recommends the use of the listed methods in application of DIN ISO 17025 [18].

Codex methods are elaborated by international organizations occupying themselves with a food or a group of foods and selected by the Codex Commission for Analysis [19]. Within this selection preference is given to those test methods that meet the criteria by the Codex Commission for Analysis for accuracy, precision, selectivity, limit of detection, sensitivity etc. The selection of methods also takes into account practicability and applicability under normal laboratory conditions. Consequently, preference will also be given to methods which have applicability for routine use and which are applicable uniformly to various groups of commodities. [19]. It should be mentioned that the majority of the analytical methods cited in Codex standards are those of the AOAC [22]. This is due to the fact that AOAC has had official observer status in Codex Alimentarius since its foundation and has given input on the development of Codex standards [22].

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) sets standards for purity of food additives. It provides a compendium of food additive specifications comprised of four volumes [20]. The first three volumes are the food additive specifications themselves in alphabetical order while the last volume includes the revised and updated analytical methods, test procedures and laboratory solutions used by and referenced in the specifications. JECFA specifications include guidance on the analytical methods that should be used for testing according to the JECFA specification. Wherever possible, this is done by reference to the fourth volume of the compendium which includes the revised and updated analytical methods [20]. Otherwise details of the test procedures are described in the individual specifications monographs.

As JECFA specifications have been elaborated for a worldwide use, the referenced methods require the use of apparatus and equipment that is available in most laboratories [20]. According to JECFA, methods involving more recently developed techniques or equipment will not normally be quoted until such techniques are accepted internationally and are generally available at reasonable cost. Taking the advances in analytical chemistry in consideration JECFA is reviewing the analytical methods from time to time. In principle, it is possible to deviate from the JECFA methods, however, provided that the use of such other method or the modification to a JECFA method gives results of equivalent accuracy and specificity to those referenced in the respective JECFA specification [20].

Official methods for food analysis in the US

The FDA has established food definitions and standards which are published in 21 CFR 100–169 [2, 21] including standards of identity and quality. The standards of identity have been set for a wide variety of food products establishing specifically the ingredients a food must contain at minimum levels for expensive ingredients as well as at maximum levels for inexpensive ingredients (like e.g. water). Those standards mostly specify official analytical methods which are to be used for analysis. These methods have been elaborated by the international scientific organizations like the AOAC or by US organizations like e.g. the AACC (American Association of Cereal Chemists) or the AOCS (American Oil Chemists Society).

The Compendium Official Methods of Analysis of the AOAC International contains over 3000 methods adopted by the AOAC appropriate for a wide variety of food products and other materials [2, 22]. 21 CFR Section 2.19 defines the AOAC methods as “official methods” which are to be utilized by the FDA in case there is no analytical method described in a regulation [22, 23]. Accordingly, the FDA, and the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) are using the AOAC methods to check if the food complies to the specific legal requirements, like nutritional labeling information on foods, presence or absence of undesirable residues or residue levels [21]. The AOAC reviews, selects and also develops methods. Once a method is selected by the AOAC it undergoes single lab validation and a full collaborative study involving 8–10 laboratories [22]. After a successful completion and approval by the AOAC Official Methods Board, the AOAC method will be published in AOAC Compendium and Journal [22].

Food chemicals codex (FCC) is a compendium containing standards for identification and purity for known food additives and chemicals (including provision of the respective analytical methods) used in food products either in the United States or internationally. Although the FCC standards have been developed in cooperation with the FDA and industry in the United States and elsewhere, the FCC does not provide information about the regulatory status of a food additives and chemicals [24]. However, some countries other than the US (e.g. Australia, Canada, New Zealand) recognize the FCC standards as legal requirements for food additives [24]. The FCC monographs include general information about the use of the particular food additive, chemical data, minimum standards for identity, purity, and quality of food additive and validated testing methods for verifying the purity and quality of the quoted food additive.

Official methods for food analysis in the EU

Existing official methods within the EU can be found e.g. in the German official collection of food analysis methods according § 64 to the German Food and Feed Act (so called Official Collection of § 64 LFGB Methods) including more than 1300 analytical procedures for food analysis [25, 26]. Experts from food control, science and the food industry develop analytical procedures and assess performance, reliability, and comparability of such methods. They decide for which field of application the methods are to be used. Before a method is included in the Official Collection, the methods are statistically tested by a number of labs in course of a inter-laboratory comparison test and are standardized [26].

In Germany, the Official Collection of § 64 LFGB Methods can be applied without any further justification [25, 26]. However, if a lab wants to use a different method instead, such deviation needs to be justified.

In EU legislation, official methods are often specified in case of product specific regulations to ensure that a certain standard of food quality can be maintained. An example is 'Regulation 2548/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis' which contains a number of official methods [27].

In EU food legislation, however, the traditional approach for food analyses which foresees the use of such official or routine methods is more and more pushed aside by the so called performance criteria approach.

The Performance Criteria approach within the EU

As a result of advances in analytical chemistry the concept of routine methods and reference methods is more and more superseded by the so-called Performance Criteria approach, in which performance criteria for the analytical method and procedures for the validation of screening and confirmatory methods are established [11, 28].

Such change of paradigms is considered in EU Regulation 882/2004 [28]. This Regulation describes the principle requirements for sampling and analytical methods as conducted by the official laboratories of the EU member states. Laboratories involved in the analysis of official samples need to work in accordance with internationally approved procedures (e.g. DIN, CEN, ISO, IUPAC, Codex Alimentarius) or criteria-based performance standards and use methods of analysis that have been validated in accordance with e.g. IUPAC Harmonised Guidelines [11].

This has already been reflected in the EU Regulations 401/2006 [29], 333/2007 [30], 1882/2006 [31] for sampling and analytical methods for contaminants like nitrate, heavy metals, benz(a)pyrene and 3-MCPD. While these regulations describe in detail the sampling procedures (requirements for the personnel, number of samples per lot, precautions to be taken etc.), the regulation does not include (strong) recommendation for analytical methods, but specific requirements for the performance criteria like precision, recovery rate, measurement uncertainty etc. are defined and must be fulfilled by the used validated analytical methods. Also requirements regarding the laboratory quality management system to be applied are included.

For pharmacologically-active residues of authorized and non-authorized veterinarian medicinal products, e.g. certain antibiotics (like chloramphenicol) or substances having an anabolic effect (e.g. Clenbuterol), analytical methods can be selected by the respective lab but also need to be validated and to fulfill the performance criteria as given by EU Decision 2002/657 [32, 33]. The lab itself must comply with the quality norms as requested by EU Regulation 882/2004. However, the National Reference Laboratories develop and validate suitable analytical methods which can be used for analysis of veterinary drugs residues in food as described by the relevant EU legislation [33].

The above described measures aim to ensure the reliability of analytical results for food analysis. In the following compilation, analytical applications for food analysis will be described which may contribute to the reliability of analytical data by simplifying the sample preparation procedures.

References and useful links

Food legislation is a very dynamic legislation. Therefore, food regulations change frequently or are often amended. The given links to food legislation may not in all cases provide the most up-to-date version of the regulation. The most up-to-date legislation is to be found on the respective government websites.

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<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31996L0023:EN:NOT>

European Union:

Eurlex:

<http://eur-lex.europa.eu/de/index.htm>

This website allows you to search for the Official Journal of the EU and to get access to the text of the regulations (also accessible as consolidated versions)

European Commission:

http://ec.europa.eu/food/food/index_en.htm

European Food safety Agency

http://ec.europa.eu/food/efsa_en.htm

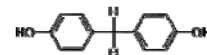
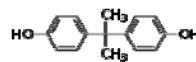
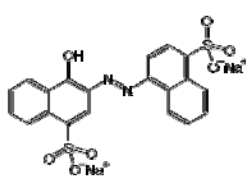
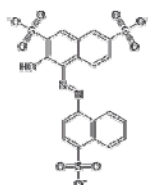
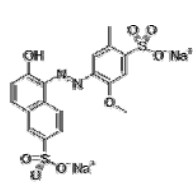
USA:

FDA (Food) homepage

<http://www.fda.gov/Food/default.htm>

US-DA homepage. The US-DA is responsible for safety of food derived from agriculture

http://www.usda.gov/wps/portal/usda/usdahome?navid=FOOD_SAFETY



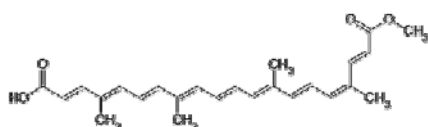
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Amaranth (E123)

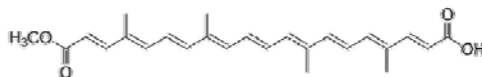
Azorubine (E 122)

Bisphenol A (BPF)

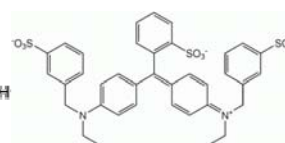
Bisphenol F (BPF)



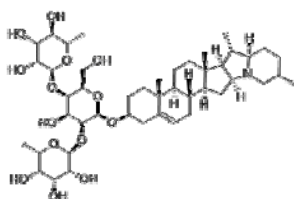
Cis-Bixin



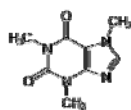
trans-Bixin



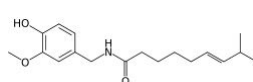
Brilliant blue



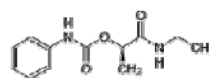
Cacoline



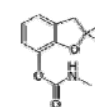
Caffeine



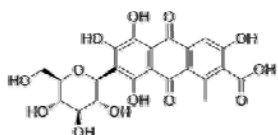
Capsaicin



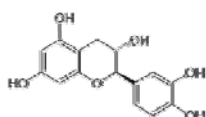
Carbetamid



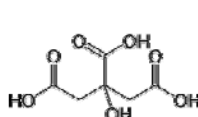
Carbofuran



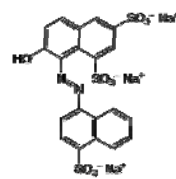
Carmine red



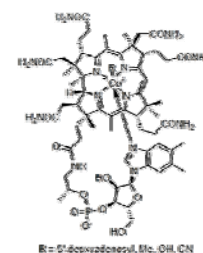
Catechin



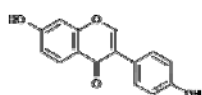
Citric acid



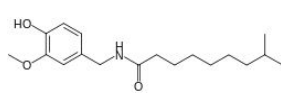
Cochineal Red A (E 124)



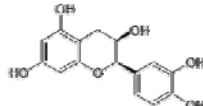
Cyanocobalamin



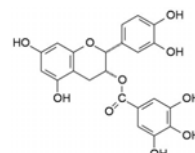
Daidzein



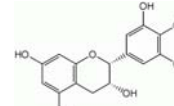
Dihydrocapsaicin



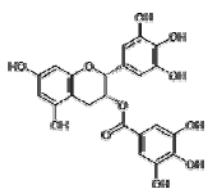
Epicatechin



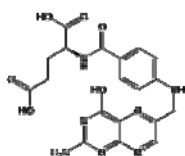
Epicatechin gallate



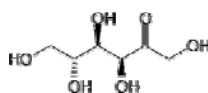
Epigallocatechin



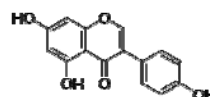
Epigallocatechin gallate



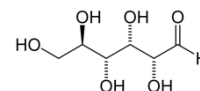
Folic acid



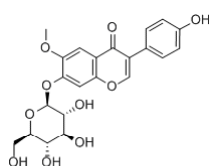
Fructose



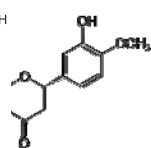
Genistein



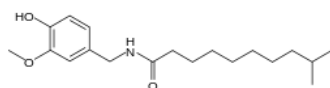
Glucose



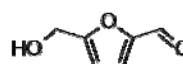
Glycitin



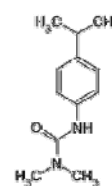
Hesperetin



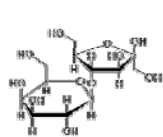
Homodihydrocapsaicin



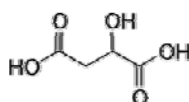
Hydroxymethylfurfural



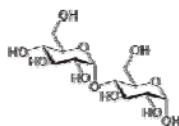
Isoproturon



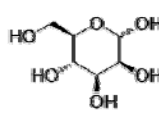
Lactulose



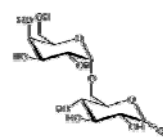
Malic acid



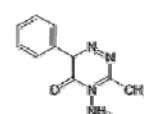
Maltose



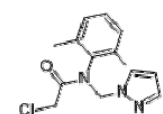
Mannose



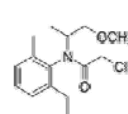
Melibiose



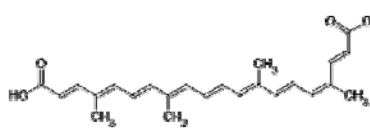
Metamitron



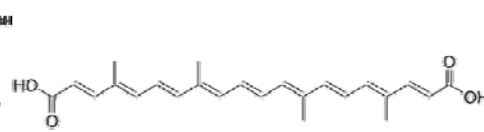
Metazachlor



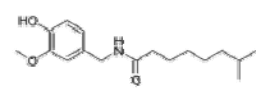
Metolachlor



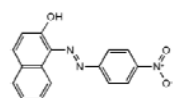
cis-Norbixin



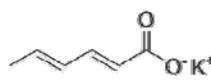
trans-Norbixin



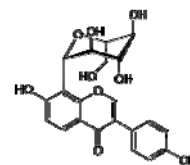
Nordihydrocapsaicin



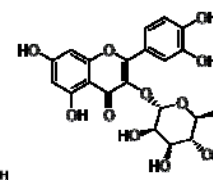
Para Red



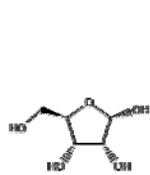
Potassium sorbate



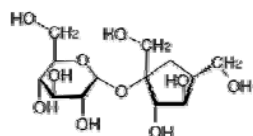
Puerarin



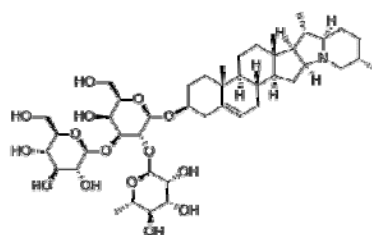
Quercetin



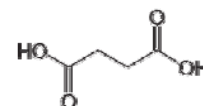
Ribose



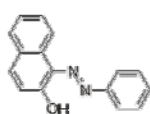
Saccharose



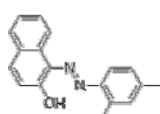
Solanine



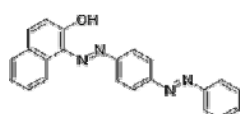
Succinic acid



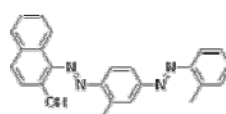
Sudan I



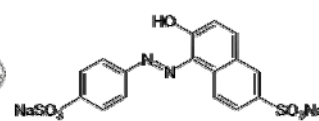
Sudan II



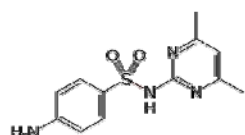
Sudan III



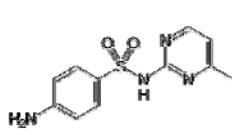
Sudan IV



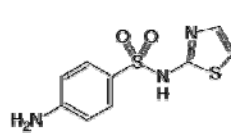
Sunset yellow



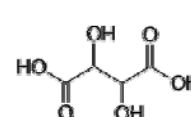
Sulfadimidine



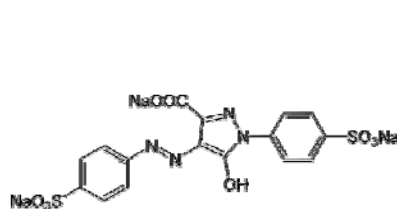
Sulfamerazine



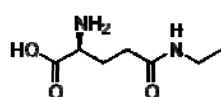
Sulfathiazole



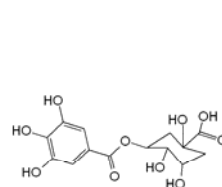
Tartaric acid



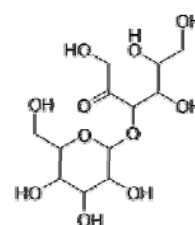
Tartrazine



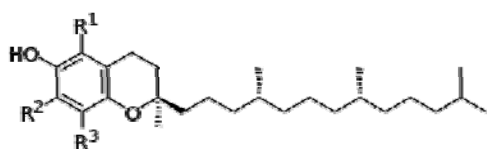
Theanine



Theogallin



Turanose



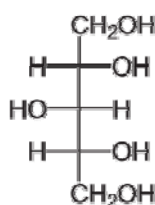
Tocopherols:

α : $R^1 = R^2 = R^3 = CH_3$;

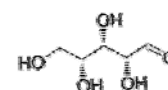
β : $R^1 = R^3 = CH_3$, $R^2 = H$;

γ : $R^1 = H$, $R^2 = R^3 = CH_3$;

δ : $R^1 = R^2 = H$, $R^3 = CH_3$.



Xylitol



Xylose

Chromolith® Robustness Test 1

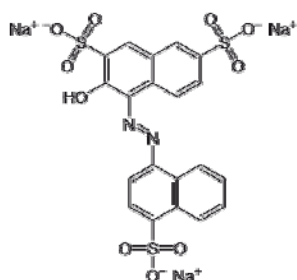
Food Colorants in Alcohol Beverages

A method for analysis of two food colorants, E 123 (Amaranth) and E 129 (Allura Red AC), in an alcohol beverage was developed to illustrate long-term performance and method robustness obtained with monolith columns. More than 8000 samples were analyzed on a 50x2.0 mm RP-18 endcapped Chromolith® column.

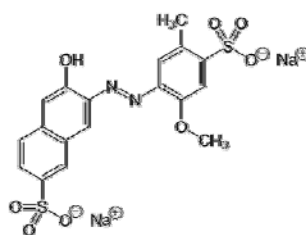
An alcohol beverage based on rum was utilized as sample matrix.
According to the specification, this beverage contains:

Rum
Saccharose
Aroma
Citric acid
Sodium citrate
Potassium sorbate
Potassium benzoate
Caramel E150d
Colorants E129 (Allura Red) and E123 (Amaranth)
E444 (saccharose acetate isobutyrate)
E445 (Glycerol esters of wood resins)

Prior to HPLC analysis the sample was filtered using a 0.45 µm syringe.



E 123 (Amaranth)



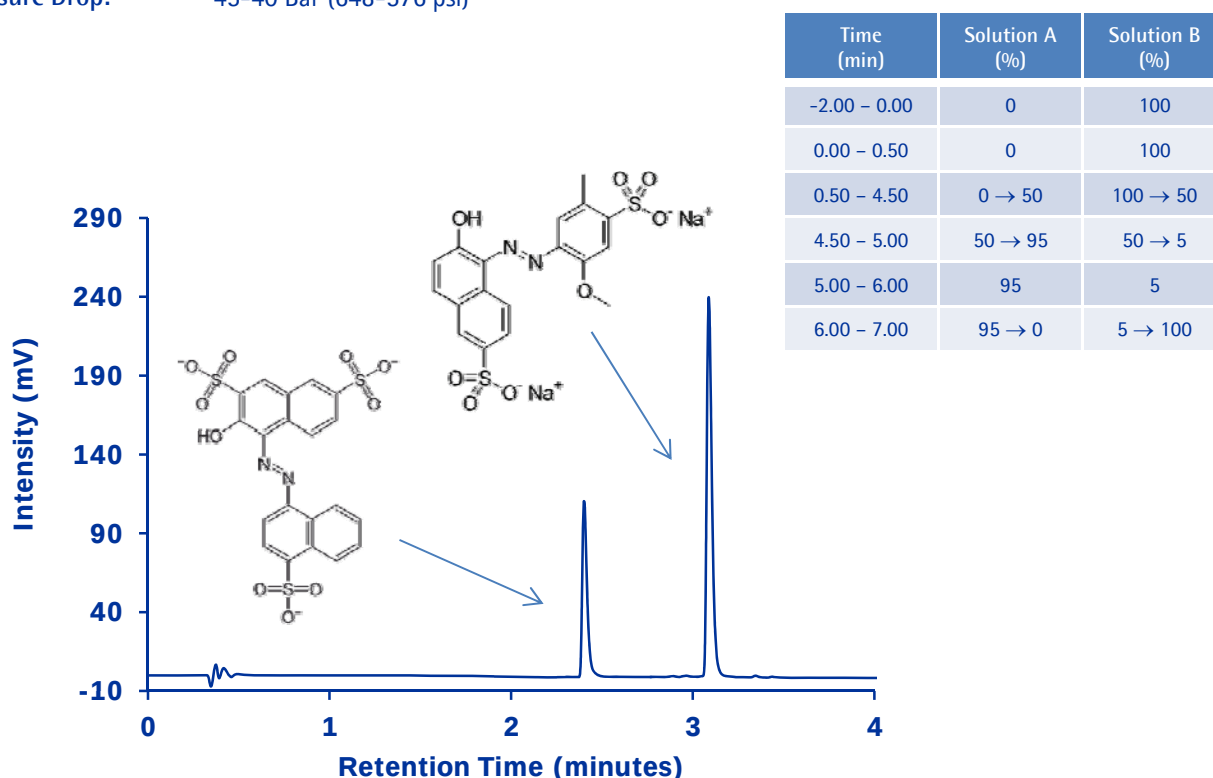
E 129 (Allura Red AC)

Food Colorants in Alcopop Beverage

Chromolith® Performance RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® Performance RP-18 endcapped 50x2.0 mm	(1.52007.0001)
Injection:	2 µl	
Detection:	UV, 500 nm	
Cell:	1.4 µL	
Flow Rate:	0.4 mL/min	
Mobile Phase (v/v):	A: Acetonitrile B: 20 mM ammonium acetate, pH 4.70	
Gradient Program:	See table	
Temperature:	25 °C	
Sample:	Alcopop beverage. Filtered through 0.45 µm Millex®-filter prior to analysis.	
Pressure Drop:	45-40 Bar (648-576 psi)	



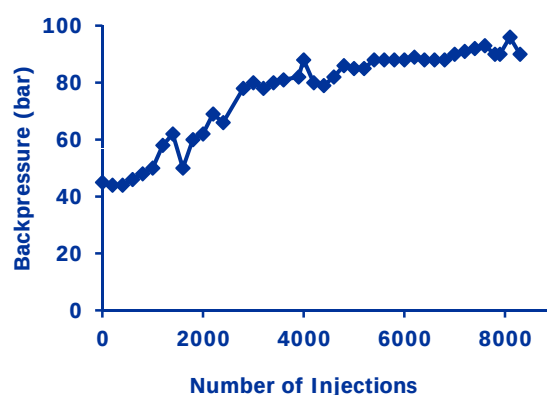
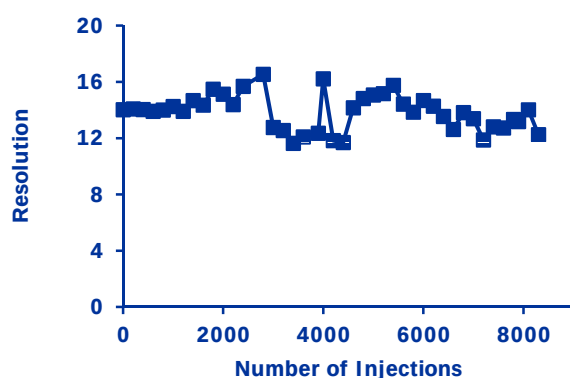
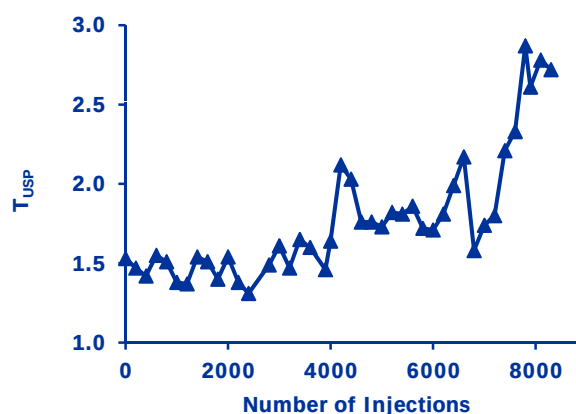
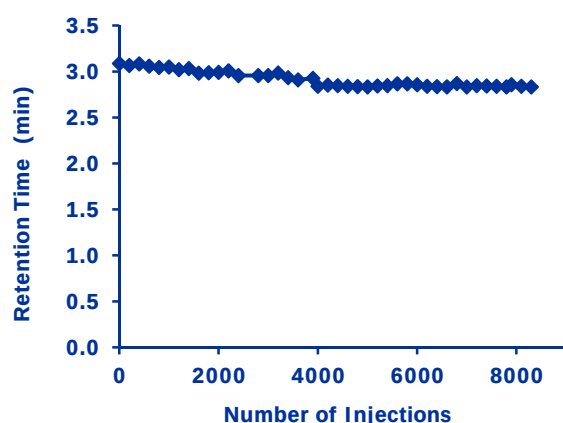
Chromatographic Data

No.	Compound	Retention Time (min)	T _{USP}
1	E123 (Amaranth)	2.4	1.5
2	E129 (Allura Red)	3.1	1.5

Chromolith® Robustness Test 1

Food Colorants in Alcohol Beverages

The figures below illustrate how column backpressure and retention (Allura Red), peak shape (Allura Red), and chromatographic resolution between E129 (Allura Red) and E123 (Amaranth) are affected with time. 8300 samples were analysed. The largest effects are seen on peak shape and backpressure. Almost same T_{USP} value is obtained over the first 4000 injections, after which a deterioration is observed. Despite aging of the column peak integration and thereby accurate quantitation of the two analytes is no problem. The column backpressure increases with time as sample matrix is accumulated in the column but never reaches over 100 bar (1450 psi). The chromatographic resolution between the analytes is substantial ($R_s > 10$) with good overall retentivity and no additional disturbing peaks are found in the chromatogram (UV detection at 500 nm). Using a Chromolith® column for this assay it is possible to keep a low cost per analysis compared with a similar method with particle packed columns and keep longer interval between column exchange.



Chromolith® Robustness Test 2

Bisphenol A in Baby Milk Formulation

Bisphenol A (BPA) is an organic compound with the chemical formula $(\text{CH}_3)_2\text{C}(\text{C}_6\text{H}_4\text{OH})_2$ belonging to the group of diphenylmethane derivatives and bisphenols, and it is used to make certain plastics and epoxy resins. BPA-based plastic is clear and tough, and is used to make a variety of common consumer goods (such as baby and water bottles). In addition, epoxy resins containing BPA are used as coatings on the inside of many food and beverage cans. However, BPA exhibits hormone-like properties and several governments have questioned its safety. In September 2010, Canada became the first country to declare BPA a toxic substance. The European Union, Canada, and recently the United States have banned BPA use in baby bottles.

To facilitate the monitoring of BPA we have developed a rapid and inexpensive method for the analysis of baby milk formulations, and the data presented here illustrates the excellent long term performance and method robustness obtained with Chromolith® columns. More than 2700 samples were analyzed.

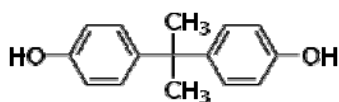
The analysed milk powder contained the following:

Protein (11%), Carbohydrates (55%, of which 48% is lactose), Fat (27%), Fiber (2.3%) and Vitamins (A, D, E, K, C, Thiamine, Riboflavin, Niacin, Vitamin B6, Biotin), Panthothenic acid, Choline, Inositol, Taurine, L-Carnithine, Minerals (Na, K, Cl, Ca, P, Fe, Mg, Zn, I, Cu, Mn, F, Se) and Nucleotides (AMP, CMP, GMP, IMP, UMP)

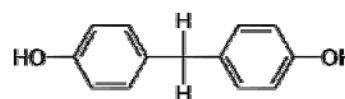
Preparation of 100 mL baby milk formulation

Boil water and transfer 100 mL into plastic milk bottle.

Add 3 spoons (12 g) of powder and mix thoroughly by shaking.



Bisphenol A (BPA)



Bisphenol F (BPF)

Chromolith® Robustness Test 2

Bisphenol A in Baby Milk Formulation

Recommended column:

Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm (1.52022.0001)

Recommended solvents and reagents

Acetonitrile: grade for liquid chromatography LiChrosolv® Reag. Ph Eur (1.00030)

Water: Water for chromatography LiChrosolv® (1.15333)
or freshly purified water from Milli-Q® water purification system

Hydrochloric acid: fuming 37% for analysis EMSURE® ACS,ISO,Reag. Ph Eur (1.00317)

Recommended filtration tools:

Mobile phase filtration:

PTFE coated with funnel, base, stopper clamp (XX1004720)

Omnipore™ PTFE membrane filter 0.45µm (JHWP04700)

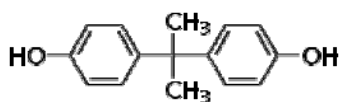
Sample filtration:

Millex® Smplicity filters, 0.20 µm, Hydrophilic, PTFE (SAMPLG001)

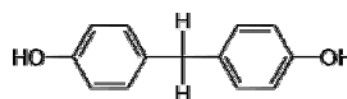
Bold blue Smplicity system bundle with 0.2 µm PTFE Millex® Smplicity filters (SAMPLG0BL)

HPLC Sample Preparation

Prior to analysis the 100 mL milk sample was acidified with 100 µL of concentrated (37%) hydrochloric acid. 5 mL of the acidified milk solution was then transferred into a 20 mL plastic vial and mixed with 10 mL of mobile phase and shaken for 5 minutes. Centrifugation of samples were performed for 7 minutes at 6400 rpm and 15°C, and finally filtered using a Merck Millipore Smplicity sample preparation unit with 0.20 µm hydrophilic PTFE Millex® Smplicity filters. The clear sample solution was transferred to HPLC vials prior to analysis.



Bisphenol A (BPA)



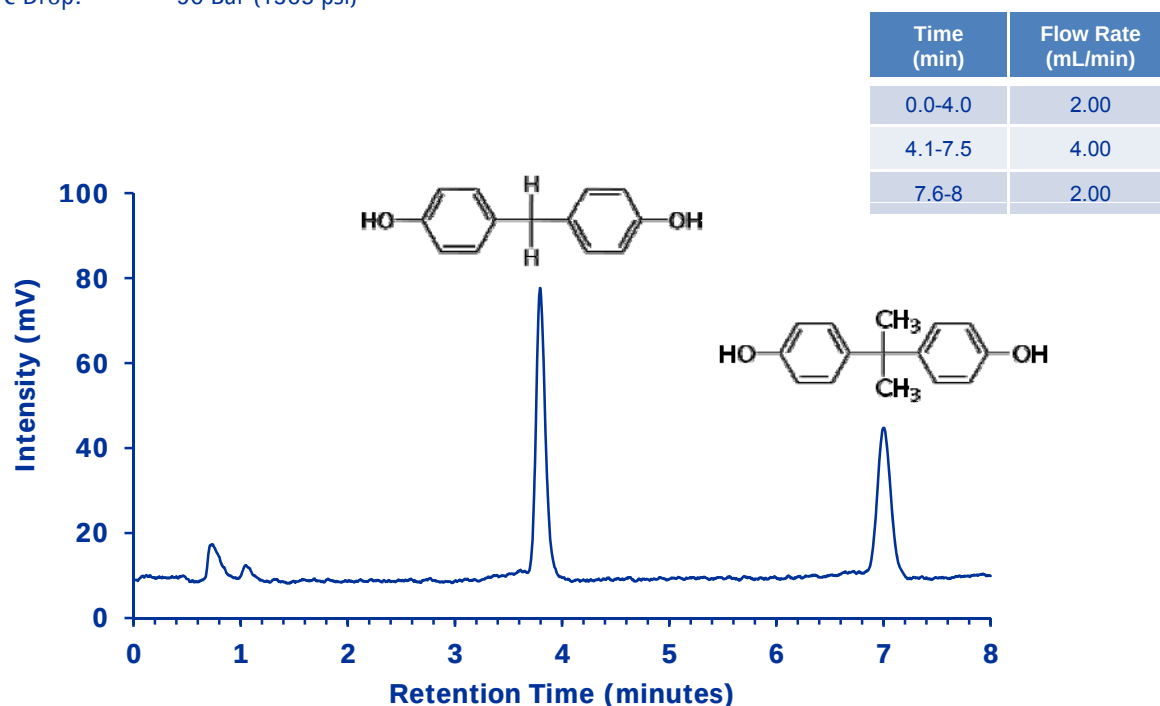
Bisphenol F (BPF)

Analysis of Bisphenol A in Milk Powder

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm	1.52022.0001
Injection:	10 µL	
Detection:	Fluorescence Detector, $\lambda_{\text{ex}}=230$; $\lambda_{\text{em}}=316$ nm	
Cell:	13 µL	
Flow Rate:	See table	
Mobile Phase:	Acetonitrile and Water 25:75 (v/v)	
Temperature:	35 °C	
Diluent	Mobile phase	
Sample:	150 ppb Bisphenol A and 60 ppb Bisphenol F (internal standard) dissolved in Methanol	
Pressure Drop:	90 Bar (1305 psi)	



Chromatographic Data

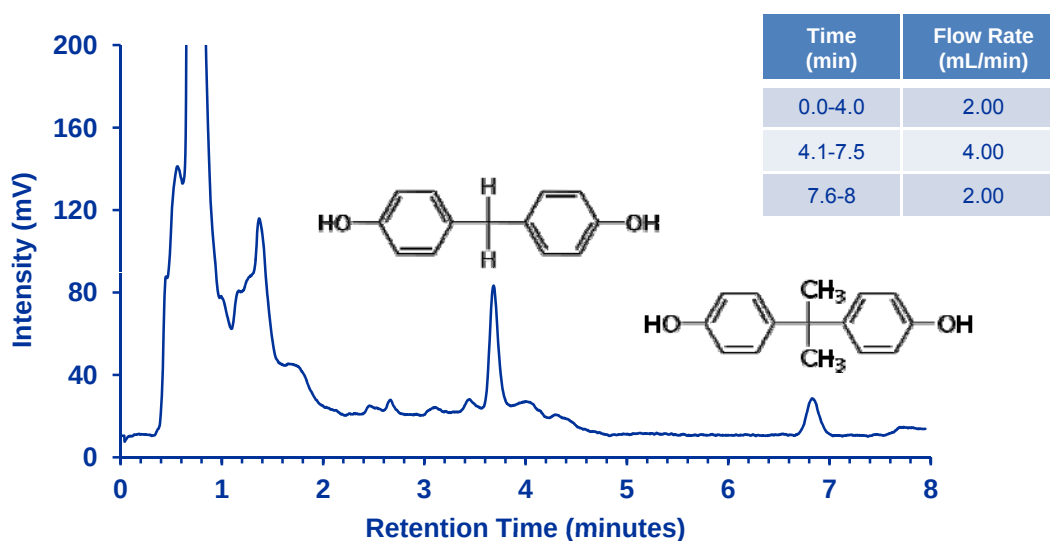
No.	Compound	Retention Time (min)	Retention factor	Asymmetry
1	Void volume	0.7	–	–
2	Bisphenol F	3.7	4.3	1.1
3	Bisphenol A	6.9	8.9	1.1

Analysis of Bisphenol A in Milk Powder

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm	1.52022.0001
Injection:	10 µL	
Detection:	Fluorescence Detector, $\lambda_{\text{ex}}=230$; $\lambda_{\text{em}}=316$ nm	
Cell:	13 µL	
Flow Rate:	Flow Rate gradient	
Mobile Phase:	Acetonitrile and Water 25:75 (v/v)	
Temperature:	35 °C	
Diluent:	Mobile phase	
Sample:	12.1 g of milk powder dissolved in 100 mL hot water. Spike 300 ppb of BPA to the solution, Add 0.1% of Hydrochloric acid and 10% acetonitrile. Add Internal standard BPF 60 ppb. Centrifuge and filter the sample with Millex® Simplicity Filter prior analysis.	
Pressure Drop:	90-185 Bar (1305-2682.5 psi)	



Chromatographic Data

No.	Compound	Retention Time (min)	Retention factor	Asymmetry
1	Void volume	0.7	-	-
2	Bisphenol F	3.7	4.3	1.1
3	Bisphenol A	6.9	8.9	1.1

Analysis of Bisphenol A in Milk Powder

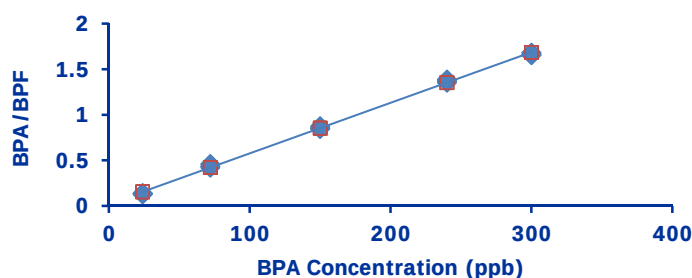
Chromolith® HighResolution RP-18 endcapped

Calibration Curve Data – Bisphenol A (BPA) and internal Standard Bisphenol F (BPF)

Day	Concentration (ppb)	Mean Response (Arbitrary Area Units)		Area Ratio BPA/BPF	N
		BPA	BPF		
1	24	186235 ± 3566 (±1.9%)	1387888 ± 49942 (±3.6%)	0.134 ± 0.006 (±4.2%)	5
	72	602534 ± 23059 (±3.8%)	1371728 ± 57740 (±4.2%)	0.440 ± 0.017 (±3.8%)	5
	150	1202378 ± 42234 (±3.5%)	1404348 ± 47496 (±3.4%)	0.856 ± 0.012 (±1.4%)	5
	240	1998729 ± 18626 (±0.9%)	1457002 ± 16444 (±1.1%)	1.372 ± 0.014 (±1.0%)	5
	300	2419051 ± 30986 (±1.3%)	1451407 ± 15079 (±1.0%)	1.667 ± 0.013 (±0.8%)	5
2	24	186704 ± 16737 (±9.0%)	1345296 ± 53101 (±3.9%)	0.139 ± 0.012 (±8.6%)	5
	72	613300 ± 12934 (±2.1%)	1263577 ± 37349 (±3.0%)	0.486 ± 0.011 (±2.2%)	5
	150	1275206 ± 42264 (±3.6%)	1357203 ± 11786 (±0.9%)	0.940 ± 0.040 (±4.3%)	5
	240	1956603 ± 47937 (±2.4%)	1299501 ± 19864 (±1.5%)	1.506 ± 0.016 (±1.1%)	5
	300	2459503 ± 114807 (±4.7%)	1338443 ± 41307 (±3.1%)	1.837 ± 0.054 (±2.9%)	5
3	24	200878 ± 13074 (±6.5%)	1307442 ± 38724 (±3.0%)	0.154 ± 0.013 (±8.7%)	5
	72	582906 ± 9193 (±1.6%)	1309387 ± 31802 (±2.4%)	0.445 ± 0.015 (±3.3%)	5
	150	1312398 ± 12769 (±1.0%)	1228963 ± 36470 (±3.0%)	1.069 ± 0.040 (±3.8%)	5
	240	1988977 ± 39203 (±2.0%)	1363312 ± 41316 (±3.0%)	1.460 ± 0.046 (±3.2%)	5
	300	2582306 ± 27184 (±1.0%)	1325420 ± 7119 (±0.5%)	1.948 ± 0.022 (±1.1%)	5

Five replicate injections of BPA standard solution (n=5) were analyzed at five concentration levels (24–300 ppb) while keeping the internal standard (BPF) concentration constant at 60 ppb. to determine the method repeatability, within and between day method reproducibility. The relative standard deviation, given in %, is presented in brackets for every calibration point.

The calibration graph from day 1 of analysis is presented below along with a table summarizing the results for all three calibration curves. Based on this data, the method can be considered as robust and reliable.



Curve Equation and Regression Coefficient		
Day	Curve Equation	(R ²)
1	y = 0.0055x + 0.0218	0.9987
2	y = 0.0061x + 0.0184	0.9969
3	y = 0.0064x + 0.0145	0.9898

Analysis of Bisphenol A in Milk Powder

Chromolith® HighResolution RP-18 endcapped

Baby milk formulation was spiked with BPA at four different concentration levels (100–500 ppb) to determine total method recovery. Internal standard (BPF) at a concentration of 60 ppb was added to improve method robustness. Six individual samples were prepared and injected in triplicate (n=18) at each spiking level. The recovery of BPA was found to be ranging between 85–91%. The method performance greatly improved with use of internal standard, see table. The detection limit (LOD) was determined to 1.4 ppb (ng/mL) based on analysis of low calibration standards and calibration curve data.

Recovery – baby Milk formulation spiked with Bisphenol A (BPA)

Spiked BPA Concentration (ppb)	BPA Mean Response (Arbitrary Area Units)	Area Ratio (BPA/BPF)	Recovery (%)	N
100	616010 ± 25186 (±4.1%)	0.511± 0.014 (±2.8%)	89.0± 2.5	18
200	1226325 ± 45048 (±3.7%)	0.976± 0.018 (±1.8%)	86.7± 1.6	18
300	1955164 ± 54922 (±2.8%)	1.467± 0.002 (±0.1%)	87.6± 1.5	18
500	3239985 ± 70881 (±2.2%)	2.376± 0.007 (±0.3%)	85.6± 1.0	18

The relative standard deviation, given in %, is presented in brackets for every calibration point.

Method robustness and column longevity were evaluated by performing long term analysis of extracted milk samples and evaluated by means of retention time and peak area measurement for BPA and BPF. Over 2700 injections were carried out over a period of three weeks. The relative standard deviation on retention time was better than 2.5 % for both compounds. In total 65 L of mobile phase was flushed through the column (24 mL per injection) before peak shape started to deteriorate and we stopped further analysis.

Retention time reproducibility

Bisphenol F (BPA)			Bisphenol A (BPA)			N
Retention Time (min)	S.D. (min)	R.S.D (%)	Retention Time (min)	S.D. (min)	R.S.D (%)	
3.7	0.08	2.1	6.9	0.10	1.5	2707*

This method allows quantitative analysis of BPA above 1 ppb (1 ng/mL) in baby milk samples. The method sensitivity can be further improved if required by increasing lamp energy (from medium to high). The detection limit is sufficient and compared well with other reported non-MS based methods and can be used for BPA screening purposes. For regulatory food sample analysis the acquired data should be confirmed by MS methods, and more specifically isotope dilution methods based on MS or tandem mass spectrometry (MS/MS).

Carbohydrates

Carbohydrates (saccharides) are a major source of nutrition and a key form of energy for most organisms as well as being structural components of plants. Carbohydrates is one of the major classes of biomolecules and consist of carbon, hydrogen and oxygen atoms with the general formula $C_n(H_2O)_n$. Saccharides contain two carbonyl groups that are either of aldehyde or ketone type. Carbohydrates are furthermore divided into four chemical groups:

- 1) Monosaccharides (examples: Glucose and Fructose)
- 2) Disaccharides (examples: Lactose, Isomaltose and Trehalose)
- 3) Oligosaccharides
- 4) Polysaccharides

In general, the monosaccharides and disaccharides, lower molecular weight carbohydrates, are commonly referred to as sugars. Beside monosaccharides and disaccharides there are also neutral sugars, acidic sugars, amino sugars, sugar alcohols, and their various isomers. Low molecular weight saccharides are common in food, such as fruits, honey and sweets. The separation and identification of saccharides is challenging, especially for compounds having the same chemical formula and only small differences in their molecular structure, i.e. disaccharides maltose and isomaltose. In addition, carbohydrates from simple sugars to oligo- and polysaccharides represent a detection challenge in that they are lacking chromophores. RI and UV (195 – 205nm), are problematic to use due to issues of poor sensitivity, long detector equilibration times and their inability to handle gradient elution. Evaporative light scattering detection (ELSD) is a viable alternative, but just as with RI detectors, ELSD is sensitive to changes in the mobile phase composition making gradient elution difficult.

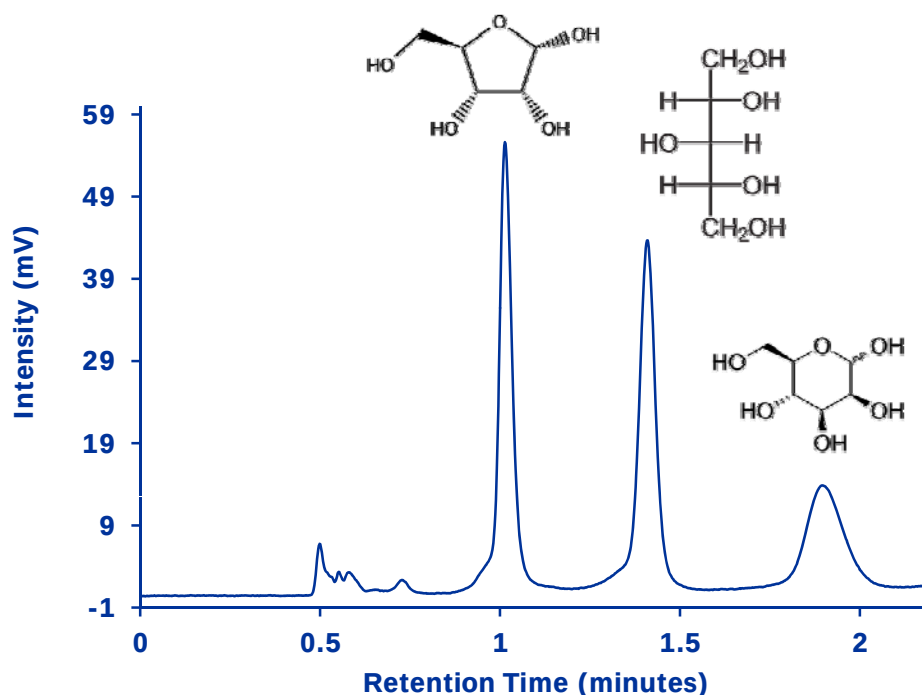
Various separation techniques have been used for carbohydrate analysis; anion chromatography, size exclusion chromatography, reversed phase chromatography, hydrophilic interaction liquid chromatography (HILIC), GC and TLC. HPLC using aminopropyl functionalized columns is one of the more common techniques for analysis of saccharides. In this technique, a mixture of acetonitrile and water is used as the mobile phase and the retention increases with reduction of water content just as in HILIC. In addition, the higher the molecular weight of the sugar, the longer it takes to elute. The aldehyde radicals in sugars can react with the amino radicals in the stationary phase to create Schiff bases, which can sometimes cause significant tailing on peaks for pentasaccharides (such as arabinose and ribose). This can be inhibited by adding a salt to the mobile phase. An advantage with an amino column is that it catalyze the mutarotation of reducing sugars effectively causing the retention time of the sugar to be the average of its two anomers, showing as only one peak in the chromatogram. This application compilation illustrates how Chromolith® NH2 columns can be used for separation of monosaccharides, disaccharides and glycoalkaloids (alkaloids with sugar moieties).

Monosaccharides – Ribose, Xylitol, Mannose

Chromolith® NH2

Chromatographic Conditions

Column:	Chromolith® NH2, 100x4.6 mm	(1.52028.0001)
Injection:	5 µl	
Detection:	UV, 190nm	
Flow Rate:	3.0 mL/min	
Mobile Phase (v/v):	Acetonitrile and Water 85:15 (v/v)	
Temperature:	23 °C	
Sample:	Ribose 5.9 mg/mL, Xylitol 8.1mg/mL, Mannose 7.6mg/mL in mobile phase	
Pressure Drop:	47 Bar (682 psi)	



Chromatographic Data

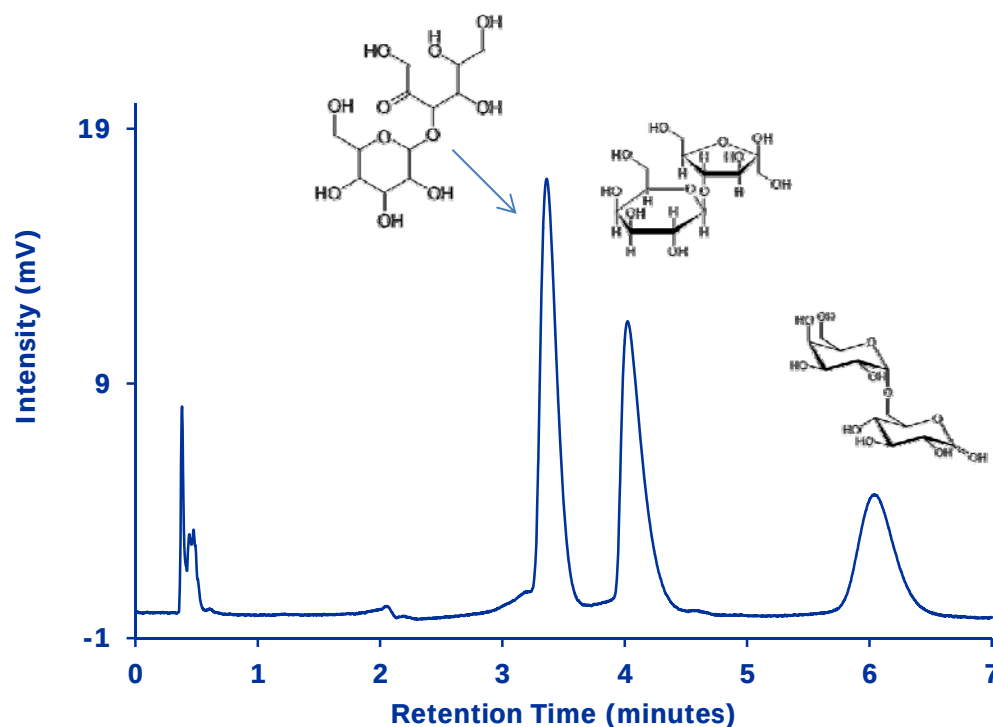
No.	Compound	Retention Time (min)	T _{USP}	Theoretical Plate
1	Void Volume	0.6	-	-
2	Ribose	1.0	0.9	4055
3	Xylitol	1.4	0.9	4656
4	Mannose	1.9	1.1	1584

Disaccharides – Turanose, Lactulose, Melibiose

Chromolith® NH2

Chromatographic Conditions

Column:	Chromolith® NH2, 100x4.6 mm	(1.52028.0001)
Injection:	10 µl	
Detection:	UV, 190nm	
Flow Rate:	4.0 mL/min	
Mobile Phase (v/v):	Acetonitrile and Water 85:15 (v/v)	
Temperature:	23 °C	
Sample:	Turanose 7.7mg/mL, Lactulose 8.0 mg/mL, Melibiose 10.6mg/mL in mobile phase	
Pressure Drop:	76 Bar (1102 psi)	



Chromatographic Data

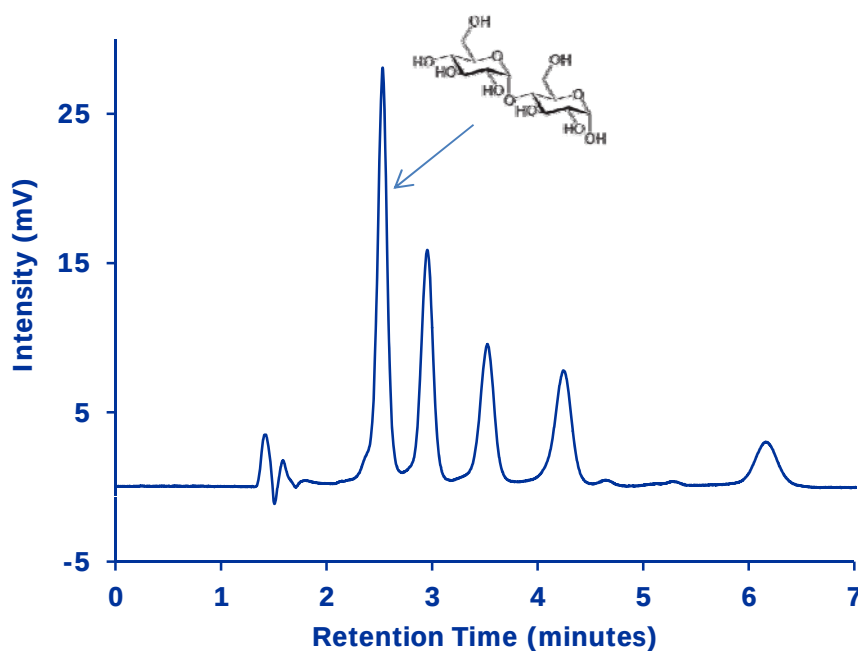
No.	Compound	Retention Time (min)	T _{USP}	Theoretical Plate
1	Void Volume	0.4	–	–
2	Turanose	3.4	1.4	2928
3	Lactulose	4.0	2.2	2761
4	Melibiose	6.0	1.2	1543

Linear Oligosaccharides

Chromolith® NH2

Chromatographic Conditions

Column:	Chromolith® NH2, 100x4.6 mm	(1.52028.0001)
Injection:	2 µl	
Detection:	UV, 190nm	
Flow Rate:	1.0 mL/min	
Mobile Phase (v/v):	Acetonitrile and Water 65:35 (v/v)	
Temperature:	23 °C	
Sample:	Maltose 21.6 mg/mL, Maltotriose 19.5 mg/mL, Maltotetraose 15.7 mg/mL, Maltopentaose 16.2 mg/mL, Maltoheptaose 10.6 mg/mL in mobile phase	
Pressure Drop:	26 Bar (377 psi)	



Chromatographic Data

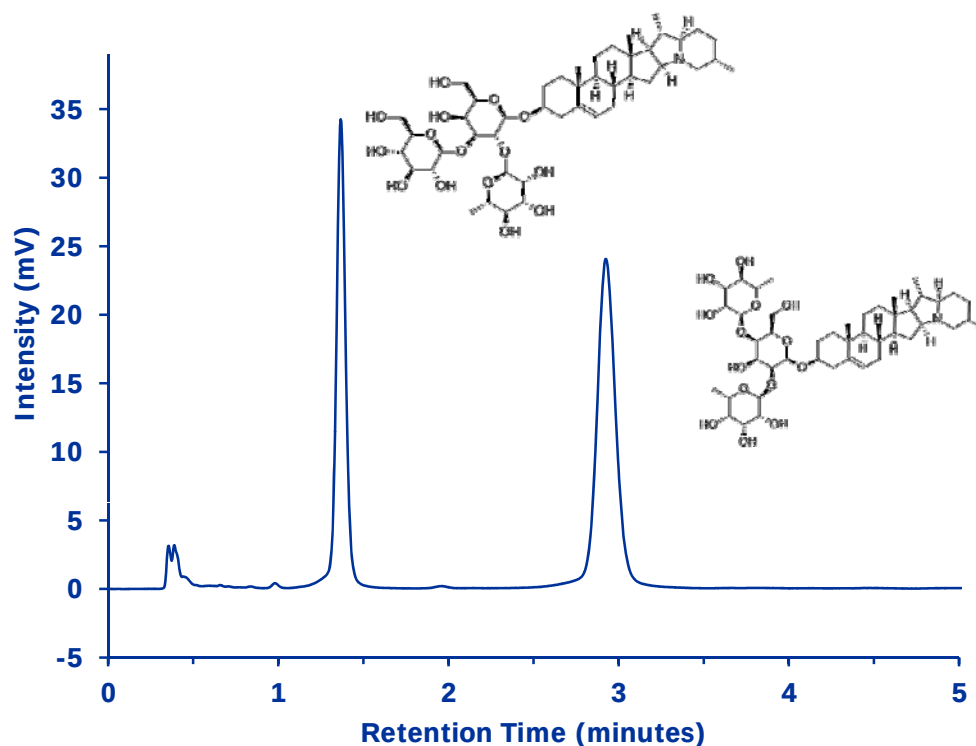
No.	Compound	Retention Time (min)	T _{USP}	Theoretical Plate
1	Void Volume	1.4	-	-
2	Maltose	2.5	0.8	3555
3	Maltotriose	3.0	1.0	3271
4	Maltotetraose	3.5	0.9	3303
5	Maltopentaose	4.2	0.9	3149
6	Maltoheptaose	6.2	1.0	3507

Glycoalkaloids – Solanine and Cacoline

Chromolith® NH2

Chromatographic Conditions

Column:	Chromolith® NH2,, 50x4.6 mm	(1.52027.0001)
Injection:	8 µl	
Detection:	UV, 208nm	
Flow Rate:	2.0 mL/min	
Mobile Phase (v/v):	Acetonitrile and phosphate buffer, pH 6; 80:20 (v/v)	
Temperature:	23 °C	
Sample:	200 ppm (0.2 mg/mL) of each Solanine and Cacoline in mobile phase	
Pressure Drop:	12 Bar (174 psi)	



Chromatographic Data

No.	Compound	Retention Time (min)	T _{USP}	Theoretical Plate
1	Void Volume	0.4	–	–
2	Solanine	1.4	1.1	3187
3	Cacoline	2.9	1.1	3505

Constituents and Additives

Food products are analyzed for a variety of reasons, e.g., compliance with legal and labeling requirements, assessment of product quality, determination of nutritive value, and detection of adulteration, etc. According to the Codex Alimentarius Commission – "Food Additive" means any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value. The term "Food additive" does not include contaminants or substances added to food for maintaining or improving its nutritive value. "Food additives" do not include use of vitamins, minerals, herbs, salt, spices, yeast, hops, starter cultures, malt extract, etc. "Food additives" are intentionally added to food and must be safe for a lifetime of consumption based on current toxicological evaluation.

"Food additives" are classified on the basis of their functional use and are grouped as:

Colors	Preservatives	Acidity Regulators
Antioxidants	Anti-caking agents	Antifoaming Agents
Artificial sweeteners	Enzymes	Emulsifiers
Emulsifying agents	Flavors	Flavor enhancers
Modified Starches	Phosphates	Stabilizers
Thickening and jelling agents.		

Antioxidants

Antioxidants refers to non-nutrient compounds in foods, which have antioxidant capacity. Other than for dietary antioxidant vitamins – vitamin A, vitamin C and vitamin E (applications found under vitamins)– no food compounds have been proved with antioxidant effect in vivo. For this reason, the US Food and Drug Administration and the European Food Safety Authority (EFSA) have published guidance disallowing food product labels to claim "antioxidant" effects when no such physiological evidence exists.

Spices, herbs, essential oils and cocoa are rich in antioxidant properties in the plant itself and in vitro, but the serving size is too small to supply antioxidants via the diet. Examples are clove, cinnamon, oregano, turmeric, cumin, parsley, basil, curry powder, mustard seed, ginger, pepper, chili powder, paprika, garlic, coriander, onion and cardamom. Typical herbs are sage, thyme, marjoram, tarragon, peppermint, oregano, savory, basil and dill weed.

Dried fruits are another good source of antioxidants and examples are pears, apples, plums, raisins, peaches, figs and dates. Red wine and dried raisins have high polyphenol levels. Fruits with lots of pigment; i.e. cranberries, blueberries, plums, blackberries, raspberries, strawberries, cherries, blackcurrants, figs, guava, oranges, mango, grape juice and pomegranate juice are also rich in antioxidants. Same is true for vegetables such as artichokes, cabbage, broccoli, asparagus, avocados, beetroot and spinach.

In this compilation, a number of "Antioxidant" applications have been included, such as separation of capsaicin in chili, isoflavones and flavonoids in green tea.

Colorants

Food colorant, or color additive, is any dye, pigment or substance that imparts color when it is added to food or drink. Natural colors are not required to be certified by a number of regulatory bodies throughout the world. Colorants are used in foods for many reasons including:

- a) offset color loss due to exposure to light, air, temperature extremes, moisture and storage conditions
- b) correct natural variations in color
- c) enhance colors that occur naturally
- d) provide color to colorless and "fun" foods

Food colorings are tested for safety by various regulatory bodies. In the United States, FDC numbers are given to approved synthetic food, while E numbers are used within the European union for all additives, both synthetic and natural, that are approved in food applications.

Natural food dyes

Because of consumer concerns about synthetic dyes a growing number of natural colorants are being commercially produced, exemplified herein with an application example of Annatto (E160b), a reddish-orange dye made from the seed of the achiote (*Bixa orellana*).

Permitted artificial colorants:

FDC Blue No. 1 – Brilliant Blue FCF, E133 (blue shade)
FDC Blue No. 2 – Indigotine, E132 (indigo shade)
FDC Green No. 3 – Fast Green FCF, E143 (turquoise shade)
FDC Red No. 40 – Allura Red AC, E129 (red shade)
FDC Red No. 3 – Erythrosine, E127 (pink shade)
FDC Yellow No. 5 – Tartrazine, E102 (yellow shade)
FDC Yellow No. 6 – Sunset Yellow FCF, E110 (orange shade)

Some artificial colorants are only allowed to be used for limited use and others are banned or delisted. For instance Orange B (red shade) is allowed only for use in hot dog and sausage casings, whereas FDC Red No. 2 (Amaranth) is banned for use in food in US.

In this compilation, several examples are included to illustrate the potential with Chromolith columns for analysis of colorants in food and beverage samples.

Determination of Artificial Colorants in Dried Fruit and Soft Drinks

Recommended column:

Chromolith® HighResolution RP-18endcapped, 100x4.6 mm (1.52022.0001)

Recommended solvents and reagents

Methanol: gradient grade for liquid chromatography LiChrosolv® (1.06007)

Water: Water for chromatography LiChrosolv® (1.15333)
or freshly purified water from Milli-Q® water purification system

Ammonium Acetate: for analysis EMSURE® ACS, Reag. Ph Eur. (1.01116)

Recommended filtration tools:

Mobile phase filtration:

PTFE coated with funnel, base, stopper clamp (XX1004720)
Omnipore™ PTFE membrane filter 0.45µm (JHWP04700)

Sample filtration:

Millex® -LG, 0.20 µm, Hydrophilic, PTFE, 13 mm, non-sterile (SLLGH13NL)

Millex® Smplicity filters, 0.20 µm, Hydrophilic, PTFE (SAMPLG001)

Bold blue Smplicity system bundle with 0.2 µm PTFE Millex® Smplicity filters (SAMPLG0BL)

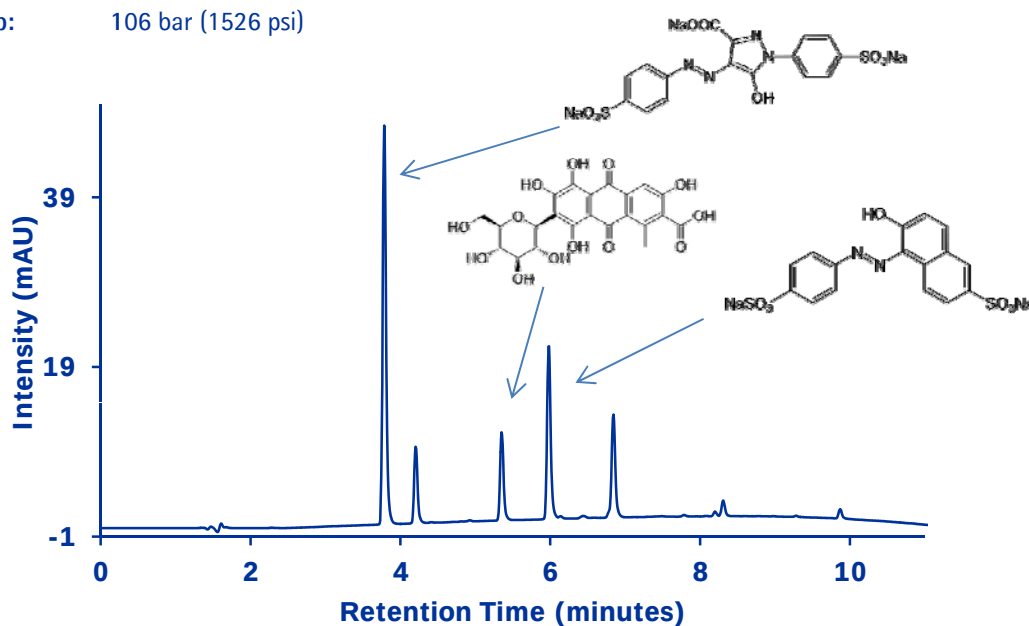
Artificial Food Colorants – UV 428 nm

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped, 100x4.6mm	(1.52022.0001)
Injection:	20 µL	
Detection:	UV, 428 nm	
Cell:	1 µL/10 mm	
Flow Rate:	1.0 mL/min	
Mobile Phase:	A: 20 mM ammonium acetate pH 6.8 in water B: Methanol	
Gradient:	See Table.	
Temperature:	30 °C	
Diluent (v/v):	Methanol and water (50:50)	
Sample:	0.05 mg/mL of each colorant in diluent.	
Pressure Drop:	106 bar (1526 psi)	

Time (min)	Solution A (%)	Solution B (%)
0.0-6.00	95→50	5→50
6.01-10.00	50→0	50→100
10.0-11.0	0	100
11.01	95	5



Chromatographic Data

No.	Compound	Retention Time (min)	Peak Asymmetry
1	Tartrazine	3.8	1.2
2	Amaranth	4.2	1.2
3	Carmine red	5.3	1.2
4	Sunset yellow	6.0	1.3
5	Fancy red	6.8	1.1

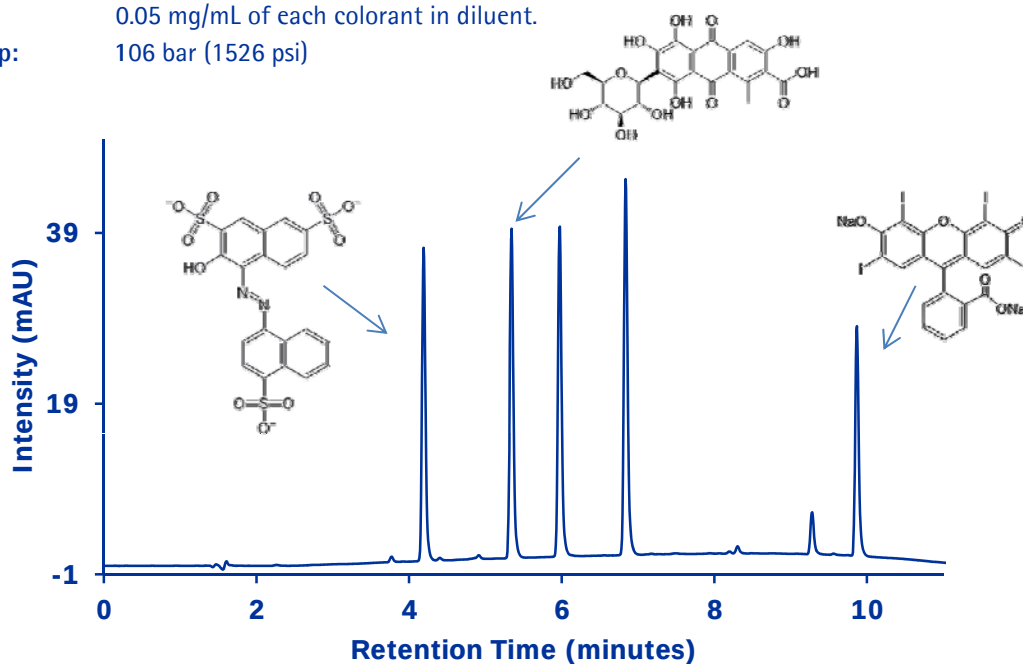
Artificial Food Colorants – UV 510 nm

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped 100x4.6mm	(1.52022.0001)
Injection:	20 µL	
Detection:	UV, 510 nm	
Cell:	1 µL/10 mm	
Flow Rate:	1.0 mL/min	
Mobile Phase:	A: 20 mM ammonium acetate pH 6.8 in water B: Methanol	
Gradient:	See Table.	
Temperature:	30 °C	
Diluent (v/v):	Methanol and water (50:50)	
Sample:	0.05 mg/mL of each colorant in diluent.	
Pressure Drop:	106 bar (1526 psi)	

Time (min)	Solution A (%)	Solution B (%)
0.0-6.00	95→50	5→50
6.01-10.00	50→0	50→100
10.0-11.0	0	100
11.01	95	5



Chromatographic Data

No.	Compound	Retention Time (min)	Peak Asymmetry
1	Amaranth	4.2	1.2
2	Carmine red	5.3	1.2
3	Sunset yellow	6.0	1.2
4	Fancy red	6.8	1.2
5	Erythrosine	9.9	2.3

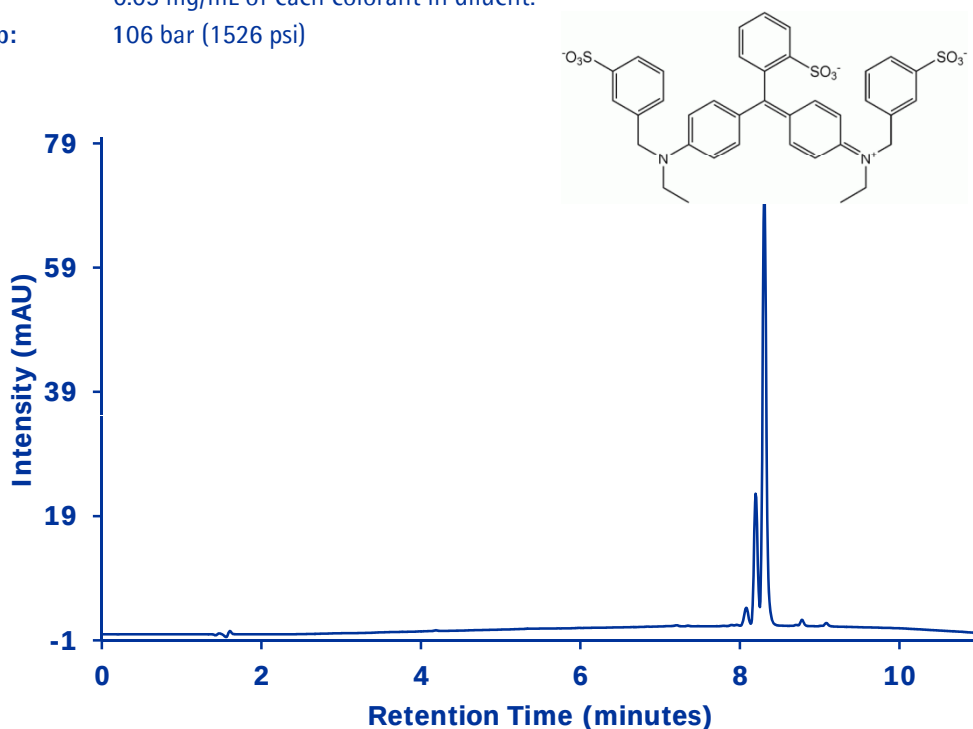
Artificial Food Colorants – UV 628 nm

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped 100x4.6mm	(1.52022.0001)
Injection:	20 µL	
Detection:	UV, 628 nm	
Cell:	1 µL/10 mm	
Flow Rate:	1.0 mL/min	
Mobile Phase:	A: 20 mM ammonium acetate pH 6.8 in water B: Methanol	
Gradient:	See Table.	
Temperature:	30 °C	
Diluent (v/v):	Methanol and water (50:50)	
Sample:	0.05 mg/mL of each colorant in diluent.	
Pressure Drop:	106 bar (1526 psi)	

Time (min)	Solution A (%)	Solution B (%)
0.0-6.00	95→50	5→50
6.01-10.00	50→0	50→100
10.0-11.0	0	100
11.01	95	5



Chromatographic Data

No.	Compound	Retention Time (min)	Peak Asymmetry
1	Brilliant Blue	8.2	1.1

Analysis Protocol for LC-UV Method Colorants

Chromolith® HighResolution RP-18 endcapped

Repeatability

No.	Compound	Mean Response (Arbitrary Area Units)	Relative Standard Deviation (%)	N	Wavelength (nm)
1	Tartrazine	150.7	0.05	5	428
2	Amaranth	28.9	0.31	5	428
3	Carmine red	34.0	0.40	5	428
4	Sunset yellow	69.8	0.84	5	428
5	Fancy red	43.2	0.25	5	428
6	Amaranth	117.7	0.04	5	510
7	Carmine red	126.6	0.12	5	510
8	Sunset yellow	131.7	0.20	5	510
9	Fancy red	148.8	0.03	5	510
10	Brilliant blue	233.7	0.05	5	628

5 Replicate injections of standard solution (n=5) were analyzed to determine the method injection repeatability. Sample contained 0.05 mg/mL (50 ppm) of each analyte in mobile phase.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

No.	Compound	LOD (ppm)	LOQ (ppm)	Curve Equation	Regression Coefficient (R ²)	Wavelength (nm)
1	Tartrazine	3	10	$y = 3061.9x - 0.3927$	0.9993	428
2	Amaranth	3	10	$y = 584.55x - 0.0082$	0.9992	428
3	Carmine red	3	10	$y = 698.05x - 0.1666$	0.9992	428
4	Sunset yellow	3	10	$y = 1420.5x - 0.4732$	0.9993	428
5	Fancy red	3	10	$y = 879.73x - 0.1172$	0.9993	428
6	Amaranth	3	10	$y = 2389.7x - 0.2932$	0.9993	510
7	Carmine red	3	9	$y = 2596.2x - 0.7792$	0.9995	510
8	Sunset yellow	3	9	$y = 2693.4x - 0.5937$	0.9994	510
9	Fancy red	3	9	$y = 3050.7x - 0.6635$	0.9994	510

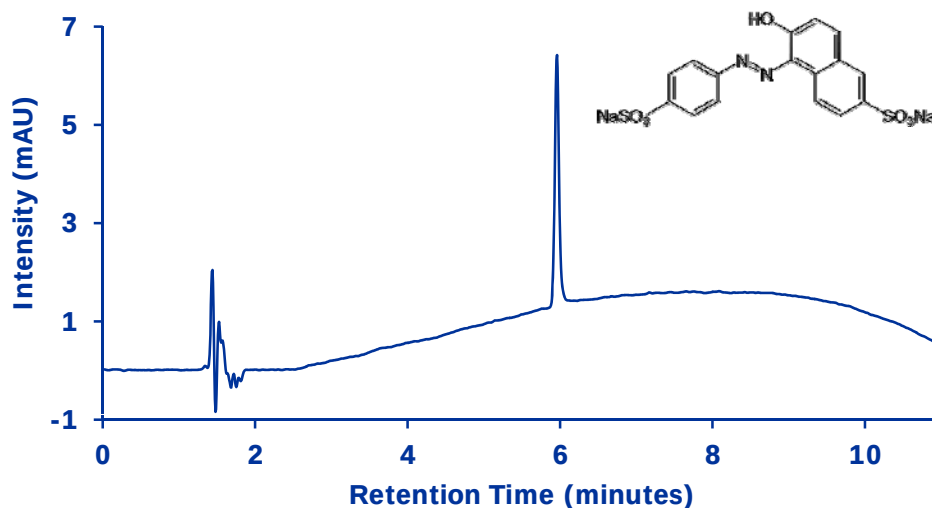
Injection of five different concentrations (each with 5 replicate injections) from LOQ level to 150 % of standard concentration (0.005-0.1 mg/mL) to determine the linearity of the method.

Artificial Food Colorants in Dried Plums

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped, 100x4.6mm	(1.52022.0001)
Injection:	20 µL	
Detection:	UV, 510 nm	
Cell:	1 µL/10 mm	
Flow Rate:	1.0 mL/min	
Mobile Phase:	A: 20 mM ammonium acetate pH 6.8 in water B: Methanol	
Gradient:	See Table.	
Temperature:	30 °C	
Diluent (v/v):	Methanol and water (50:50)	
Sample:	5 g of powdered dried fruit was transferred to a 50 mL volumetric flask. 45 mL of diluent was added into the flask. The flask was shaken for 30 s, then sonicated for 10 min. Diluent was added to the volume after the flask was cold. The solution was transferred to a 50 mL PP tube, centrifuged at 4000 rpm for 10 min, thereafter the clear solution was filtered by 0.45 µm PTFE syringe filter into a 2 mL autosampler vial prior to injection.	
Pressure Drop:	106 bar (1526 psi)	



Chromatographic Data

No.	Compound	Retention Time (min)	Peak Asymmetry
1	Sunset yellow	6.0	1.2

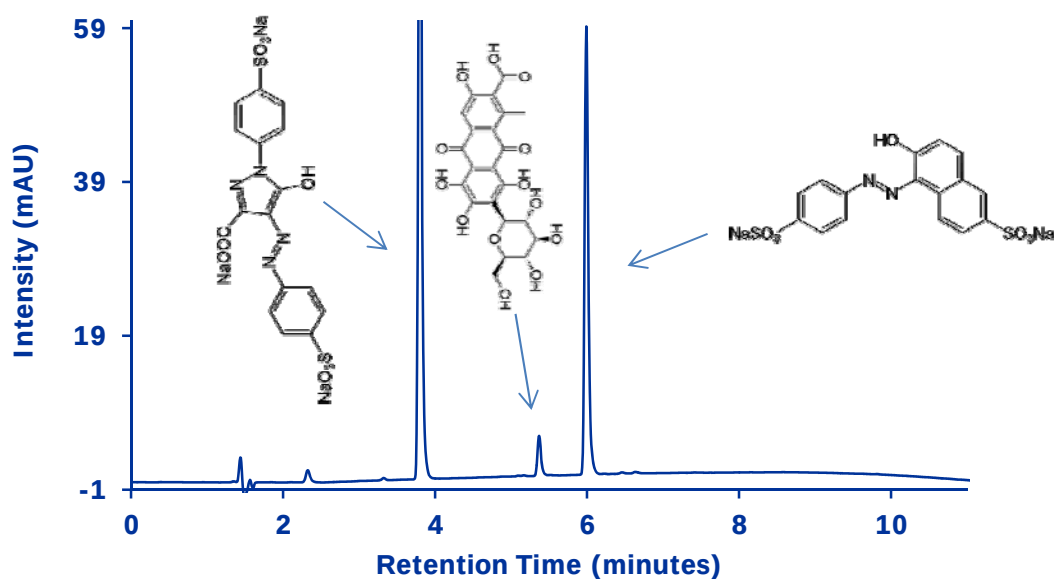
Artificial Food Colorants in Orange Soft Drink

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped, 100x4.6mm	(1.52022.0001)
Injection:	20 µL	
Detection:	UV, 428 nm	
Cell:	1 µL/10 mm	
Flow Rate:	1.0 mL/min	
Mobile Phase:	A: 20 mM ammonium acetate pH 6.8 in water B: Methanol	
Gradient:	See Table.	
Temperature:	30 °C	
Diluent (v/v):	water	
Sample:	5 mL of the beverage was transferred to a 10 mL volumetric flask, sonicated for 5 minutes, then pure water was added to the volume. The solution was filtered by 0.45 µm PTFE syringe filter into a 2mL autosampler vial prior to injection.	
Pressure Drop:	106 bar (1526 psi)	

Time (min)	Solution A (%)	Solution B (%)
0.0-6.00	95→50	5→50
6.01-10.00	50→0	50→100
10.0-11.0	0	100
11.01	95	5



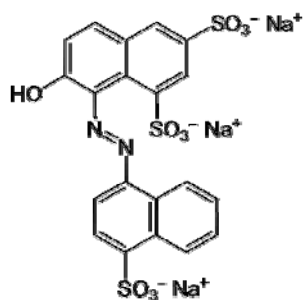
Chromatographic Data

No.	Compound	Retention Time (min)	Peak Asymmetry
1	Tartrazine	3.8	1.2
3	Carmine red	5.4	1.2
4	Sunset yellow	6.0	1.3

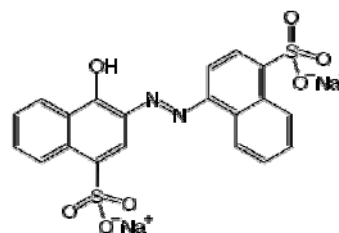
Artificial Food Colorants in Yoghurt

Method Transfer LC-UV to LC-MS

In many instances rapid, simple yet robust and inexpensive methods are requested for food and beverage testing. HPLC with mass spectrometric detection has more recently entered as a viable analytical technique, especially for determination of adulterants and trace analysis of certain molecules. In the following two applications, an example is presented for analysis of artificial food colorants (E 124 - Cochineal Red A, and E 122 - Azorubine) in yoghurt.



E 124 - Cochineal Red A



E 122 - Azorubine

The purpose of the two applications is to illustrate that short 50x2.0 mm Chromolith® columns can be used in a LC-UV HPLC system to screen samples for certain colorants, and thereafter do confirmatory analysis with LC-MS using same experimental conditions. Hence when a new method is being developed the experimental conditions chosen should ideally be considered to fit multiple detection modes to fit further needs and to minimize need for multiple methods for same purpose.

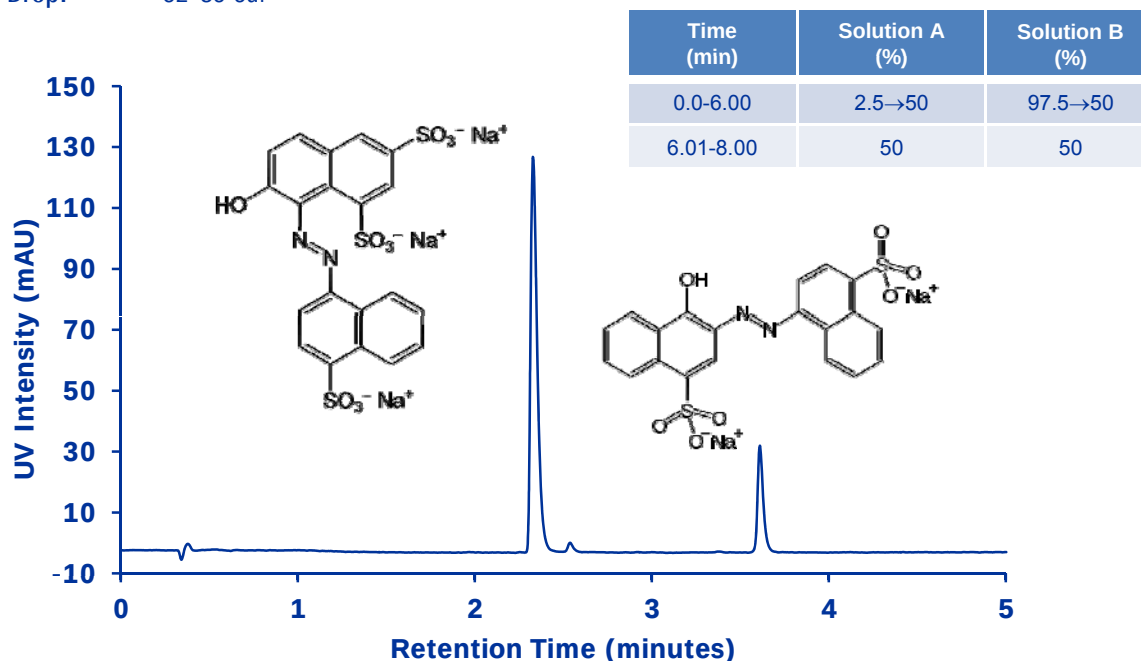
When comparing the results among the two applications, obviously the sensitivity is better with LC-MS and more impurities and sample components can be monitored. The chromatographic performance is though similar in both applications.

Artificial Food Colorants in Yoghurt – LC-UV

Chromolith® FastGradient RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® FastGradient RP-18 endcapped, 50x2.0mm	(1.52007.0001)
Injection:	1 µL	
Detection:	UV, 480 nm	
Cell:	1.4 µL	
Flow Rate:	0.4 mL/min	
Mobile Phase:	A: Acetonitrile, B: 20 mM ammonium acetate pH 4.7 in water	
Gradient:	See Table.	
Temperature:	25 °C	
Diluent (v/v):	water	
Sample:	Mix 10 g of yoghurt with 30 mL ACN, stir for 30 min and centrifuge for 20 min at 4500 rpm. Remove ACN (TurboVap II, 90 min, 40 °C, 1 bar nitrogen) and filter solution (0.45 µm filter). Spike after sample treatment with E 124 (Cochineal Red A, 69.44 mg in 50 mL water) and E122 (Azorubine, 3.46 mg in 10 mL water) stock solutions in a 10:2:3 ratio (v/v).	
Pressure Drop:	62-59 bar	



Chromatographic Data

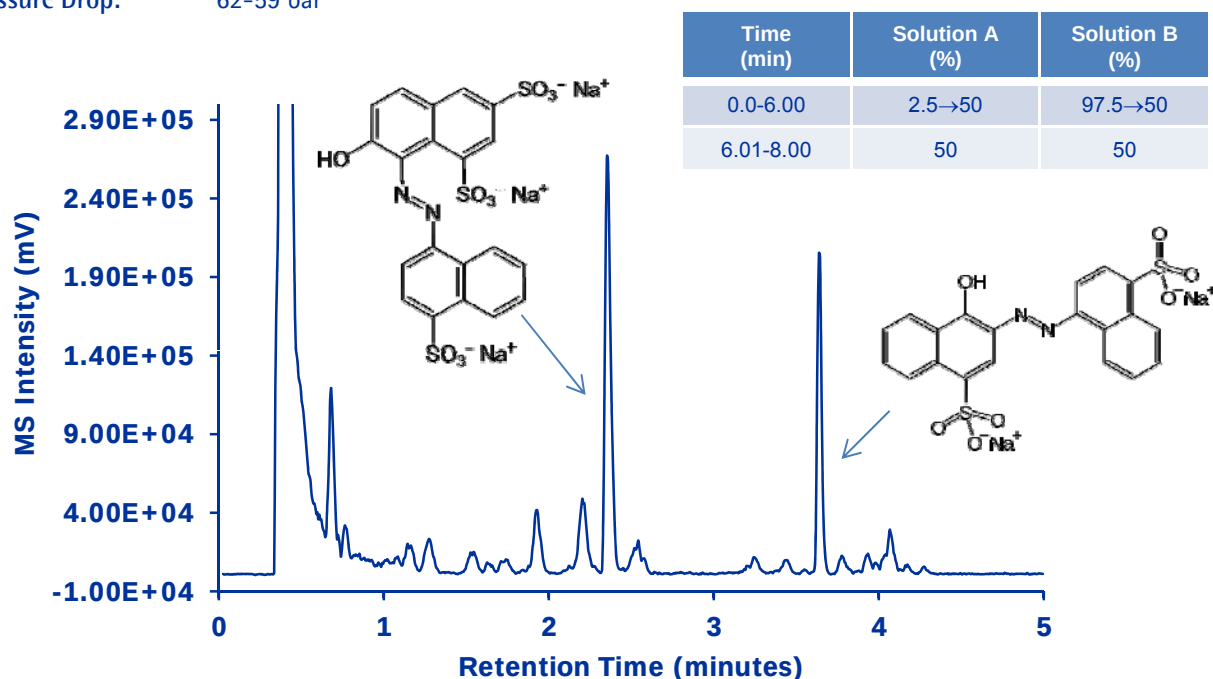
No.	Compound	Retention Time (min)
1	E 124 (Cochineal Red A)	2.3
2	E 122 (Azorubine)	3.6

Artificial Food Colorants in Yoghurt – LC-MS

Chromolith® FastGradient RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® FastGradient RP-18 endcapped 50x2.0mm	(1.52007.0001)
Injection:	1 µL	
Detection:	Negative ESI-MS (m/z range 100–550), BPC	
Cell:	1.4 µL	
Flow Rate:	0.4 mL/min	
Mobile Phase:	A: Acetonitrile, B: 20 mM ammonium acetate pH 4.7 in water	
Gradient:	See Table.	
Temperature:	25 °C	
Diluent (v/v):	water	
Sample:	Mix 10 g of yoghurt with 30 mL ACN, stir for 30 min and centrifuge for 20 min at 4500 rpm. Remove ACN (TurboVap II, 90 min, 40 °C, 1 bar nitrogen) and filter solution (0.45 µm filter). Spike after sample treatment with E 124 (Cochineal Red A, 69.44 mg in 50 mL water) and E122 (Azorubine, 3.46 mg in 10 mL water) stock solutions in a 10:2:3 ratio (v/v).	
Pressure Drop:	62–59 bar	



Chromatographic Data

No.	Compound	Retention Time (min)	[M+H] ⁺ (m/z)
1	E 124 (Cochineal Red A)	2.4	537.0
2	E 122 (Azorubine)	3.6	459.0

Nano LC-MS Analysis of Dyes in Chili

Chili powder (spice) is an important ingredient used by the food manufacturing industry. The spice quality is often judged only by its color (brightness, purity and intensity). Unscrupulous companies trading spices may utilize this and add illegal banned dyes to “boost” the appearance and perceived quality. A number of incidents have been reported all over the world where chili powders and derivative foods were found to be adulterated with illegal dyes such as Sudan I-IV and/or related dyes.

Sudan I (also known as CI Solvent Yellow 14 and Solvent Orange R), is a lysochrome, a diazo-conjugate dye with the chemical formula of 1-phenylazo-2-naphthol), Sudan III, and Sudan IV have been classified as category 3 carcinogens. Other dyes are suspected to be genotoxic and or carcinogenic and is thus not permitted for use in food.

The following two applications describes the simple and fast quantification of Sudan I-IV, Para Red and capsaicinoids extracted from hot sauces used to spice up sausages. The investigated molecules were separated on a robust reversed-phase monolithic silica capillary column (CapRod) and then directly transferred to a MS detector. The first application is showing the separation of a standard solution containing Para Red and Sudan I-IV, and the other example show a separation of above mentioned illegal dyes along with four capsaicinoids in an extracted hot chili sauce sample.

In a recent publication Chromolith® CapRod RP-18 endcapped capillaries were used to analyze a set of 10 different Chili sauces, and where it was found that the two main analytes, capsaicin and dihydrocapsaicin contribute to 90% of chilli heat*.

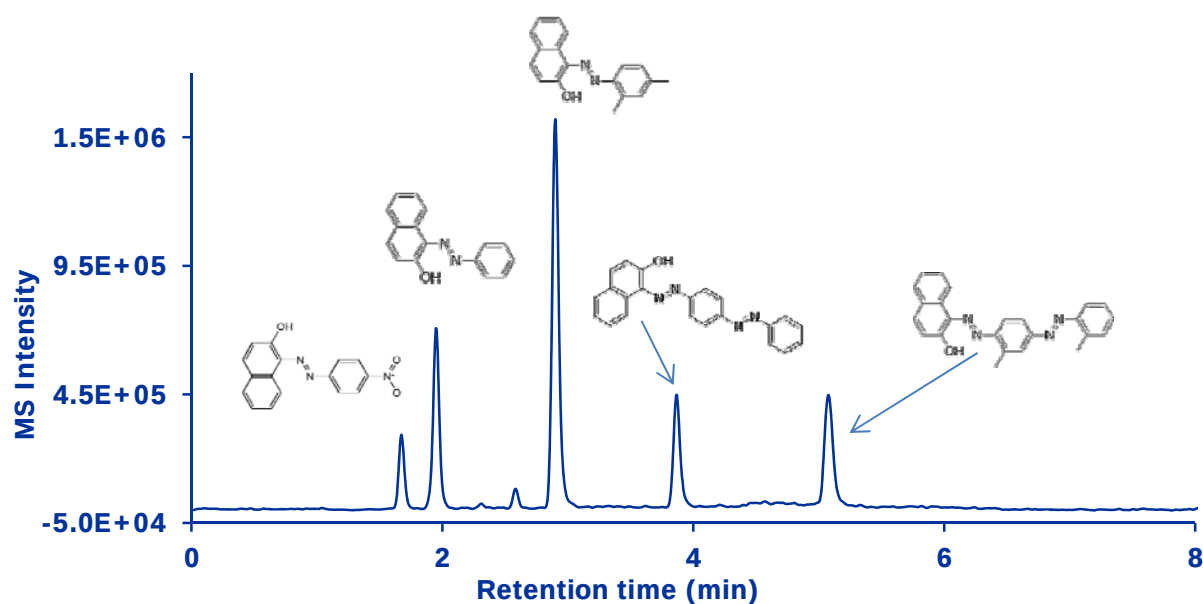
*"Simple and Fast Quantification of Capsaicinoids in Hot Sauces Using Monolithic Silica Capillaries and LC-MS"
S. Forster and S. Altmaier, LCGC NORTH AMERICA VOLUME 31 NUMBER 3 MARCH 2013, page 218.

Sudan Dyes

Chromolith® CapRod® RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® CapRod RP-18 endcapped 150x0.1 mm	(1.50402.0001)
Injection:	2.5 nL	
Detection:	nano-ESI(+) 240-390 m/z	
Flow Rate:	1.24 µL/min	
Mobile Phase :	A: water + 0.1 % formic acid B: acetonitrile + 0.1 % formic acid	
Gradient:	70 % B to 95 % B in 5 min	
Temperature:	Ambient	
Diluent	Acetonitrile	
Sample:	Para red, Sudan I, Sudan II, Sudan III, Sudan IV	
Pressure Drop:	76 Bar (1100 psi)	



Chromatographic Data

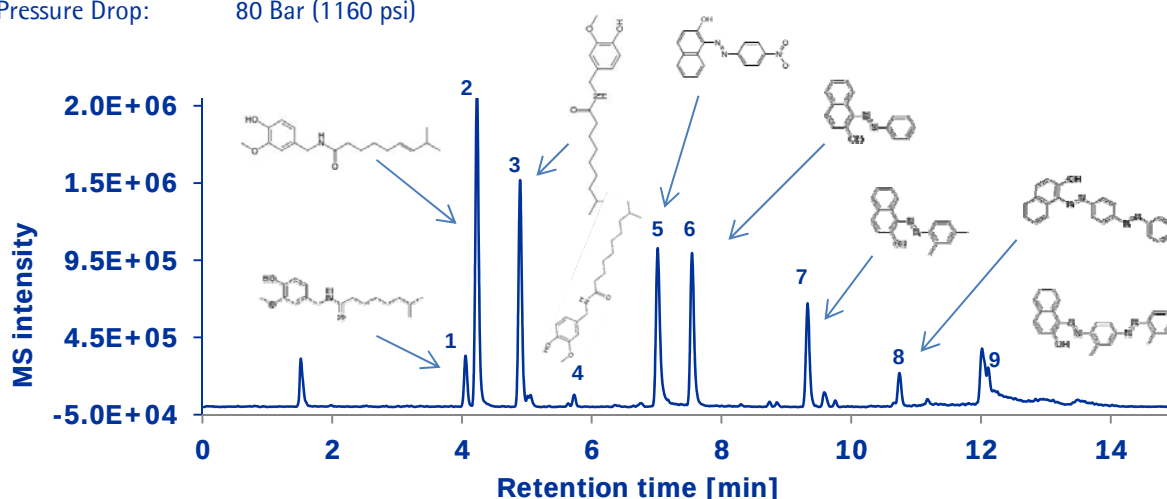
No.	Compound	Retention Time (min)	Monoisotopic mass (g/mol)	[M+H] ⁺ (m/z)
1	Para Red	1.67	293.08	294.01
2	Sudan I	1.95	248.09	249.01
3	Sudan II	2.90	276.13	277.09
4	Sudan III	3.87	352.13	353.08
5	Sudan IV	5.08	380.16	381.11

Sudan Dyes and Capsaicinoids in Chili Sauce

Chromolith® CapRod® RP-18 endcapped

Chromatographic Conditions

Column: Chromolith® CapRod RP-18 endcapped, 150x0.1 mm (1.50402.0001)
 Injection: 2.5 nL
 Detection: nano-ESI(+) 100-600 m/z
 Flow Rate: 1.24 µL/min
 Mobile Phase : A: water + 0.1 % formic acid
 B: acetonitrile + 0.1 % formic acid
 Gradient: 35 % B to 95 % B in 12 min
 Temperature: Ambient
 Diluent Acetonitrile
 Sample: Sudan dyes and capsaicinoids in hot chili sauce (see table)
 Pressure Drop: 80 Bar (1160 psi)



Chromatographic Data

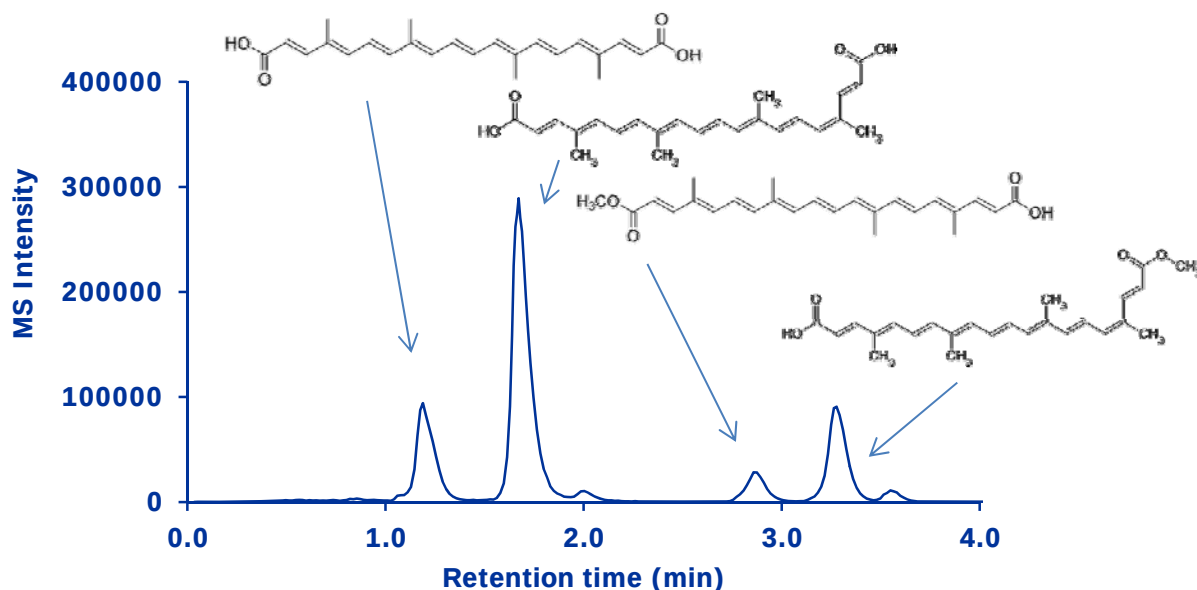
No.	Compound	Retention Time (min)	Monoisotopic mass (g/mol)	[M+H] ⁺ (m/z)
1	Nordihydrocapsaicin	4.05	293.20	294.11
2	Capsaicin	4.23	305.20	306.18
3	Dihydrocapsaicin	4.90	307.21	308.19
4	Homodihydrocapsaicin	5.73	321.23	322.11
5	Para red	7.02	293.08	294.05
6	Sudan I	7.55	248.09	249.05
7	Sudan II	9.33	276.13	277.10
8	Sudan III	10.74	352.13	353.11
9	Sudan IV	12.11	380.16	381.21

Annato (or Roucou or Achiote)

Chromolith® RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® FastGradient RP-18 endcapped, 50x2.0 mm	(1.52007.0001)	
Injection:	10 µL		
Detection:	Positive ESI-MS (m/z range 378–396), BPC		
Flow Rate:	0.4 mL/min		
Mobile Phase :	A: water + 0.1 % formic acid B: acetonitrile + 0.1 % formic acid		
Gradient:	See table		
Temperature:	25° C		
Diluent	Acetonitrile and water (50:50 v/v)		
Sample:	2.2 mg/mL cis-Norbixin and 0.12 mg/mL cis-Bixin in diluent. After dissolution of the analytes the solution was cooked for 5.5 h at 75 °C under reflux to speed up transformation of cis- to trans-Isomers and thereafter diluted 1:10 with diluent.		
Pressure Drop:	39–22 Bar (562–317 psi)		



Chromatographic Data

No.	Compound	Retention Time (min)
1	trans-Norbixin	1.19
2	cis-Norbixin	1.67
3	trans-Bixin	2.87
4	cis-Bixin	3.27

Flavonoids and Isoflavones

Flavonoids (from the Latin word flavus meaning yellow, their color in nature) are a class of plant secondary metabolites. Flavonoids are all ketone-containing compounds and the IUPAC nomenclature classify flavonoids as:

Flavones from 2-phenylchromen-4-one (2-phenyl-1,4-benzopyrone) structure (examples: quercetin, rutin).

Isoflavonoids from 3-phenylchromen-4-one (3-phenyl-1,4-benzopyrone) structure

Neoflavonoids, from 4-phenylcoumarine (4-phenyl-1,2-benzopyrone) structure.

Food manufacturers have become interested in flavonoids for their possible medicinal properties. Although no physiological evidence is yet established, the beneficial effects of fruits, vegetables, tea, and red wine have sometimes been attributed to flavonoid compounds. Good sources of flavonoids include all citrus fruits, berries, ginkgo biloba, onions, parsley, pulses, tea (especially white and green), red wine, seabuckthorn, and dark chocolate (with > 70% cocoa content).

Quercetin, a flavonoid and more specifically a flavonol, is the aglycone (non-sugar) form of other flavonoid glycosides, found in citrus fruit, buckwheat and onions. The glycosides quercitrin and rutin are formed from quercetin reacting with rhamnose and rutinose, respectively. Epicatechin may improve blood flow. Cocoa, the major ingredient of dark chocolate, contains relatively high amounts of epicatechin. Daidzein can be found in food such as soybeans and soy products like tofu and textured vegetable protein.

To analyze flavonoids in fruit, vegetables and other flavonoid rich solid samples problems may or will arise from sample matrix. The presence and concentration levels among different interesting marker molecules (analytes) differ from high to low concentrations, and their presence differ among sample types (i.e. cocoa bean and lemon). The analysis of flavonoids thus constitute problems with finding suitable sample preparation, to resolve all interesting compounds from each other chromatographically, and to detect them accordingly.

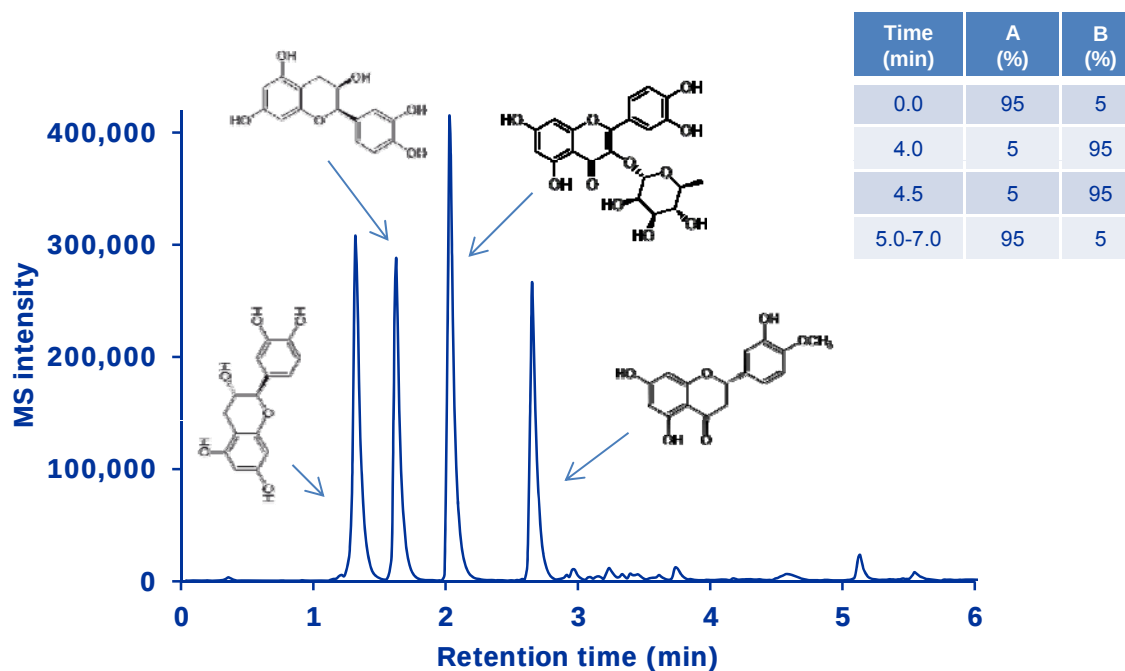
A few examples are presented herein to illustrate that Chromolith® columns are ideal tools for this purpose, and combined with MS detection it is possible to develop sensitive methods for monitoring flavonoids and isoflavones in food and beverage samples.

Flavonoids – LC-MS

Chromolith® RP-18 endcapped

Chromatographic Conditions

Column: Chromolith® FastGradient RP-18 endcapped, 50x2.0 mm (1.52007.0001)
 Injection: 1 µL
 Detection: Positive ESI-MS (m/z range 100–455)
 Flow Rate: 0.4 mL/min
 Mobile Phase : A: water + 0.1 % formic acid
 B: acetonitrile + 0.1 % formic acid
 Gradient: See table
 Temperature: 25° C
 Diluent: water
 Sample: 317 ppb Catechin, 270 ppb epicatechin, 198 ppb quercetin and 177 ppb hesperetin in diluent.
 Pressure Drop: 47–17 Bar (677–245 psi)



Chromatographic Data

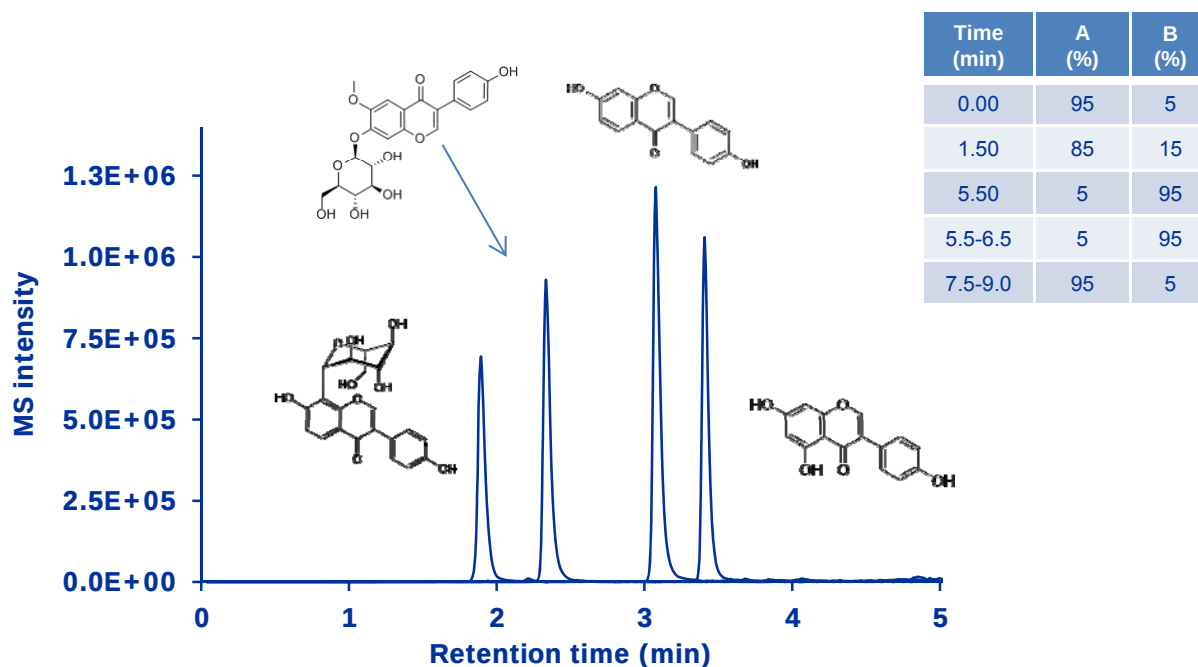
No.	Compound	Retention Time (min)	[M+H] ⁺ (m/z)
1	(+)-Catechin	1.3	291.1
2	(-)-Epicatechin	1.6	291.1
3	Quercitrin	2.0	449.1
4	Hesperetin	2.7	303.1

Isoflavones – LC-MS

Chromolith® RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® FastGradient RP-18 endcapped, 50x2.0 mm	(1.52007.0001)
Injection:	1 µL	
Detection:	Positive ESI-MS (m/z range 100-600), overlay of four EICs (m/z 271.0, 255.0, 447.1, 417.1)	
Flow Rate:	0.5 mL/min	
Mobile Phase :	A: water + 0.1 % formic acid B: acetonitrile + 0.1 % formic acid	
Gradient:	See table	
Temperature:	25° C	
Diluent	Methanol and water (50:50 v/v)	
Sample:	10 ppb (10 µg/mL) of each Genistein, Daidzein, Glycitin, and 20 ppb of Puerarin in diluent	
Pressure Drop:	57-22 Bar (821-317 psi)	



Chromatographic Data

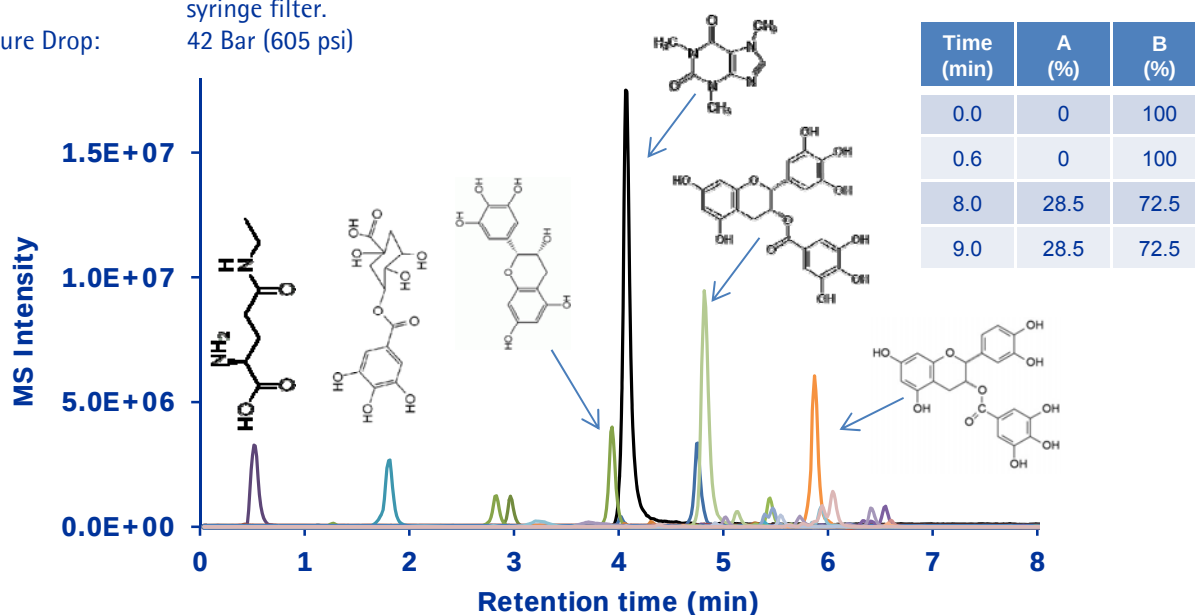
No.	Compound	Retention Time (min)	[M+H] ⁺ (m/z)
1	Puerarin	1.9	417.1
2	Glycitin	2.3	447.1
3	Daidzein	3.1	255.0
4	Genistein	3.4	271.0

Analysis of Green Tea – LC-MS

Chromolith® RP-18 endcapped

Chromatographic Conditions

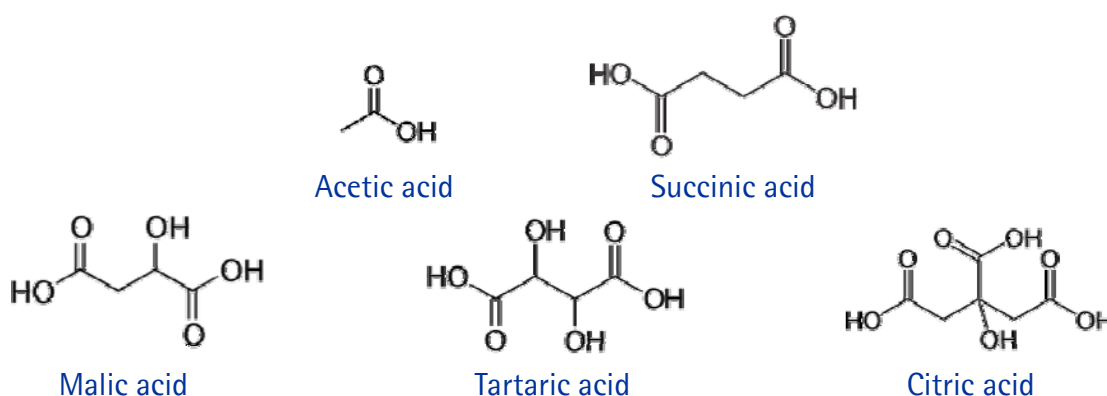
Column: Chromolith® FastGradient RP-18 endcapped, 50x2.0 mm (1.52007.0001)
 Injection: 1 µL
 Detection: Positive ESI-MS (m/z range 150-950), BPC and overlay of EICs
 Flow Rate: 0.4 mL/min
 Mobile Phase : A: acetonitrile + 0.1 % formic acid
 B: water + 0.1 % formic acid
 Gradient: See table
 Temperature: 25° C
 Diluent: Water
 Sample: 1.75 g of green tea was extracted for three minutes using 100 mL of boiling water. Prior to injection both samples were centrifuged at 4500 rpm for 10 min and cleaned using a 0.45 µm syringe filter.
 Pressure Drop: 42 Bar (605 psi)



Chromatographic Data

No.	Compound	Retention Time (min)	[M+H] ⁺ (m/z)
1	Theanine	0.5	175.1
2	Theogallin	1.8	345.1
3	Epigallocatechin	3.9	307.1
4	Caffeine	4.1	195.1
5	Epigallocatechin gallate	4.8	459.1
6	Epicatechin gallate	5.9	443.1

Organic Acids



Organic acids are organic compounds with acidic properties where the carboxylic acids are the most common; being weak acids that do not dissociate completely in water. The predominant organic acids in grapes are tartaric and malic acid while succinic and citric acids are present in minor proportions. In winemaking a common differentiation is made between acids which come directly from the grape (tartaric, malic and citric acids) and those that are produced in the fermentation process (succinic, lactic and acetic acids). Organic acids are also used in food preservation because they can penetrate bacteria's cell wall and disrupt their normal physiology. Hence, organic acids are present in every meal we eat, and there is necessary to have analytical methods able of accurate determination (both quantitatively and qualitatively).

Organic acids are hydrophilic compounds, and to be retained in reversed phase mode it is a requirement to either add ion-pairing reagents, work at low pH, and or use completely aqueous mobile phases. In this compilation, we have included a method for determination of tartaric acid, malic acid, citric acid and succinic acid following the current Chinese Standard method: GB/T 5009.157-2003 Determination of organic acid in foods and applied said method for determination of citric acid.

Analysis of Organic Acids in Beverages

Recommended column:

Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm (1.52022.0001)

Recommended solvents and reagents

Water: Water for chromatography LiChrosolv® (1.15333)
or freshly purified water from Milli-Q® water purification system

Di-ammonium hydrogenphosphate for analysis EMSURE® ACS,ISO,Reag. Ph Eur (1.01207)

ortho-Phosphoric acid 85% for analysis EMSURE® ACS,ISO,Reag. Ph Eur (1.00573)

Sample Preparation

Sample: Commercial orangeade (soft drink)

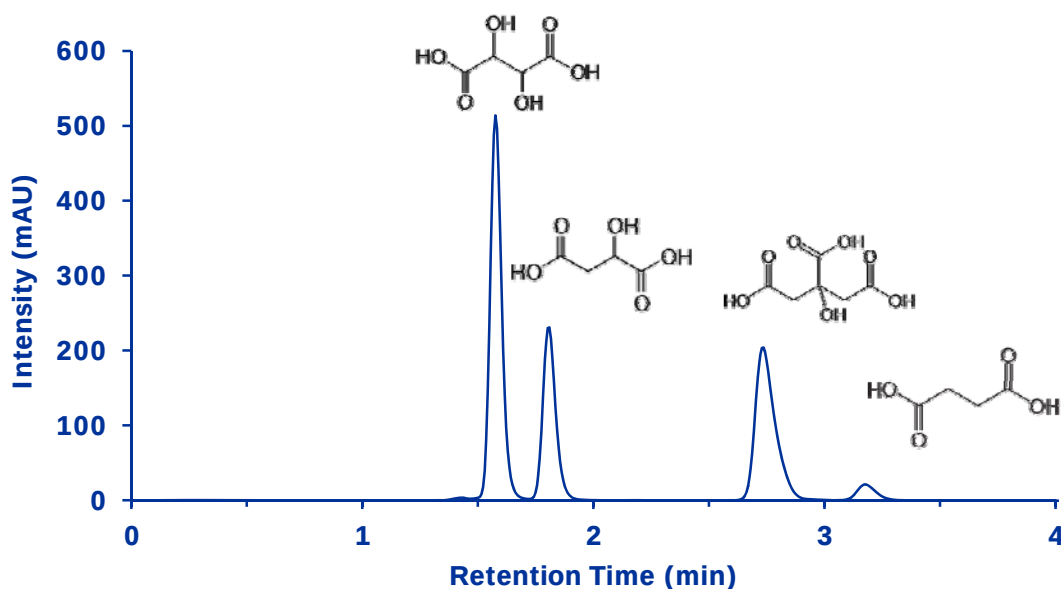
Take 5 mL of orangeade and sonicate for 5 minutes, thereafter add 0.2 mL of phosphoric acid solution (1M) and make up to a final volume of 10mL by adding water. This gives a dilution factor of 2 for sample.

Analysis of Organic Acids – Standards

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm	(1.52022.0001)
Injection:	20 µL	
Detection:	UV, 210 nm	
Cell:	1 µL/10 mm	
Flow Rate:	1.0 mL/min	
Mobile Phase:	10 mM Di-ammonium hydrogen phosphate solution (pH 2.7)	
Temperature:	30 °C	
Diluent	water	
Sample:	Standard solution with 1mg/ml of tartaric acid, malic acid and citric acid, and 0.2mg/ml of succinic acid diluted in water	
Pressure Drop:	32 Bar (464psi)	



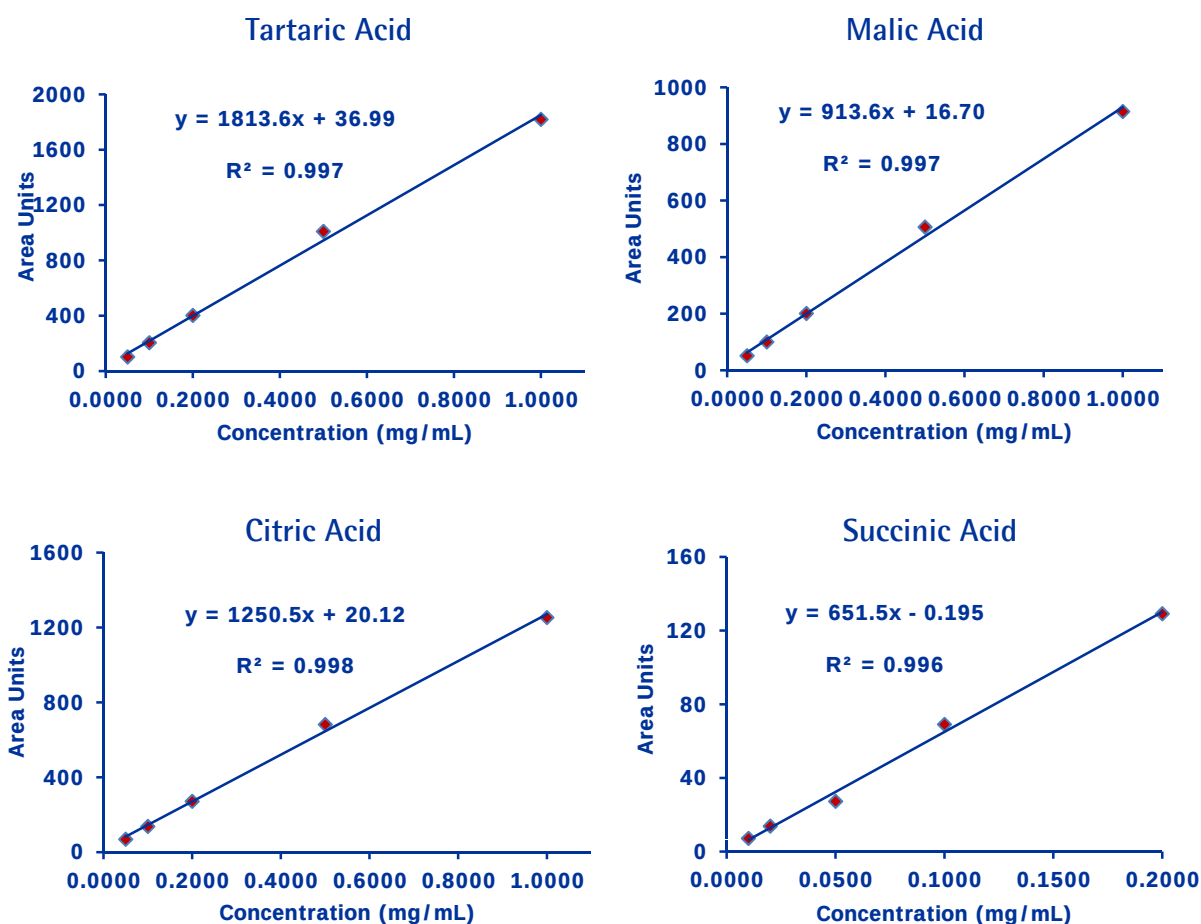
Chromatographic Data

No.	Compound	Retention Time (min)	Retention factor	Asymmetry
1	Tartaric Acid	1.6	0.6	1.2
2	Malic Acid	1.8	0.8	1.2
3	Citric Acid	2.7	1.7	1.4
4	Succinic Acid	3.2	2.2	1.2

Analysis of Organic Acids in Beverages

Chromolith® HighResolution RP-18 endcapped

Calibration curves were constructed in the range 0.005–1.0 mg/mL for tartaric, malic and citric acid, while the calibration range for succinic acid was 0.001–0.20 mg/mL. Five (n=5) replicate injections of standard solution were analyzed at the five different concentration levels to determine the method linearity. The relative standard deviation for replicate injections at all concentration levels was better or equal to 1% for all four compounds.



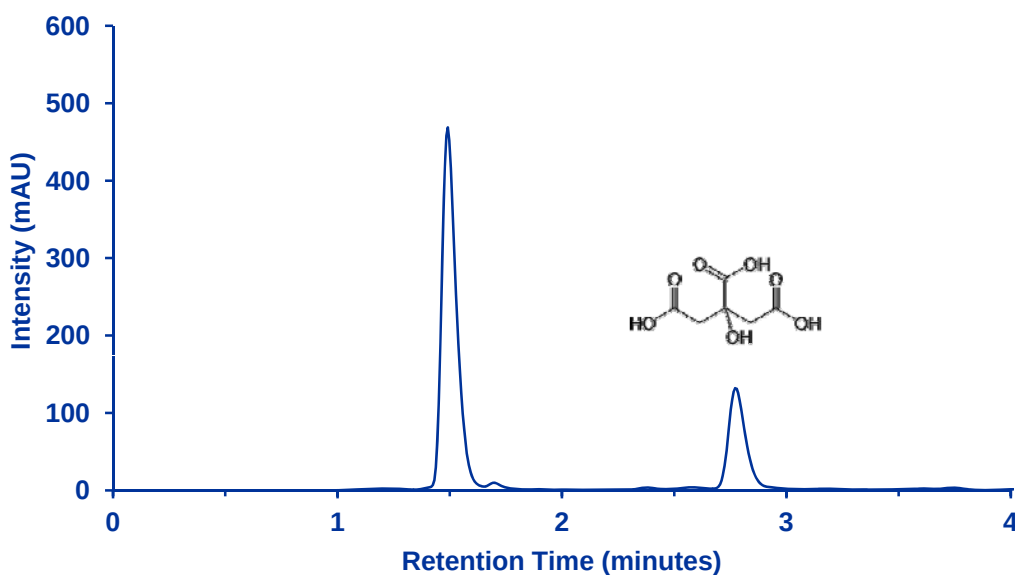
As a final test of the method, a commercial orangeade was analysed and as can be seen on next page only citric acid was found in the beverage. The citric acid concentration was determined to 1.1 mg/mL

Analysis of Organic Acids in Beverages

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm	(1.52022.0001)
Injection:	20 µL	
Detection:	UV, 210 nm	
Cell:	1 µL/10 mm	
Flow Rate:	1.0 mL/min	
Mobile Phase:	10 mM Di-ammonium hydrogen phosphate solution (pH 2.7)	
Temperature:	30 °C	
Diluent	water	
Sample:	5mL of orangeade was sonicated for 5 minutes. Thereafter 0.2ml of phosphoric acid solution (1M) was added. Finally the solution was diluted to 10mL by water.	
Pressure Drop:	32 Bar (464psi)	



Chromatographic Data

No.	Compound	Retention Time (min)	Theoretical plates	Asymmetry
1	Citric Acid	2.8	6366	1.4

Preservatives

A preservative is a naturally occurring or synthetically produced substance that is added to food and beverages to prevent decomposition (either by microbial growth or chemical changes). Preservatives in food can be compounds used alone or combined with other methods of food preservation. Rosemary extract, hops, salt, sugar, vinegar, alcohol, diatomaceous earth and castor oil are used as food preservatives. They are examples of natural food preservatives.

Preservatives can be classified in two groups, Class I and II, where the former are represented by common household products such as vinegar, salt, sugar, honey, and vegetable oil. Class II preservatives refers to those preservatives which are chemically manufactured.

There are antimicrobial preservatives (inhibit growth of bacteria or fungi) such as sorbic acid and its salts, benzoic acid and its salts, calcium propionate, sodium nitrite/sodium nitrate, sulfites (sulfur dioxide, sodium bisulfite, potassium hydrogen sulfite, etc.) and disodium EDTA. There are also antioxidants (inhibit oxidation) such as BHA (Butylated hydroxyanisole), BHT (Butylated hydroxytoluene), TBHQ (tert-Butylhydroquinone) and propyl gallate. Other preservatives include ethanol and methylchloroisothiazolinone. Freezing, pickling, smoking and salting techniques can also be used to preserve food.

On the next pages, a method is presented for determination of caffeine in a commercial caffeine containing cola, and caffeine and potassium sorbate in an energy beverage.

Caffeine and Potassium Sorbate

Recommended column:

Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm

(1.52022.0001)

Recommended solvents and reagents

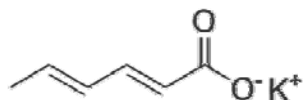
Water: Water for chromatography LiChrosolv® (1.15333)
or freshly purified water from Milli-Q® water purification system

Di-ammonium hydrogen phosphate for analysis EMSURE® ACS,ISO,Reag. Ph Eur (1.01207)

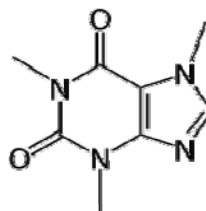
ortho-Phosphoric acid 85% for analysis EMSURE® ACS,ISO,Reag. Ph Eur (1.00573)

Sample Preparation

Take 5 mL of an commercial caffeine containing cola and sonicate for 5 minutes, thereafter add 0.2 mL of phosphoric acid solution (1M) and make up to a final volume of 10mL by adding water. This gives a dilution factor of 2 for sample.



Potassium Sorbate



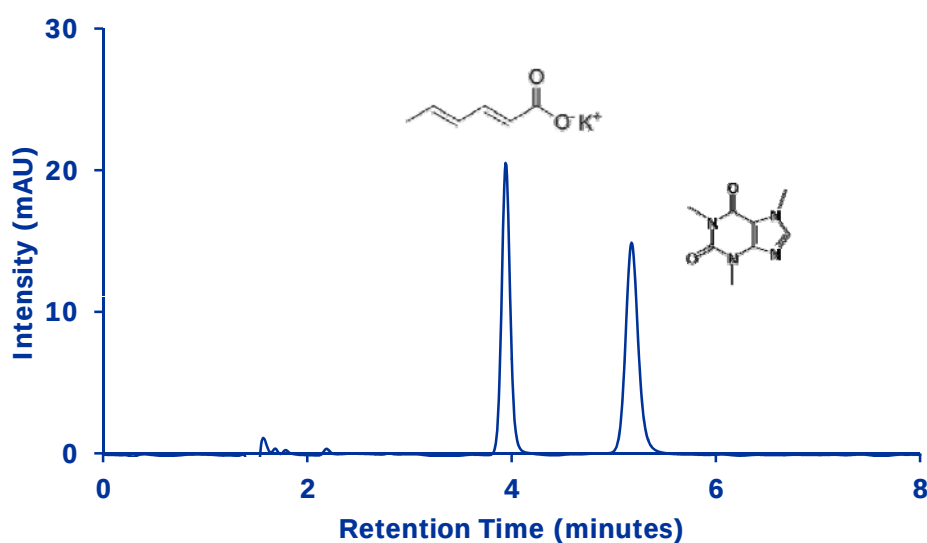
Caffeine

Caffeine and Potassium Sorbate – Standards

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm	(1.52022.0001)
Injection:	10 µL	
Detection:	UV, 220 nm	
Cell:	1 µL/10 mm	
Flow Rate:	1.0 mL/min	
Mobile Phase:	A: 15 mM ammonium acetate and 50 mM potassium di-hydrogen phosphate 40:60 v/v B: Acetonitrile C: Methanol Composition: A:B:C= 85:3:12 (v/v)	
Temperature:	30 °C	
Diluent	water	
Sample:	5µg/mL of potassium sorbate and caffeine diluted in water	
Pressure Drop:	47 Bar (681 psi)	



Chromatographic Data

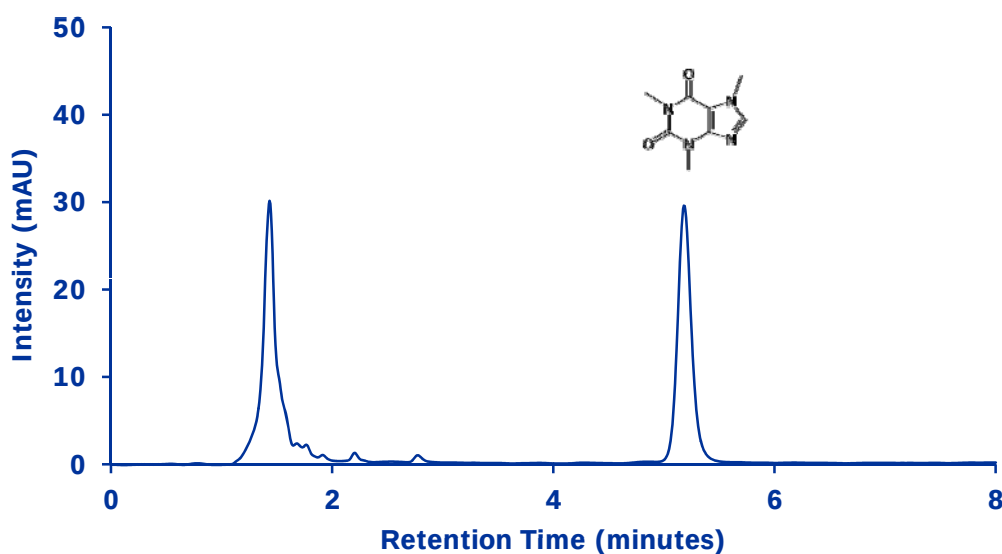
No.	Compound	Retention Time (min)	Theoretical plate	Asymmetry
1	Potassium sorbate	3.9	10886	1.1
2	Caffeine	5.2	8600	1.1

Caffeine in Beverage

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column: Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm (1.52022.0001)
 Injection: 10 µL
 Detection: UV, 220 nm
 Cell: 1 µL/10 mm
 Flow Rate: 1.0 mL/min
 Mobile Phase: A: 15 mM ammonium acetate and 50 mM potassium di-hydrogen phosphate 40:60 v/v
 B: Acetonitrile
 C: Methanol
 Composition: A:B:C= 85:3:12 (v/v)
 Temperature: 30 °C
 Diluent: water
 Sample: 10mL of a commercial caffeine containing cola was sonicated for 5 minutes.
 1ml of the degassed cola was thereafter diluted to 10mL by water. (sample is diluted 10 times)
 Pressure Drop: 47 Bar (681 psi)



Chromatographic Data

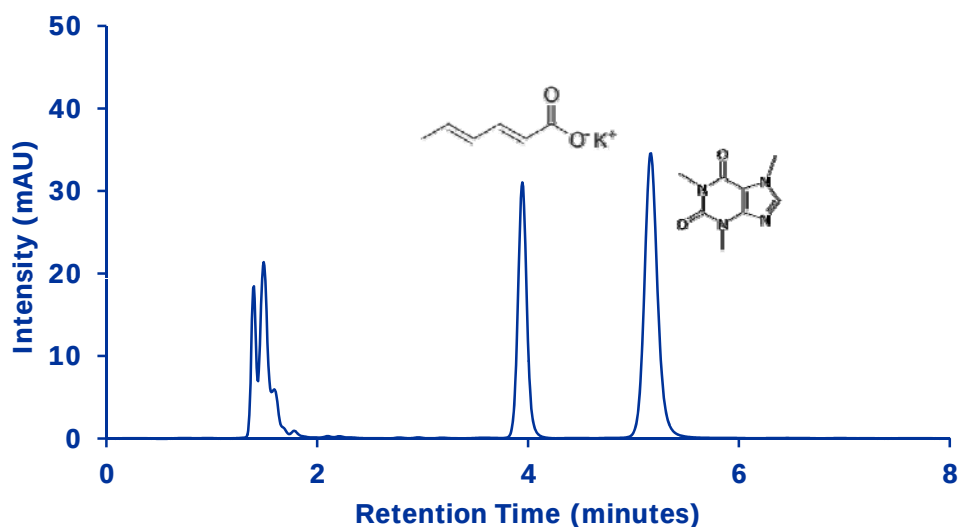
No.	Compound	Retention Time (min)	Theoretical plate	Asymmetry
1	Caffeine	5.2	8600	1.1

Potassium Sorbate and Caffeine in Beverages

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column: Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm (1.52022.0001)
 Injection: 10 µL
 Detection: UV, 220 nm
 Cell: 1 µL/10 mm
 Flow Rate: 1.0 mL/min
 Mobile Phase: A: 15 mM ammonium acetate and 50 mM potassium di-hydrogen phosphate 40:60 v/v
 B: Acetonitrile
 C: Methanol
 Composition: A:B:C= 85:3:12 (v/v)
 Temperature: 30 °C
 Diluent: water
 Sample: 10mL of the beverage was sonicated for 5minutes.
 Pressure Drop: 1ml of the degassed beverage was diluted to 25 mL by water (sample is diluted 25 times).
 47 Bar (681 psi)



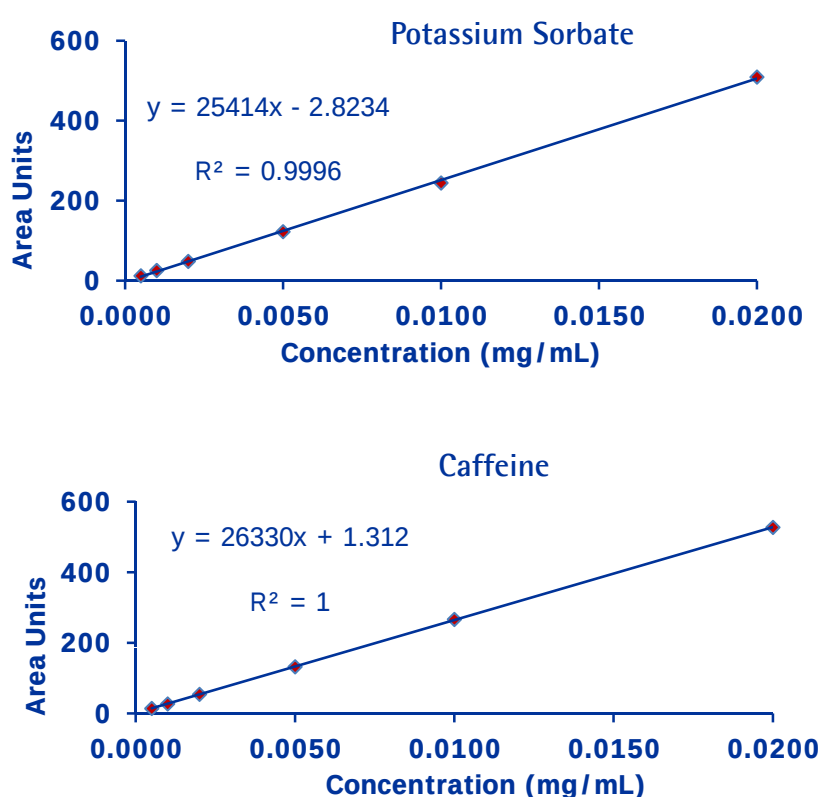
Chromatographic Data

No.	Compound	Retention Time (min)	Theoretical plate	Asymmetry
1	Potassium sorbate	3.9	10700	1.1
2	Caffeine	5.2	8600	1.1

Potassium Sorbate and Caffeine in Beverages

Chromolith® HighResolution RP-18 endcapped

Calibration curves were constructed in the range 5–20 ppb ($\mu\text{g/mL}$) for potassium sorbate and caffeine. Five ($n=5$) replicate injections of standard solution were analyzed at the five different concentration levels to determine the method linearity. The relative standard deviation for replicate injections at all concentration levels was better or equal to 1.5% for both compounds.

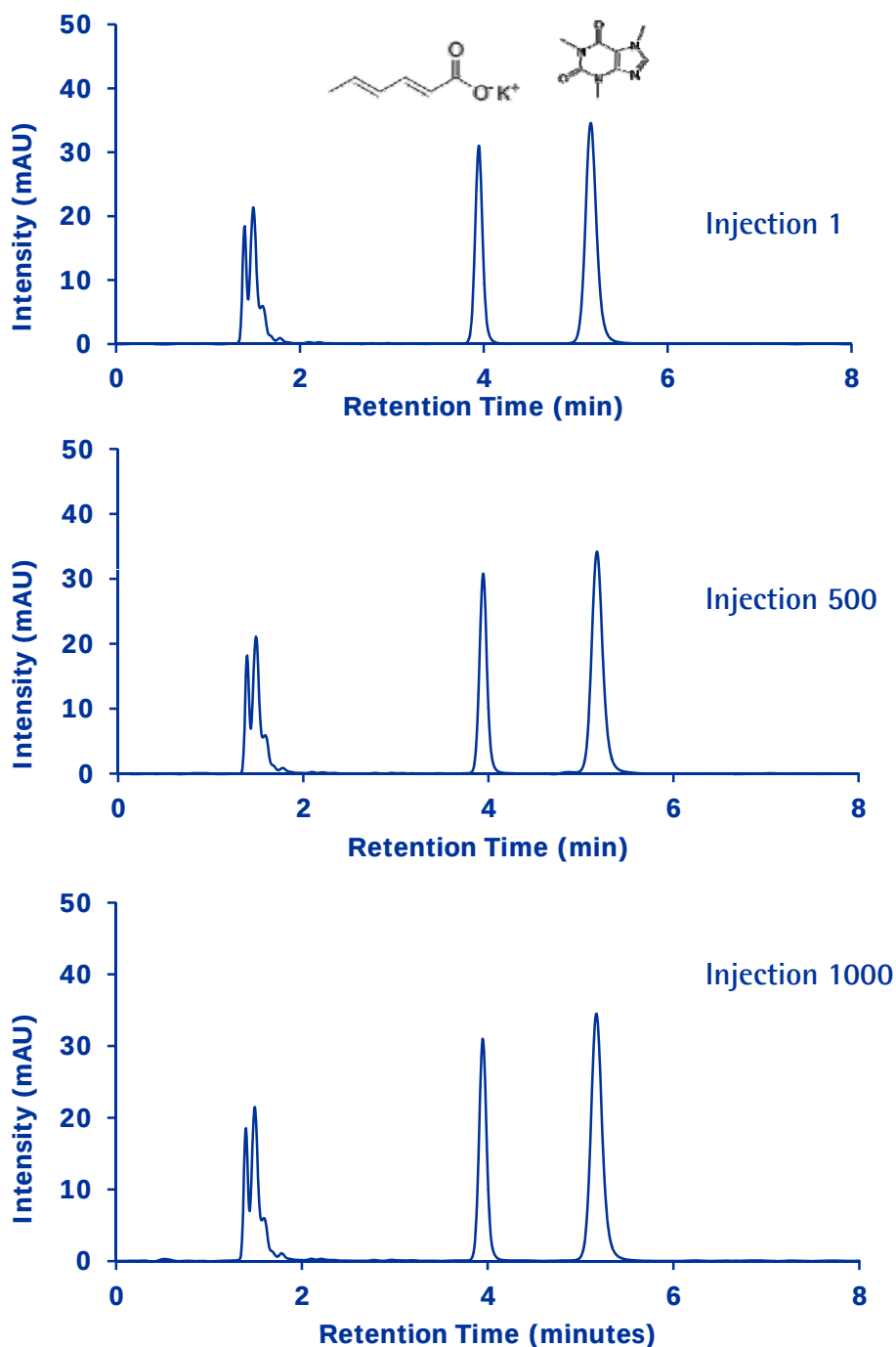


The detection limits (LOD's) and the quantitation limits (LOQ's) were 0.54/1.63 ppb for potassium sorbate and 0.14/0.42 ppb for caffeine, respectively. The energy drink contained 0.18 mg/mL of potassium sorbate and 0.29 mg/mL caffeine.

In total 1000 injections of standards and diluted energy drink samples were analysed and on the following page sample injection 1, 500 and 1000 are shown. The method is robust and can be used for quantitation of potassium sorbate and caffeine in beverages.

Potassium Sorbate and Caffeine in Beverages

Chromolith® HighResolution RP-18 endcapped



Enzymes

Milk contains proteins, specifically caseins, that maintain its liquid form. Proteases are enzymes that are added to milk during cheese production, to hydrolyze caseins, specifically kappa casein, which stabilizes micelle formation preventing coagulation. Rennet and rennin are general terms for any enzyme used to coagulate milk.

Lysozyme is used to prevent growth of Clostridia in aged, hard cheeses which contribute to flavor and structural defects such as late blowing. It is approved for use in numerous countries including the EU where it may be used as an additive (E-1105) and must be labeled on the final product.

On the following pages, an application based on current ISO standard method* for determination of lysozyme in hard cheese has been tested using a Chromolith® HighResolution column and UV detection.

*Milk and milk products – Determination of hen's egg white lysozyme by HPLC
ISO/TS 27105:2009 (IDF 216: 2009)
www.iso.org

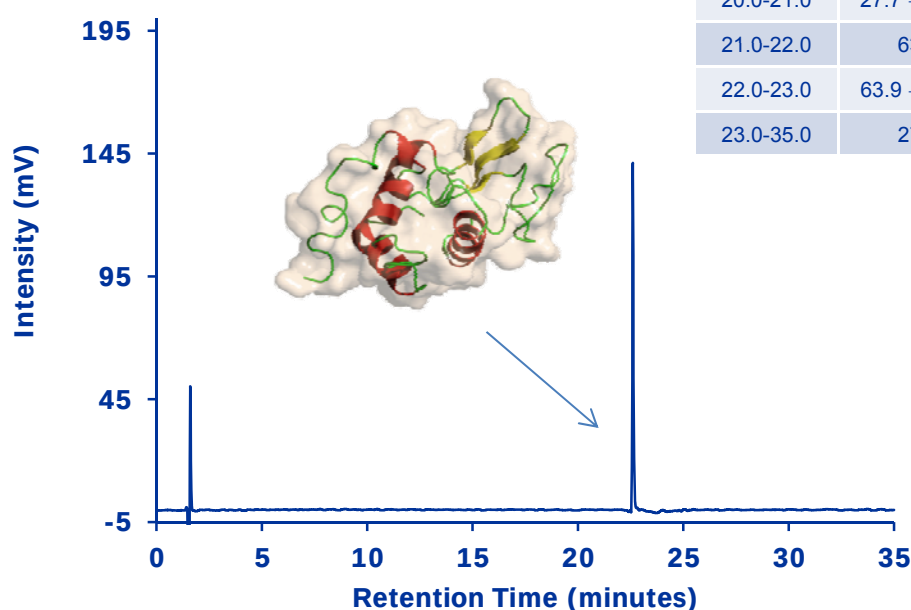
Lysozyme

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column: Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm (1.52022.0001)
 Injection: 1 µL
 Detection: UV, 214nm
 Flow Rate: 1.0 mL/min
 Mobile Phase: A: 100% Acetonitrile + 0,1%TFA
 B: 100% Water + 0,1% TFA
 Gradient: See table
 Temperature: Ambient
 Diluent: 1M NaCl
 Sample: 8480 ppm (8.48 mg/mL) lysozyme in diluent.
 Pressure Drop: 55Bar (798 psi)

Time (min)	A (%)	B (%)
0.0-20.0	27.7	72.3
20.0-21.0	27.7 → 63.9	72.3 → 36.1
21.0-22.0	63.9	36.1
22.0-23.0	63.9 → 27.7	36.1 → 72.3
23.0-35.0	27.7	72.3



Chromatographic Data

No.	Compound	Retention Time (min)	Asymmetry
1	Void volume	1.6	-
2	Lysozyme	22.6	1.3

Vitamins

The term vitamin is derived from "vitamine," a combination of vital and amine, i.e. amine of life. 100 years ago it was believed that organic micronutrient food factors that prevent dietary-deficiency diseases might be chemical amines. Later this proved incorrect. Today it is well established that a vitamin is an organic compound being a vital nutrient in tiny amounts, not endogenously synthesized in enough quantities by the organism, and hence must be obtained from the diet. There are thirteen vitamins, classified by their biological and chemical activity, not their structure,, four are fat-soluble (A, D, E, and K) and the other nine are water-soluble (B1, B2, B3, B5, B6, B7, B9, B12, C). Ascorbic acid (vitamin C) is a vitamin for human, but not for most other animals. The largest number of vitamins (e.g., B complex vitamins) function as precursors for enzyme cofactors, that help enzymes in their work as catalysts in metabolism.

This application compilation focuses on the more hydrophobic water soluble vitamins that are rather "neutral" such as folic acid (logP -0.02), and cyanocobalamin but also the more hydrophobic vitamin E family.

Further reading:

<http://www.nlm.nih.gov/medlineplus/ency/article/002399.htm>

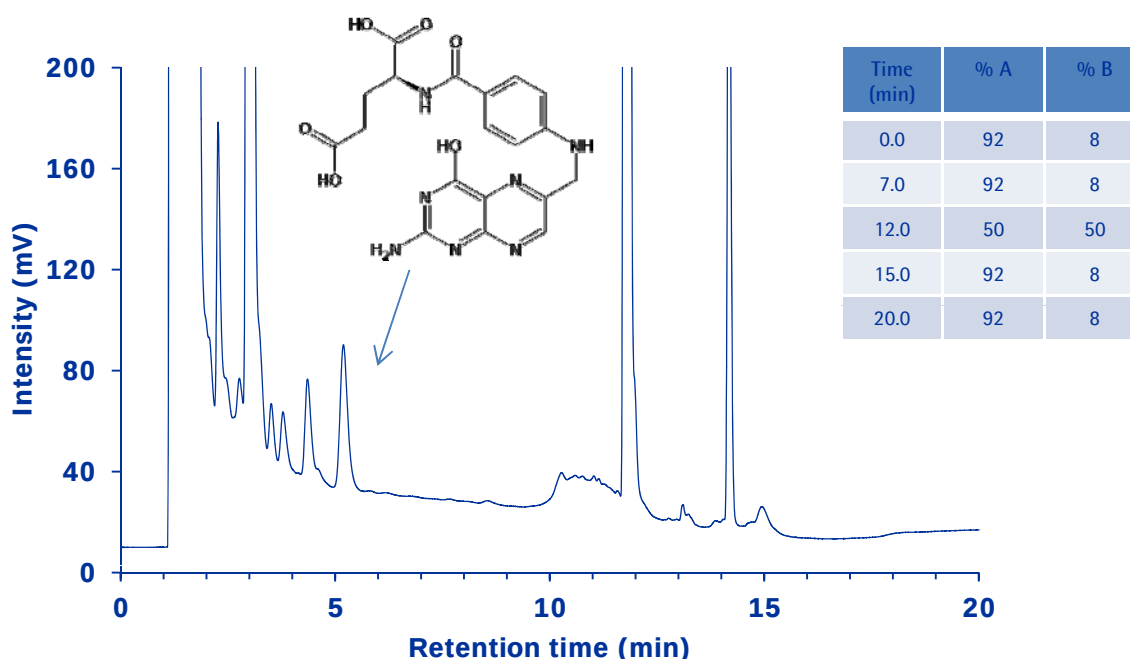
<http://en.wikipedia.org/wiki/Vitamin>

Folic Acid (Vitamin B₉) in a Syrup Product

Chromolith® HighResolution RP-18endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18endcapped, 100x4.6 mm	(1.52022.0001)
Injection:	10 µl	
Detection:	UV, 277 nm	
Cell:	10 µL	
Flow Rate:	1.0 mL/min	
Mobile Phase (v/v):	A: Dissolve 1.5 g of hexane 1-sulfonate in 1000 ml water cont. 10 ml of glacial acetic acid. B: Acetonitrile	
Gradient Program:	See table	
Temperature:	30 °C	
Diluent:	Dissolve 6.8 g of KH ₂ PO ₄ in 1000 ml water. Adjust pH to 6.8 with 10% NaOH solution	
Standard:	10 ppm in diluent	
Sample:	Pipette out 15 ml of "syrup product" in 50 ml volumetric flask. Add diluent to make up the volume. Sonicate for 30 min. Filter through 0.2 µm filter prior to analysis	
Pressure Drop:	83 Bar (1203 psi)	



Chromatographic Data

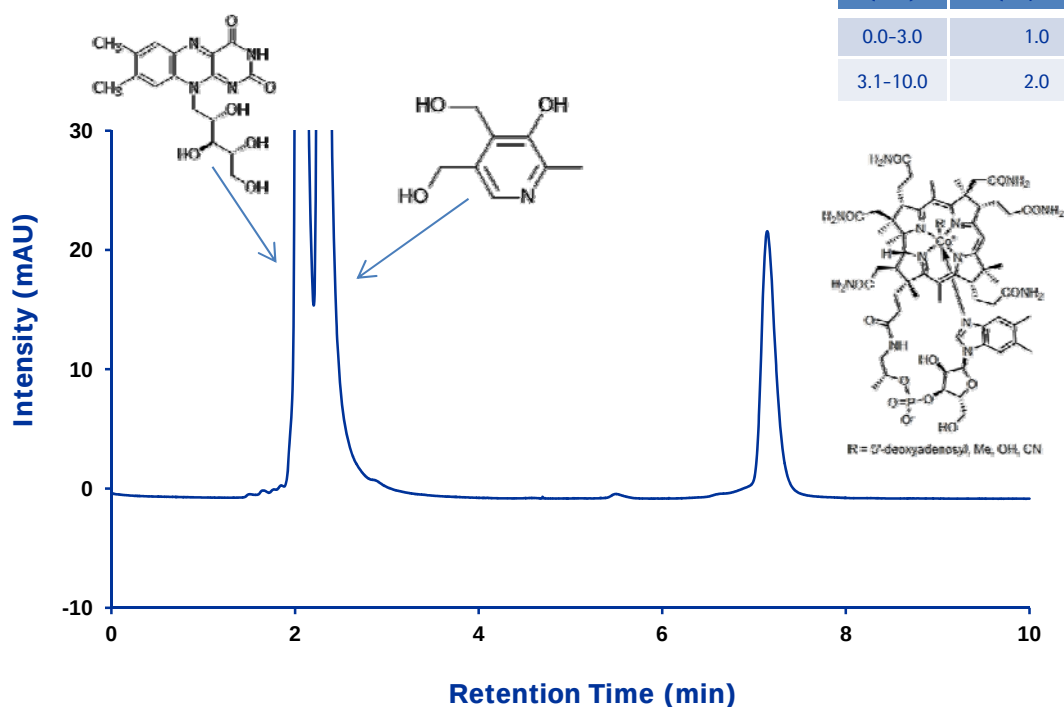
No.	Compound	Time (min)	T _{USP}	Theoretical Plate
1	Folic acid	5.2	1.2	4200

Cyanocobalamin (Vitamin B₁₂)

Chromolith® NH2

Chromatographic Conditions

Column:	Chromolith® NH2, 100x4.6 mm	(1.52028.0001)
Injection:	2 µl	
Detection:	UV, 280nm	
Cell:	11 µL	
Flow Rate:	See table	
Mobile Phase (v/v):	Acetonitrile and water (20:80 v/v)	
Temperature:	Ambient	
Sample:	880 ppm Riboflavin (0.88 mg/mL), 550 ppm pyridoxine (0.55 mg/mL), and 520 ppm cyanocobalamin (0.52mg/mL) dissolved in mobile phase.	
Pressure Drop:	7-20bar (101-289 psi)	



Chromatographic Data

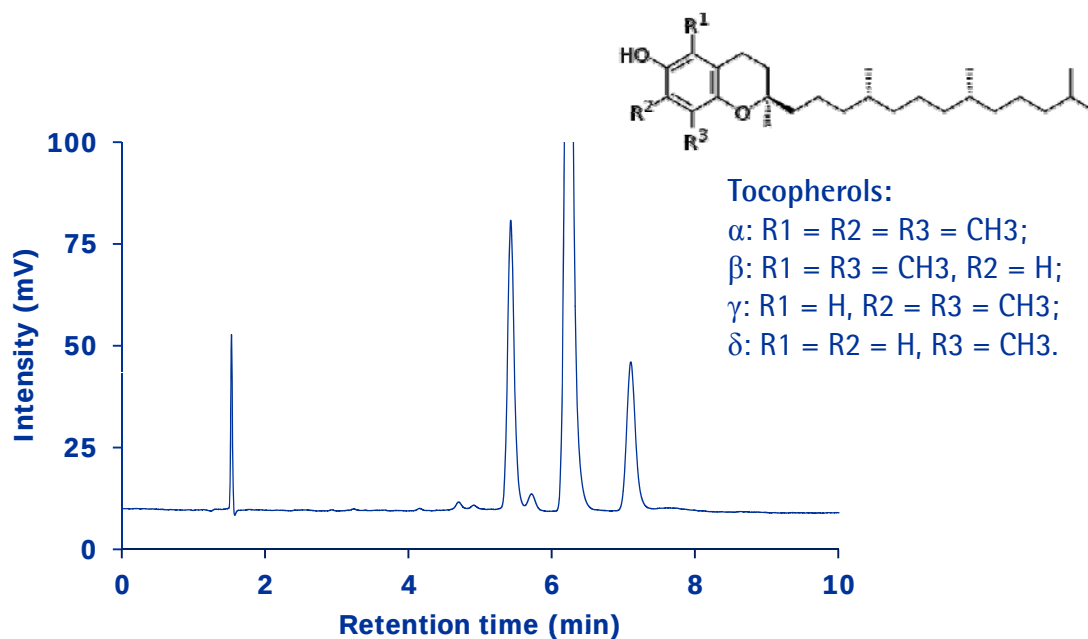
No.	Compound	Time (min)	T _{USP}	Retention Factor
1	Void volume	1.9	-	-
2	Riboflavin	2.0	-	0.1
3	Pyridoxine	2.3	-	0.2
4	Cyanocobalamin	7.1	1.3	2.9

Vitamin E

Chromolith® HighResolution RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18 endcapped, 100x4.6 mm	(1.52022.0001)
Injection:	5 µL	
Detection:	UV, 230 nm	
Cell:	10 µL	
Flow Rate:	1.0 mL/min	
Mobile Phase (v/v):	Methanol and Acetonitrile 8:92 (v/v)	
Temperature:	25 °C	
Diluent:	Methanol	
Sample:	1000 ppm sample in mobile phase	
Pressure Drop:	22 Bar (319 psi)	



Chromatographic Data

No.	Compound	Time (min)	T _{USP}	Theoretical Plate
1	δ -Tocopherol	5.4	1.2	16600
2	β -Tocopherol	5.7	1.1	18300
3	γ -Tocopherol	6.2	1.3	15800
4	α -Tocopherol	7.1	1.1	16100

Honey Analysis

An overview of honey analysis, with the regulatory requirements, ends the analytical part of this compilation. The intention is to highlight where HPLC methods are being regarded official for Honey analysis and how the Merck Millipore chromatography team can aid you in this field.

Codex Alimentarius Honey Standard – CODEX STAN 12-1981

"1.1 Part One of this Standard applies to all honeys produced by honey bees and covers all styles of honey presentations which are processed and ultimately intended for direct consumption. Part Two covers honey for industrial uses or as an ingredient in other foods.

1.2 Part Two of this Standard also covers honey which is packed for sale in bulk containers, which may be repacked into retail packs."

In this standard a few tests may or can be based on HPLC analysis. On the following pages, a number of application notes with relevance to aforementioned text are included.

Sugars content (sum of both fructose and glucose, and another measure for sucrose content)

If you are interested in an application with all these three sugars in same run please visit the analytical application finder – www.merckmillipore.com/AAF or the chromatography application world at www.merckmillipore.com/chromatography

Hydroxymethylfurfural content (AOAC 980.23)

" The hydroxymethylfurfural content of honey after processing and/or blending shall not be more than 40 mg/kg. However, in the case of honey of declared origin from countries or regions with tropical ambient temperatures, and blends of these honeys, the HMF content shall not be more than 80 mg/kg."

Residues of pesticides and veterinary drugs

" The products covered by this standard shall comply with those maximum residue limits for honey established by the Codex Alimentarius Commission."

Herein exemplified by an application on sulfonamides and another on pesticides.

Further reading:

<http://teca.fao.org/resource/codex-alimentarius-honey-standard>

CODEX STAN 12-1981 – www.codexalimentarius.org

AOC 980.23

For more information about the Codex Alimentarius, visit the website:

http://www.codexalimentarius.net/web/index_en.jsp

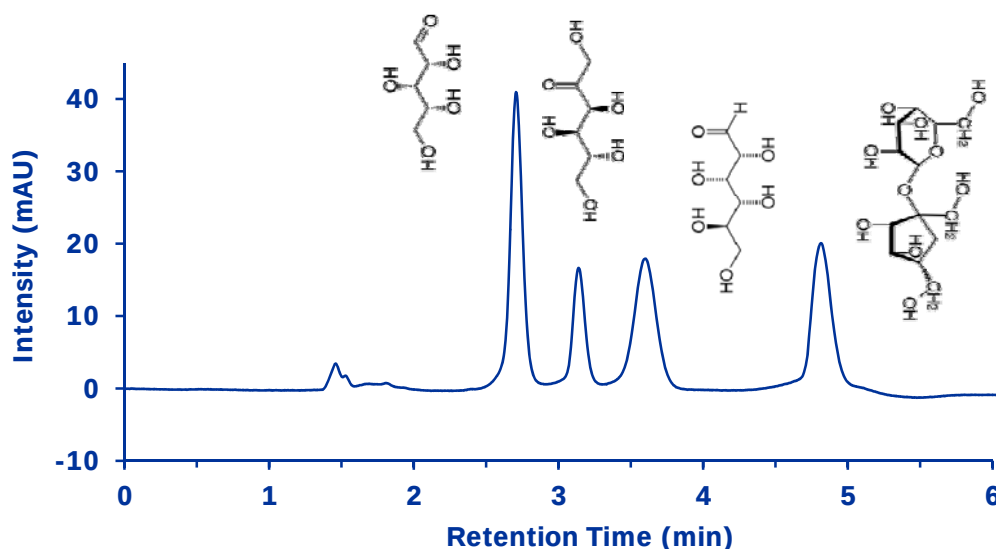
Xylose, Fructose, Glucose and Saccharose

Chromolith® NH2

Chromatographic Conditions

Column:	Chromolith® NH2, 100x4.6 mm	(1.52028.0001)
Injection:	2 µl	
Detection:	UV, 190 nm	
Cell:	11 µL	
Flow Rate:	1.0 mL/min	
Mobile Phase (v/v):	Acetonitrile and water (75:25 v/v)	
Temperature:	23° C	
Sample:	Xylose 2.28 mg/mL, Fructose 6.45 mg/mL, Glucose 9.20 mg /mL, and Saccharose 9.42 mg/mL diluted in mobile phase.	
Pressure Drop:	11 bar (160 psi)	

Time (min)	Flowrate (mL/min)
0.0-3.0	1.0
3.1-10.0	2.0



Chromatographic Data

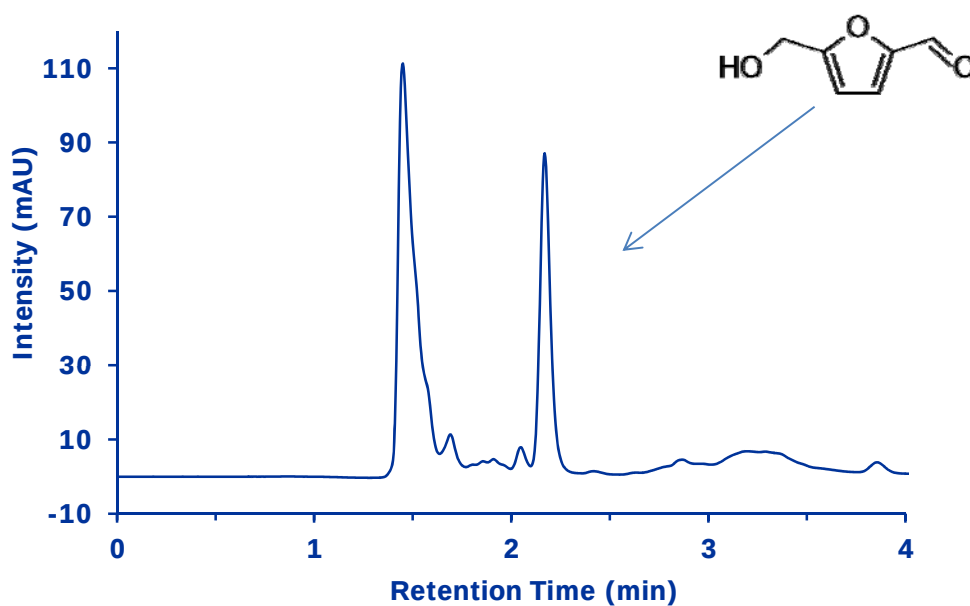
No.	Compound	Time (min)	Retention Factor	T _{USP}
1	Void volume	1.5	-	-
2	Xylose	2.7	0.9	1.0
3	Fructose	3.1	1.2	0.9
4	Glucose	3.6	1.5	0.9
5	Saccharose	4.8	2.3	1.0

Hydroxymethylfurfural (HMF) in Honey

Chromolith® HighResolution RP-18endcapped

Chromatographic Conditions

Column:	Chromolith® HighResolution RP-18endcapped, 100x4.6 mm	(1.52022.0001)
Injection:	5 µl	
Detection:	UV, 277nm	
Cell:	11 µL	
Flow Rate:	1.0 mL/min	
Mobile Phase (v/v):	Acetonitrile and water 10:90 (v/v)	
Temperature:	Ambient	
Diluent:	Methanol	
Sample:	2.5 g honey diluted in 10 mL phosphoric acid (1M solution)	
Pressure Drop:	62 bar (868 psi)	



Chromatographic Data

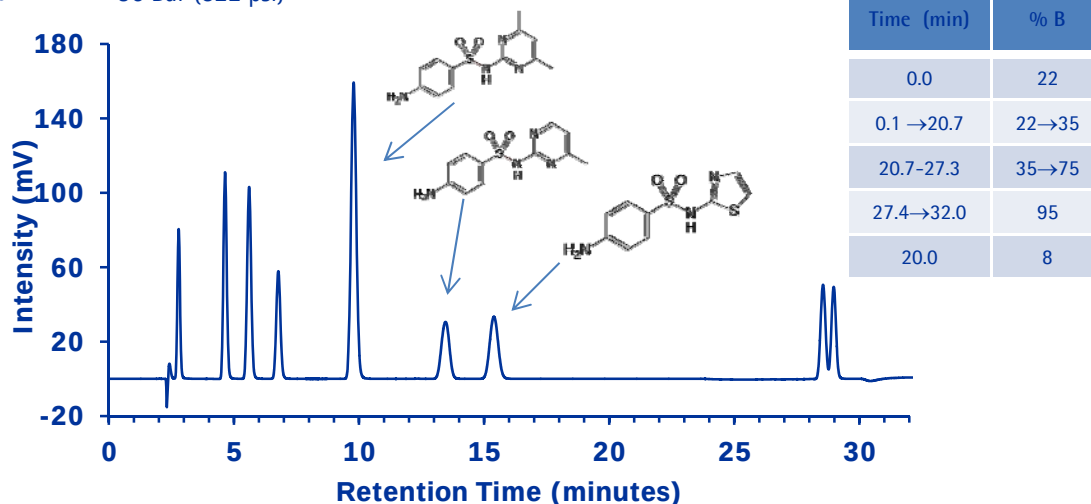
No.	Compound	Time (min)	Retention Factor	T _{USP}
1	Void volume	1.5	-	-
2	Hydroxymethylfurfural (HMF)	2.2	0.5	1.2

Sulfonamides

Chromolith® HighResolution RP-18endcapped

Chromatographic Conditions

Column: Chromolith® HighResolution RP-18endcapped, 100x4.6 mm (1.52022.0001)
Injection: 5 µl
Detection: UV, 270 nm
Cell: 11 µL
Flow Rate: 0.7 mL/min
Mobile Phase (v/v): A: Acetonitrile and 20mM ammonium acetate, pH 4.75 (2:98 v/v)
 B: Acetonitrile and 20mM ammonium acetate, pH4.75 (32:68 v/v)
Temperature: 40° C
Diluent: Acetonitrile/Water 50/50 v/v
Sample: Sulfadiazine 1.1 mg/mL, Sulfamethoxazole 0.8 mg/mL, Sulfadimethoxyine, 0.8 mg/mL,
 Sulfaquinoxaline 1.0 mg/mL, Sulfadimidine 2.6 mg/mL, Sulfamerazine 1.0 mg/mL,
 Sulfathiazole 1.5 mg/mL, Sulfanilamide 0.7 mg/mL, Sulfisoxazole 1.0 mg/mL in diluent
Pressure Drop: 36 Bar (522 psi)



Chromatographic Data

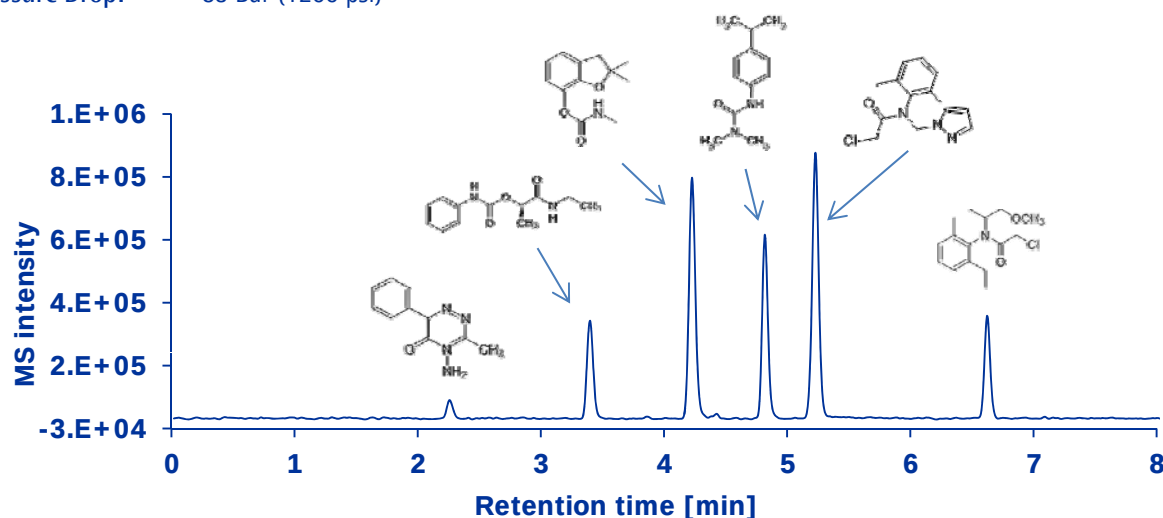
No.	Compound	Time (min)	Area (mAU*min)	T _{USP}
1	Sulfadiazine	2.8	23.959	1.8
2	Sulfamethoxazole	4.7	17.374	1.0
3	Sulfadimethoxin	5.6	19.411	1.0
4	Sulfaquinoxaline	6.8	11.267	1.1
5	Sulfadimidine	9.8	39.760	1.1
6	Sulfamerazine	13.5	11.340	1.0
7	Sulfathiazole	15.4	13.062	1.0
8	Sulfonilamide	28.6	11.788	-
9	Sulfisoxazole	29.0	10.789	-

Pesticides in porcine kidney

Chromolith® CapRod® RP-18 endcapped

Chromatographic Conditions

Column:	Chromolith® CapRod RP-18 endcapped, 150x0.1 mm	(1.50402.0001)
Injection:	2.5 nL	
Detection:	nano-ESI(+) MS, 100–600 m/z	
Flow Rate:	1.24 µL/min	
Mobile Phase :	A: water + 0.1 % formic acid B: acetonitrile + 0.1 % formic acid	
Gradient:	20 % B to 90 % B in 10 min	
Temperature:	Ambient	
Diluent	Acetonitrile	
Sample:	1 g porcine kidney macerated and spiked with 2 mL spike solution (Metamitron, Carbetamid, Carbofuran, Isoproturon, Metazachlor, and Metolachlor – 60–500 µg/mL in acetonitrile). Extraction with water/acetonitrile 80:20 (v/v), then SPE with LiChrolut® cartridge (RP-18). Finally 0.45 µm filtration prior to analysis..	
Pressure Drop:	83 Bar (1200 psi)	



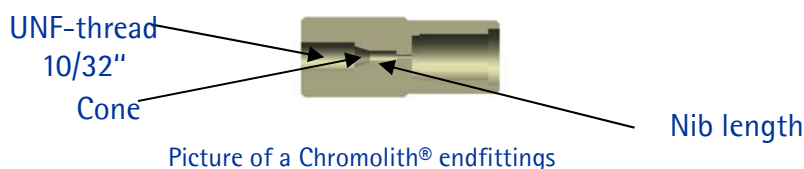
Chromatographic Data

No.	Compound	Retention Time (min)	[M+H] ⁺
1	Metamitron	2.3	202.96
2	Carbetamid	3.4	237.04
3	Carbofuran	4.2	222.02
4	Isoproturon	4.8	207.05
5	Metazachlor	5.2	278.09
6	Metolachlor	6.6	284.06

Tips and Tricks

This section summarizes a few practical tips in optimizing the use of Chromolith® Columns.

1. Installation of the column – Fittings and Tubing



Merck Millipore HPLC columns have 2 mm Nib length whereas other manufacturers sometimes have different specifications. Thus if you normally use fixed steel tubing, fittings and ferrules, when changing from one column to another you may experience leakage or other problems. Metal capillaries with 1/16" outer diameter and a metal ferrule, which is fixed for a 3 mm nib length will damage Chromolith® columns!

Remedy:

Use flexible metal capillaries (0.25 mm outer diameter) with PVDF cone
Use PEEK capillaries with PEEK fittings



For flow rates between 2 and 6 mL/min tubing with inner diameter of 0.2 mm (metal) or 0.17 mm (PEEK, yellow) are ideal. For 2.0 mm i.d. columns and flow rate up to 1 mL/min 0.13 mm (PEEK, red) is recommended (and remember to keep the length of tubing as short as possible to minimize extra void volume effects).

2. Installation of the column – Use of column oven

If there is a big temperature difference between the column oven and the mobile phase there may appear a temperature gradient inside the Chromolith® column. This will influence the separation efficiency and peak shape negatively.

Reason: Chromolith® are clad with a mechanical and chemical resistant polymer (PEEK), which has significantly lower conduction coefficient compared with stainless steel (the most common material for column bodies).

Remedy:

Pre-heat the mobile phase.

Mount the tubing from the HPLC pump to the injector through the column oven. Normally a 30 cm long tubing in front of the Chromolith® column inside the column oven is sufficient. The higher the flow rate is, the more important is a pre-heated mobile phase.

Some HPLC systems are equipped with pre-heating modules for the mobile phase.

Further reading :

<http://www.merckmillipore.com/chromatography>