Food Safety and Food Quality Control

Analysis of Polar Compounds in Complex Samples

Applications for HPLC and LC-MS with SeQuant® ZIC®-HILIC columns

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Content

ZIC®-cHILIC coming soon

*The application on organic acids in wine is taking advantage of the selectivity of the new SeQuant® ZIC®-*c*HILIC columns. For more information on specifications and launch of SeQuant® ZIC®-*c*HILIC, please contact your Merck Millipore sales representative or visit www.merckmillipore.com/chromatography or www.sequant.com/zicchilic.

Introduction

This compilation covers Food Safety and Food Quality Control and is focusing on the Analysis of Polar Compounds in Complex Samples, for example melamine in milk powder formulations.

The quality of our daily food is of concern for producers, consumers and controlling authorities. The globalization process changes the demands and requirements literally as we speak. Merck Millipore contributes in this context by offering analytical solutions and prompt technical support.

The initial part of this compilation covers trends and news regarding food safety legislation and has been compiled by the Merck Millipore Regulatory Department.

The sections following give an account to why the analysis of polar compounds is important for food and beverage control. Analytical challenges are pointed out, and an explanation is given to why Hydrophilic Interaction Liquid Chromatography (HILIC) in general, and SeQuant® ZIC®-HILIC columns in particular, is the ideal tool for analysis of polar compounds in food and other complex samples.

Several methods are presented, with complete workflows, and as you will see, Merck Millipore offers virtually everything but the instrument to successfully implement these methods in your laboratory.

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Trends in food safety legislation

The aim of food legislation has always been to ensure that food is safe and that consumers get what they pay for [1].

During the last century methods of food production, processing and preservation (e.g. chemical as well as heat treatment) have been a subject of continuous and substantial improvement. In parallel there has been dramatic and profound developments regarding the analytical methods needed for food control in order to safeguard that acceptable food safety standards are met.

However, safeguarding of food safety cannot be achieved by legislation and controls alone. Ensuring compliance with food law and in particular food safety must also be the primary responsibility of the food industry. This has been acknowledged and reflected by the general food law (Regulation 178/2002/EC) of the European Union [2]. The implementation of risk management systems like the hazard analysis and critical control points (HACCP) system has become a basic requirement for the European food industry [2].

There are new challenges appearing in food safety., i.e. globalization of food production and food trade, the increase of diet-related diseases (e.g. diabetes type 2, obesity and / or chronic vascular diseases).

According to the world health organization (WHO) worldwide obesity has more than doubled since 1980 [3]. In 2010, around 43 million children under the age of five were overweight [1]. In Germany, a health survey performed in 2008 revealed that 15 % of the children under the age of seventeen are overweight [4]. Overweight and obesity are linked to more deaths worldwide than underweight. 65% of the world's population lives in countries where overweight and obesity kills more people than underweight (this includes all high-income and most middle-income countries) [3]. 346 million people worldwide have diabetes and in 2004 estimated 3.4 million people died from consequences of high blood sugar [5]. Once considered as high-income country problems, diabetes type 2, overweight and obesity are also increasing in low- and middle-income countries, particularly in urban areas [3,5]. In the light of these dramatic figures, we should not forget that obesity and diabetes type 2 are preventable diseases. Food industry and food legislation can play a significant role, for example by making dietary values transparent to consumers on basis of a mandatory nutrient declaration (with references to the recommended daily intake of the respective nutrients), or by reducing the sugar and salt content of food [3,5].

During the last decade food production has witnessed a rapid growth in globalization and global trade of food is increasing yearly [6]. As a consequence, the potential risk of international food safety related incidents increases continuously [6]. Food safety incidents can involve (intentionally) contaminated food like the melamine case in 2008 or the phthalate contamination in 2011 in Asia. In the latter incident, palm oil (more expensive) was substituted by phthalate plasticizers in the manufacture of a food additive used in soft drinks [7]. Global trade and food production contribute also to the spread of pathogens across the national borders [6] which may lead to outbreaks of foodborne diseases. In 2011, there was an outbreak in Europe with nearly 4000 cases (including 50 deaths) due to contaminated fenugreek sprouts imported from Egypt [8].

These new threats to food safety have led many countries to update their food legislations to adequately react to and mitigate the new imposed risks.

European Union – situation and news for legislation on food safety

Legal situation

The European Union consists of 27 member states. Harmonizing of food legislation within the European Union is a task of the European Commission and the European Parliament. European food legislation is structured by a set of horizontal European regulations and directives ruling general principles of food law (which apply to all types of food or include general provisions for food) and vertical regulations and directives applying to specific foodstuffs. EU Regulations are valid directly in all EU member states while directives have to be transferred into the national legislation of each EU-member state. Regulation (EC) No. 178/2002 (General Food Law Regulation, [2]) provides a framework for a harmonized European food legislation by laying down the general principles for food safety [9, 10]:

- the so-called 'farm to fork' approach regarding traceability throughout the complete food chain;
- the precautionary principle regarding information and actions to protect public health in case of food safety emergencies,
- the establishing of the European Food Safety Authority and
- the primary responsibility of the food industry for ensuring compliance with food law, and in particular the safety of the food.

It also provides the general framework for those areas which are not covered by specific harmonized rules until now [9, 10].

Food Additives

In order to improve the safety of food additives the European Union has adopted several regulations for food additives (so-called Food Improving Agents Package, FIAP) during the last few years dealing with the safe use of food enzymes, food flavors and food additives. The FIAP consists of four regulations, effective in all 27 EU member states [11, 12, 13, 14]:

- Regulation (EC) No. 1331/2008: dealing with the authorization procedure for food additives, enzymes and flavors [11]
- Regulation (EC) No. 1332/2008 including rules for safe use and declaration of food enzymes [12]
- Regulation (EC) No. 1333/2008 including rules for safe use and declaration for food additives [13]
- Regulation (EC) No. 1334/2008 dealing with the safe use and declaration rules for food flavoring [14]

The scope of the FIAP is a stronger harmonization of food additive law within the European member states. This includes a harmonized authorization procedure for new food additives, flavors and enzymes based on the evaluations and risk assessments of the European Food Safety Agency (EFSA) as well as on harmonized rules for labeling and use of flavors, enzymes and food additives.

In 2012 the EU Regulation about the Specifications for Food Additives (in force starting December 2012) has been adopted as revision of the purity criteria of food additives setting stricter limits for impurities in general in order to improve food safety [15]. The evaluation on the safe conditions of use (for example evaluation on the maximum levels of use for food additives) is still on going by the EFSA and will according the published timetable not be finalized before end of this decade.

Mandatory Nutrition Information to Consumers

The European Food information Regulation entered into force in December 2011 and shall apply from December 2014. One main objective of this regulation is to ensure that consumers are appropriately informed regarding the food they consume [16] requiring a mandatory nutrition declaration on food packages (which shall apply from December 2016). Such nutrient declaration (with references to the recommended daily intake) shall support consumers to make informed choices of food in accordance to their individual dietary needs [16]. Future application of this new regulation will require the food manufacturer to perform additional testing for nutrients like sugars, fat and saturated fatty acids, protein and sodium as well as for vitamins (dependant on the respective nutrional or health claim) in order to ensure a consistent nutrient declaration.

Contaminants, Adulterants and Residues

European Union food legislation includes encompassing rules on natural occurring potentially harmful (like mycotoxins), as well as on anthropogenic contaminants (such as heavy metals, dioxins, polychlorinated biphenyls). The latter may originate from environmental contamination or residues of herbicides, fungicides, insecticides used in agriculture, as well as to residues of pharmaceuticals used in animal husbandry like fish or beef cattle, but also includes adulteration of food using substances such as melamine. Following regulations are setting maximum levels for natural and anthropogenic contaminants and residues:

- Regulation (EC) 1881/2006 setting maximum levels for nitrate, mycotoxins (aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins), metals (lead, cadmium, mercury, inorganic tin), 3-MCPD, dioxins and dioxin-like PCBs and polycyclic aromatic hydrocarbons (benzo(a)pyrene and melamine) [17];

- Regulation 396/2005/EC setting maximum residue levels for pesticide residues[18]

More adequate and sensitive testing methods are developed, and new contaminants are being identified. Regulations are therefore updated on a regular basis to include limits for previously not controlled contaminants (e.g. melamine), or to set stricter limits for those already listed to improve consumer safety. The European Commission is planning to include melamine to the European Contaminants Regulation [19]. The limit for melamine shall be 2.5 mg per kg food and 1 mg per kg baby food (powder).

United States of America –situation and news for legislation on food safety

There are several laws in the USA which are associated with food safety. The Food Drug and Cosmetic Act (FDCA) is the primary law dealing with safety and quality of food [20], and it prohibits adulteration and misbranding of food [20]. The FDCA is part of the US Code and can be found in Title 21, Chapter 9. Since its adoption in 1938, the FDCA has been amended many times [20]. The Prohibited Acts are described FDCA Section 301 and the conditions under which a food is rendered to be adulterated are given by FDCA section 402 [20]. Besides the FDCA there are also other acts dealing with food safety, e.g. the Federal Meat Inspection Act or the Poultry Products Inspection Act [20].

Food Safety Modernization Act

In 2011 the Food Safety Modernization Act (FSMA) was enacted [21]. It is an enormous reform program to improve food safety within the whole supply chain, also involving labs and enhancing the authority of the Food and Drug Agency (FDA). It takes into account the risks resulting from globalization of food production and trade. Certain provisions of the FSMA are already in effect, other will take effect later in 2012 or 2013 [22]. The FSMA includes encompassing rules and prevention measures to avoid intentional as well as non-intentional food adulterations. It relates therefore not only to manufacture, but also to packaging, holding, distribution, importation, receipt, testing of food. It is divided into four main parts.

Part 1 **'Improving capacity to prevent food safety problems'** deals with the ability of the food manufacturer and other in the food supply chain involved parties as well as with the capacity of the institutions like FDA to prevent food safety problems [21]. It includes among other the

- registration of food facilities (domestic and foreign facilities) as a biennial registration requirement. Suspension of registration will lead to prohibition of marketing and / or importation of food into the US as well as exporting food from the US.
- Hazard Analysis and Risk-Based Preventive Controls (HARBPC). The HARBPC-plan must be implemented by domestic and foreign facilities as a pre-condition for marketing food in the US. The HARBPC includes analysis and preventive control of known and reasonably foreseeable hazards associated with the facility (including a food allergen control plan) and additionally analysis and preventive controls of hazards that may be intentionally introduced (including acts of terrorism). Furthermore, a recall plan must be implemented.
- protection against intentional adulteration which requires a vulnerability assessment of the US food system by FDA.

The Part 2 of the FSMA called **'Improving capacity to detect and respond to food safety problems'** deals with the detection of food safety problems throughout the food supply chain [21]. It requests

- a greater frequency of FDA inspections of domestic and foreign food facilities. A successful FDA inspection of a foreign facility is the pre-requisite for exporting to the USA;
- a lab accreditation for food analysis to ensure applicable good laboratory practice including for example appropriate analytical procedures, sampling techniques, quality systems;
- an enhancement of food traceability. This shall enable a rapid and effective identification of recipients of food in order to prevent or mitigate e.g. a foodborne illness outbreak.

The third part **'Improving the safety of imported food'** includes requirements regarding the safety of imported food and can be seen as reaction on food scandals like the melamine case in 2008 [21]. It includes among other

- a foreign supplier verification program requesting by the importer (who may be an US owner or a consignee) that the imported food complies with HARBPC and is not misbranded, or adulterated;
- annual FDA inspections of importers which means that no importation into the US is possible in case of rejecting a FDA audit or if the audit has not been successful. On the other hand the FSMA is offering a 'fast lane' for importers importing food from certified foreign facilities. Foreign facilities can be certified after successful FDA audits;
- FDA inspections of foreign food facilities putting the focus on foreign suppliers, and food types that present a high risk in the opinion of the FDA.

The last part **'Miscellaneous Provisions'** includes a set of very different provisions e.g. a whistleblower protection as well as the relationship of the FSMA to international agreements.

The FDA has created a FSMA website giving update on FDA's progress [23], the full text of the law and a list of dockets open for comments [21].

Food safety - do we need a global approach?

In many countries food safety is not the task of one agency but rather where the authority and responsibility is divided among several agencies [22]. In the European Union, responsibility for food safety is allocated to the European Food Safety Authority for all European matters and general questions, and to the national agencies for matters of national concern.

The food controls are performed by the competent national food inspection boards of the respective member state according to the rules of the respective state. At first glance such system appears to be not very effective in case of food safety alerts. An information system so-called 'Rapid Alert System for Food and Feed' (RASFF) was, as a consequence, implemented between the food and feed control authorities of the EU member states already in 1979 [24]. The legal basis for the RASFF was established by the Regulation (EC) 178/2002 [24, 2]. The RASFF enables the food and feed control authorities to rapidly exchange data and information regarding serious risks and incidents detected in relation to food or feed as well as regarding the measures taken for responding to such risks. This exchange of information supports the EU member states to react more rapidly and in a coordinated way when responding to a food or feed safety incident [24].

The global production and trade of food is increasing every year representing a real challenge for national food authorities. Nowadays, they have to deal not only with the national division of responsibilities and authorities, but also with food authorities and requirements around the world. Although the EU and USA and other countries are consistently working on improving their food safety systems also with regard to globalization (for example FDA inspections of foreign food manufacturer), there is a real need for a closer collaboration between food safety agencies and for a global exchange of food safety information as for example provided by the RASFF for the European Union.

The WHO and the FAO has recognized the need for an international food safety information network and have already developed such a network (called INFOSAN) [6, 22]. INFOSAN is a network between the food safety authorities of the 177 member states for exchange of information regarding food safety related issues to promote partnership in food safety between the countries and to support countries in improving their capacity to manage food safety risks [6, 22]. However, more intensive participation and contribution of the member states as well a further development is needed for ensuring food safety in a globalised world [6].

References and useful links

Food legislation is a very dynamic legislation and is frequently changing. The given links to food legislation may not in all cases provide you with most current version of the regulation. The most current legislation is to be found on the respective government websites.

[1] "How we did get where are today?" P. Curtis and W.- Dunlap, "Guide to food laws and regulations", Blackwell Publishing, USA, 2005, 25-39.

[2] "Regulation (EC) No 178/2002 of the European Parliament and of the Council laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety", Official Journal of the European Communities, L 31/1, 2002 and subsequent amendments.

[3] "Obesity and overweight", Fact sheet N°311, World Health Organisation, 2011.

[4] "The challenge of comprehensively mapping children's health in a national-wide health survey: design of German KiGGS study", B.-M. Kurth et al., BMC Public Health, 2008, 8:196.

[5] "Diabetes", Fact sheet N°312, World Health Organisation, 2011.

[6] "Advancing food safety initiatives", 63.World Health Assembly, Agenda item 11.8, WHO, 2010.

[7] "Phthalate contamination: frequently asked questions", Excipients insight (IPEC e-newsletter), June 2011.

[8] "Samen von Bockshornklee mit hoher Wahrscheinlichkeit für EHEC O104:H4 Ausbruch verantwortlich", Bundesinstitut für Risikobewertung (BfR), 30 June 2011.
http://www.bfr.bund.de/cm/343/samen_von_bockshornklee_mit_hoher_wahrscheinlichkeit_fuer_ehec_o104_h4_ausbruch_verantwortlich.pdf.

[9] "White paper on food safety", Commission of European Communities, Brussels, January 2000.
http://ec.europa.eu/dgs/health_consumer/library/pub/pub06_en.pdf

[10] "General food law", an outline of the European Commission to Regulation 178/2002.
http://ec.europa.eu/food/food/foodlaw/index_en.htm

[11] "Regulation (EC) No. 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings", Official Journal of the European Communities, L 354/1, 2008 and subsequent amendments.

[12] "Regulation (EC) No. 1332/2008 of the European Parliament and of the Council on food enzymes", Official Journal of the European Communities, L 354/7, 2008 and subsequent amendments.

[13] "Regulation (EC) No. 1333/2008 of the European Parliament and of the Council on food additives", Official Journal of the European Communities, L 354/16, 2008 and subsequent amendments.

[14] "Regulation (EC) No. 1334/2008 of the European Parliament and of the Council on flavourings and certain food ingredients with flavouring properties for use in and on foods", Official Journal of the European Communities, L 354/34, 2008. and subsequent amendments.

[15] "Regulation (EU) No 231/2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council", Official Journal of the European Communities, L 83/1, 2012.
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:083:0001:0295:EN:PDF

[16] "Regulation (EU) No 1169/2011 of the European Parliament and of the Council on the provision of food information to consumers", Official Journal of the European Communities, L304/18, 2011.
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:304:0018:0063:EN:PDF

[17] "Regulation (EC) No 1881/2006 of the Commission setting maximum levels for certain contaminants in foodstuffs", Official Journal of the European Communities L364/5, 2006 and subsequent amendments.
[18] "Regulation (EC) No 396/2005 of the European Parliament and of the Council on maximum residue levels of pesticides in or on food and feed of plant and animal origin, Official Journal of the European Communities L70/1, 2005 and subsequent amendments.
[19] Draft Commission Regulation amending Regulation (EC) 1881/2006 as regards the maximum levels of the contaminants ochratoxin A, non dioxin-like PCBs and melamine in foodstuffs. Online: http://register.consilium.europa.eu/pdf/en/12/st08/st08478.en12.pdf
[20] "Major laws and regulations related to food safety and quality", P. Curtis and W.- Dunlap, "Guide to food laws and regulations", Blackwell Publishing, USA, 2005, 57-84.
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[24] "RASFF Preliminary Annual Report", http://ec.europa.eu/food/food/rapidalert/docs/rasff_pre-annual_report_2011_en.pdf

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

Analysis of hydrophilic compounds in food

For hydrophobic substances, reliable methods on C-18 RP columns have been developed over the past 30 years, but until relatively recent, analyzing hydrophilic compounds in a matrix such as food had been a massive undertaking, involving derivatization and complex sample preparation procedures.

One of the most used methods in food analysis is the total-nitrogen content determination using the Kjeldahl method, the non specificity of the method has shown to be a weakness since any nitrogen rich compound can be added to artificially enhance the nitrogen and thus the perceived protein content. All such nitrogen rich chemicals are hydrophilic, and most sweeteners, natural as well as artificial, are also hydrophilic. Many vitamins, new pesticides and metabolites of veterinary drugs are also hydrophilic. All these are difficult or even impossible to analyze using the traditional C-18 RP columns.

The biggest challenge is all the unknown emerging threats to our food supply.
New instrumentation have provided us with the possibility to develop comprehensive and sensitive methods. The challenge is to make them robust to work in diverse matrices. Developing methods that are fast and accurate in a single matrix is rather straightforward, but in many cases a lot of information can be lost if the samples are analyzed with only one chromatographic selectivity. Screening methods, based on complementary chromatographic selectivities together with sensitive and specific detection techniques can all together provide us with much more complete information.

This documentation will exemplify how hydrophilic interaction liquid chromatography (HILIC) in general, and in particular the bonded zwitterionic SeQuant® ZIC®-HILIC stationary phase, can and have been used for food safety screening as well as for food constituent control.

What is HILIC and why ZIC®-HILIC?

Analysis of polar molecules in complex mixtures is problematic since the separation is difficult due to their inherently poor retention in traditional reversed-phase liquid chromatography (RP-LC). As a solution, Merck Millipore have developed the high-quality SeQuant® ZIC®-HILIC range of HPLC columns. These are used in Hydrophilic Interaction Liquid Chromatography (HILIC) mode, which means buffered aqueous eluents rich in organic solvents such as acetonitrile. With this mode of operation follows also a couple of characteristic advantages such as low column back-pressure allowing high-speed separations, enhanced sensitivity when interfaced with mass spectrometry (MS), and simplified sample preparation schemes. By employing ZIC®-HILIC columns, food analysis laboratories can be more efficient and deliver more secure analysis results for polar and hydrophilic analytes. And this is regardless if it is well-equipped with sophisticated instrumentation such as LC-MS/MS, or rely on more traditional HPLC with detection by UV light absorption or ELSD (evaporative light scattering).

Separations are equally easy to develop on ZIC®-HILIC columns as on traditional RP HPLC columns since the eluents are similar, however, the difference is the effect of the water in the separation. In HILIC mode, water is the strongest solvent. To increase analyte retention, the organic portion of the mobile phase needs to be increased, and the water or buffer portions decreased. This will increase the hydrophilic partitioning into the water-enriched stationary phase, and thus increase the retention of the analyte.

The zwitterionic character of ZIC®-HILIC with a 1:1 balanced charge, gives further possibilities for selectivity by weak electrostatic interactions between the stationary phase and the molecules that are separated. This interaction can be tuned by changing buffer type and concentration, typically in the interval 5-50 mM. Buffer pH is also an important parameter to control retention, but also here the thinking is opposite to in RP mode; more ionized compounds will be more hydrophilic and thus have more retention on ZIC®-HILIC.

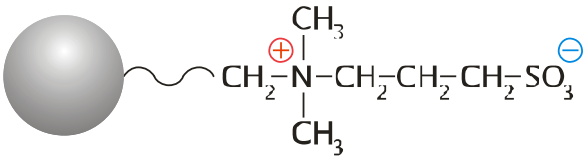
More technical details on how to develop methods with ZIC®-HILIC can be found in the booklet 'A Practical Guide to HILIC', which is available free of charge from Merck Millipore printed or in online format (www.sequant.com/hilicguide).

SeQuant® ZIC®-HILIC

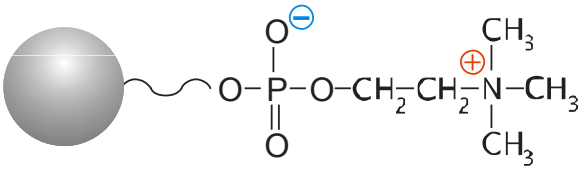
Sorbent characteristics:	high-density zwitterionic sulfobetaine modification
Charge balance:	1:1
Particle material:	high-purity type B silica
Particle type:	spherical, fully porous
Particle size:	3.5 µm, 5 µm
Pore size:	100 Å , 200 Å
pH range:	pH 3 - 8
Max temperature:	70 °C
Max pressure:	350 bar (PEEK columns) or 400 bar
Column inner diameters:	0.075, 0.1, 0.3, 1.0, 2.1, 4.6, 7.5, 10, 21.2 mm
Column lengths:	20, 30, 50, 100, 150, 250 mm

SeQuant® ZIC®-*p*HILIC

Sorbent characteristics:	high-density zwitterionic sulfobetaine modification
Charge balance:	1:1
Particle material:	high-purity polymer
Particle type:	spherical, fully porous
Particle size:	5 µm
pH range:	pH 2 - 12
Max temperature:	50 °C
Max pressure:	200 bar
Column inner diameters:	2.1, 4.6 mm
Column lengths:	50, 100, 150 mm



The stationary phase on ZIC®-HILIC and ZIC®-*p*HILIC - sulfobetaine.



The stationary phase on ZIC®-*c*HILIC - phosphorylcholine.

SeQuant® ZIC®-*c*HILIC

Sorbent characteristics:	high-density zwitterionic phosphorylcholine modification
Charge balance:	1:1
Particle material:	high-purity type B silica
Particle type:	spherical, fully porous
Particle size:	3 µm
Pore size:	100 Å
Column inner diameters:	0.1, 0.3, 1.0, 2.1, 4.6 mm
Column lengths:	50, 100, 150, 250 mm

ZIC®-*c*HILIC coming soon

*For more information on specifications and launch of SeQuant® ZIC®-*c*HILIC, please contact your Merck Millipore sales representative or visit www.merckmillipore.com/chromatography or www.sequant.com/zicchilic.

Adulterants

Definition

1. An adulterant is a chemical substance which should not be contained within other substances for legal or other reasons. The addition of adulterants is called adulteration.

2. Adulterants need to be relatively odorless, colorless and tasteless to avoid negative impact upon consumer acceptance of the fraudulent product.

3. If used as a protein substitute, the adulterant(s) also need to be commercially available in large quantities.

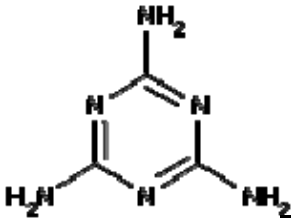
4. The ultimate goal with adulteration is economic fraud, not to injure consumers; hence food producers would be unlikely to deliberately use an acutely toxic chemical as an adulterant. However, as could be proven with melamine adulteration, even relatively non-toxic chemicals can cause unforeseen health effects.

5. Addition of adulterants must generally result in a product that costs less than the authentic food product, or otherwise there is no real economic incentive for adulteration.

6. Adulterants when used in illicit drugs are called cutting agents, while deliberate addition of toxic adulterants to food or other products for human consumption is known as poisoning.

Further reading on Food Contaminants & Adulteration:
http://www.fda.gov/food/foodsafety/foodcontaminantsadulteration

Economically Motivated Adulteration in Protein-containing Foods and Food Ingredients



Melamine

Incidents:	USA	2007
	China	2008

Melamine is an organic base and a trimer of cyanamide, with a 1,3,5-triazine skeleton. It is a small polar compound which is very rich in nitrogen (67% by mass). It has been found in milk products and animal feed, where it have been added to give a false impression of high protein content. Melamine combined with cyanuric acid can cause fatal kidney stones due to the formation of an insoluble melamine-cyanurate complex. Determination of melamine and other small nitrogen-rich compounds, is therefore of large importance to ensure food safety.

Economically Motivated Adulteration in Protein-containing Foods and Food Ingredients

In 2012, US FDA published a new analytical methodology to be considered as a powerful tool against economically motivated adulteration in protein-containing products. After incidents with deliberate addition of melamine to pet food (2007) and milk powder (2008), public awareness on food safety has increased and more efforts are taken by official organizations in order to control quality of food and beverages consumed by humans and animals. Despite this it is likely that criminal ingredient suppliers or food producers are searching for, and testing out new alternatives to melamine (other poly-nitrogenous compounds) to artificially enhance the concentrations of protein detected in their products.

Kjeldahl Method

The traditional standard technique for measuring protein content in food is the Kjeldahl method; a quantitative determination of total nitrogen content. The method consists of heating a substance with sulfuric acid, which decomposes the organic substance by oxidation to liberate the reduced nitrogen as ammonium sulfate. In this step potassium sulfate is added to increase the boiling point of the medium. Chemical decomposition of the sample is complete when the medium has become clear and colorless (initially very dark). The solution is then distilled with sodium hydroxide (added in small quantities) which converts the ammonium salt to ammonia. The amount of ammonia present (hence the amount of nitrogen present in the sample) is determined by back titration.

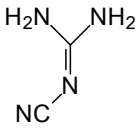
The Kjeldahl method is therefore a non-direct measurement of protein, hence it is possible to artificially enhance protein concentrations by adding nitrogen-rich chemicals, therefore any chemical compound having a high percentage of nitrogen, by weight, has the potential to be used in economically motivated adulteration of protein-containing food products.

The Canadian Food Inspection Agency's Food Safety Division has recently generated a list of potential adulterants. The Canadian list has also been shared with other food safety agencies. The US FDA has, based on the Canadian recommendations and their own intelligence, determined a number of potential compounds likely to be used in protein adulteration.

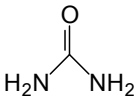
Economically Motivated Adulteration in Protein-containing Foods and Food Ingredients

FDA listed compounds most likely to be used in protein adulteration:

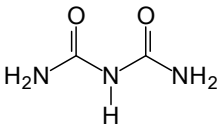
•Dicyandiamide (DC)	used in the production of melamine, as well as in fertilizers and as a fire-proofing agent
•Urea	found in fertilizers, as a non-protein nitrogen source in animal feeds, as well as in production of many commercial products
•Biuret (BU)	non-protein nitrogen source. It is the result of condensation of two molecules of urea and is a problematic impurity in urea-based fertilizers. May be used in some animal feeds
• Triuret (TU)	non-protein nitrogen source. May be used in some animal feeds
•Cyromazine (CY)	insect growth regulator. A cyclopropyl derivative of melamine produces melamine upon metabolism
•Amidinourea (AU)	used in fertilizers



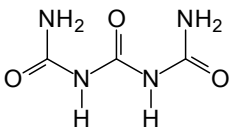
Dicyandiamide



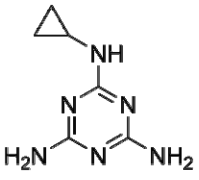
Urea



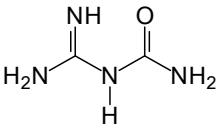
Biuret



Triuret



Cyromazine



Amidinourea

Melamine and Cyanuric Acid in Pet Food

In 2007 many brands of pet food was recalled in the United States, Europe and a few other countries, in response to reports of renal failure in pets. Initially, the recalls were associated with the consumption of mostly wet pet foods made with wheat gluten. A month after the first recall, rice protein was also identified as being contaminated, causing kidney failure in pets. On April 27, 2007, United States Food and Drug Administration (U.S. FDA) detained ALL vegetable proteins imported from China, intended for human or animal consumption. This meant wheat gluten, rice gluten, rice protein, corn gluten, soy protein, proteins (includes amino acids and protein hydrolysate), mung bean protein, and variants thereof.

It was found that melamine (MEL) and cyanuric acid (CYA), a hydrolysis by-product of melamine, had been deliberately added to pet food, to fool the Kjeldahl method for protein determination.

Melamine and cyanuric acid can cause serious health issues in human and pets. While each is relatively innocuous, they can form a complex (MEL:CYA) which is nearly insoluble and crystallizes in kidney tubules, leading to illness or death.

The determination of MEL and CYA, and the MEL:CYA complex is quite challenging because the compounds are very polar. A number of screening methods including GC and LC methods have been developed for the determination of MEL and CYA in foods in recent years. Most of these methods require complex derivatization and/or extraction procedures, and do not allow for simultaneous quantitation or provide sufficient identification confidence for regulatory action. In this compilation you will find links to the original validated ZIC®-HILIC method for the determination of melamine and cyanuric acid in animal feeds developed by scientists at the US FDA Center for Veterinary Medicine. The protocol was successfully employed for the analysis of melamine in commercial aquaculture, fish and shrimp feed. The method was developed to comply with regulatory analysis of animal feed.

http://www.fda.gov/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Melamine/default.htm

Chinese Milk Scandal in 2008

In September 2008, several Chinese companies were involved in a scandal involving milk and infant formula adulterated with melamine, leading to kidney stones and other renal failure, especially among young children. By December 2008, nearly 300,000 people had become ill, with more than 50,000 infant hospitalizations and six infant deaths.

As late as July 2010, almost 2 years after the first reports, Chinese authorities were still reporting some seizures of melamine-contaminated dairy product in some provinces. It is though unclear whether these new contaminations were a result of illegal reuse of material from the 2008 adulterations.

Food Testing

Until 2007, melamine had not routinely been tested in food, except in the perspective of plastic safety or insecticide residue. The U.S. FDA issued in October 2008 new methods for the analysis of melamine and cyanuric acid in infant formulations, see links below. Similar recommendations have been issued by other authorities, like the Japanese Ministry of Health, Labor and Welfare, both based on liquid chromatography – mass spectrometry (LC/MS) detection after hydrophilic interaction liquid chromatography (HILIC) separation.

Official Liquid Chromatographic Methodology from US FDA

Determination of Melamine and Cyanuric Acid Residues in Infant Formula using LC-MS/MS:
Laboratory Information Bulletin 4421, October 2008

Interim Method for Determination of Melamine and Cyanuric Acid Residues In Foods using LC-MS/MS:
Laboratory Information Bulletin 4422, October 2008

After the Chinese scandal, the Joint Research Centre (JRC) of the European Commission set-up a website about methods to detect melamine.
irmm.jrc.ec.europa.eu/melamine

Members of the European Union are required under Commission Decision 2008/757/EC to ensure that products containing at least 15% of milk product, originating from China, are tested before import into the Community. Products containing more than 2.5 mg/kg melamine must immediately be destroyed.

Pharmaceutical Industry Guidance on Preventing Melamine Contamination

August 6, 2009 the U.S. FDA issued a Guidance for Industry - Pharmaceutical Components at Risk for Melamine Contamination. The events involving pet and livestock food products, and milk products for infants illustrate the potential for drug components to be contaminated with melamine. This guidance says that certain pharmaceutical ingredients used in the manufacture or preparation of drug products are recommended to be screened for melamine. Hence, it is important for drug manufacturers to assure that no component used in the manufacture of any drug is contaminated with melamine. FDA recommends that compounders who use at-risk components in drugs ensure proper testing.

The guidance for pharmaceuticals recommends the use of FDA-published methods based on equipment generally available to pharmaceutical manufacturers or contract testing labs. The test method used should be suitable to assay melamine contamination down to at least 2.5 parts per million (ppm).

Recommended methods are based on liquid chromatography triple quadrupole tandem mass spectrometry (LC-MS/MS) or gas chromatography/mass spectrometry (GC-MS). The LC MS/MS method is based on HILIC and also urge the need to prevent melamine degradation during sample handling, (see FDA methods). The compounds at risk may be, but are not limited to:

Adenine
Amino acids derived from casein protein hydrolysates
Calcium pantothenate
Chlorophyllin copper complex sodium
Copovidone
Dihydroxyaluminum aminoacetate
Glucagon
Hyaluronidase
Lactose
Povidone
Protamine sulfate
Taurine
Urea
Zein
Albumin
Ammonium salts
Caseinate or sodium caseinate
Colloidal oatmeal
Crospovidone
Gelatin
Guar gum
Imidurea
Melphalan
Povidone-Iodine
Protein hydrolysate (powder) for injection
Thioguanine
Wheat bran

This list was based on the FDA Inactive Ingredient Database (IID), and is not considered to be exhaustive. It is essential that manufacturers evaluate their drug components to determine whether they are vulnerable to melamine contamination.

Adulteration of Milk - India 2012

In January 2012, it was reported that more than 67% of Indian milk is adulterated. Everything from salt to detergents have been found. Among the substances found in milk were milk powder, fat, glucose and water. The Indian Food Safety and Standards Authority conducted a survey in 33 states and found that the problem is more severe in urban India, where nearly 70% of samples were found to be contaminated, compared with about 30% in rural areas.

Of these reasons, and considering the past scandals with pet, livestock food and infant formula milk powder, more and better testing is needed. Methods, not only for melamine and cyanuric acid is required as one can expect other nitrogen rich compounds to be used in economic adulteration to enhance the nitrogen content in milk products and bulk proteins.

•Risk Assessment/Safety Assessment

•Letter to the United States Food Manufacturing Industry, Regarding Melamine October 10, 2008

•Interim Safety and Risk Assessment of Melamine and its Analogues in Food for Humans October 3, 2008

•Update: Interim Safety and Risk Assessment of Melamine and its Analogues in Food for Humans November 28, 2008

In this application compilation, we present the latest analytical method from US FDA to be used as a powerful tool against economically motivated adulteration in protein-containing products. The new method have been developed to determine the presence of six nitrogen-rich compounds, cyromazine, dicyandiamide, urea, biuret, triuret, and amidinourea together with melamine. The method has been validated in skim milk, skim milk powder, soy protein, wheat flour, wheat gluten, and corn gluten meal matrices at concentrations as low as 1 ppm.

After acidic treatment of samples, acetonitrile is added to induce precipitation of proteins. Ready samples are analyzed using a SeQuant ZIC®-HILIC column and tandem mass spectrometry (HILIC–MS/MS) using electrospray ionization (ESI).

Determination of Nitrogen-rich Adulterants in Food using HILIC-MS/MS

FDA recommended column:
SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 150×2.1 mm (1.50454.0001)

Alternative column:
SeQuant® ZIC®-HILIC (3.5 µm, 200Å) PEEK 100×2.1 mm (1.50447.0001)

Recommended solvents and reagents

Acetonitrile: hypergrade for LC-MS LiChrosolv® (1.00029)

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

Formic acid: 98-100% for analysis EMSURE® ACS, Reag. Ph Eur (1.00264)

Ammonium formate: Use ACS grade or HPLC grade.

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)
Samplicity™ starter bundle with filter 0.20µm (SAMPLG0BL)

Determination of Nitrogen-rich Adulterants in Food using HILIC-MS/MS

Mobile phase

prepare mixtures of 0.1% formic acid/10 mM ammonium formate in Milli-Q® water and acetonitrile
A: 95:5 ACN:0.1% formic acid/10 mM ammonium formate in Milli-Q® water
B: 50:50 ACN:0.1% formic acid/10 mM ammonium formate in Milli-Q® water

Gradient profile

Time (min)	Solution A (%)	Solution B (%)	Flow rate (mL/min)	Elution
0.0-5.0	100	0	0.400	isocratic
5.0-12.8	100→25	0→75	0.400	gradient
12.8-15.8	25	75	0.400	isocratic
15.8-16.0	100	0	0.400	equilibration
16.0-24.9	100	0	0.600	equilibration
24.9-25.0	100	0	0.400	equilibration

Sample preparation

Briefly:
- Mix 2 g sample with 18 mL 2% formic acid and shake immediately vigorously for 1 min
- Sonicate for 30 min and shake vigorously for 1 min
- Centrifuge at 4500 rpm for 30 min
- Take 50 µL supernatant and mix with 950 µL acetonitrile
- Centrifuge at 4500 rpm for 10 min
- Filter supernatant through 0.20 µm PTFE membrane
- (Milk samples only) Dilute 100 µL filtrate with 500 µL 95:5 acetonitrile:2% formic acid

For full details of the methods of sample preparation and HILIC-MS/MS, please refer to the original documents from FDA:

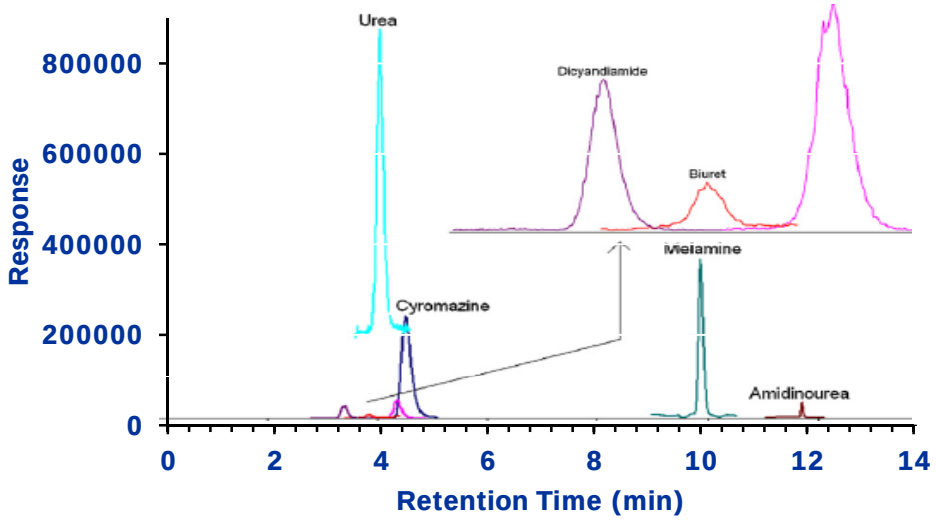
- FDA Laboratory Information Bulletin 4487 (not yet available online)
- Journal of Chromatography A 1220 (2012) 101–107
- FDA Laboratory Information Bulletin 4421

Determination of Nitrogen-rich Adulterants in Food using HILIC-MS/MS

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 150x2.1 mm	1.50454.0001
Injection:	20 µL	
Detection:	Individual LC–MS/MS quantitation ion chromatograms using a Shimadzu Prominence UFLC XR with AB Sciex 4000 QTRAP in ESI(+) mode $[M+H]^+$ m/z 85.0 for DC, 61.0 for urea, 104.1 for BU, 147.1 for TU, 167.1 for CY, 127.1 for melamine (MEL) and 103.0 for AU were the precursor ions for MS/MS;	
Flow Rate:	0.4 mL/min during separation, 0.6 mL/min during equilibration	
Mobile Phase (v/v):	A: 95:5 ACN:0.1% formic acid/10 mM ammonium formate in H2O B: 50:50 ACN:0.1% formic acid/10 mM ammonium formate in H2O	
Temperature:	Ambient	
Sample:	Extracted and spiked (1 ppm) wheat gluten sample	

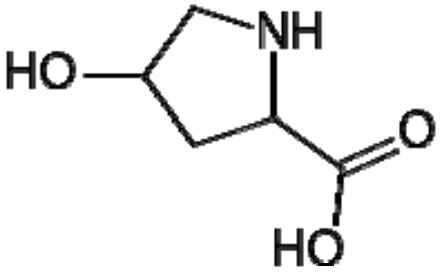


Chromatogram reproduced from J. Chromatogr. A 1220 (2012) 101-107 , with permission from Elsevier Science and Shaun MacMahon, US FDA.

Chromatographic Data

No.	Compound	Time (min)	Transition (m/z)
1	Dicyandiamide (DC)	3.6	85.0→68.0; 85.0→43.1
2	Biuret (BU)	3.9	104.1→61.0; 104.1→44.0
3	Urea	4.0	61.0→44.0
4	Triuret (TU)	4.5	147.1→130.1; 147.1→104.1; 147.1→61.1
5	Cyromazine (CY)	4.7	167.1→85.1; 167.1→125; 167.1→68.0
6	Melamine (MEL)	9.9	127.0→85.0; 127.0→68.0
7	Amidinourea (AU)	11.7	103.1→60.1; 103.1→43.1

Non-native amino acids in milk
"leather milk"



L-Hydroxyproline

Incidents: China 2011

Amino acids are molecules with both amine and carboxylic acid functionalities and a varying side-chain that defines their properties into being; hydrophilic or hydrophobic, weak acid or base. Amino acids are important to life, and have many functions. Eight amino acids are classified as "essential" for human and cannot be synthesized via metabolism from other endogenous compounds, therefore they must come from our food intake..

Amino acids serve as building blocks of proteins (chains of amino acids). For this reason, amino acid composition analysis is a classical protein analysis method, used in medical and food science research. Composition analysis of proteins is complex, comprising two steps, hydrolysis of the substrate and chromatographic separation and detection. The hydrolysis is commonly conducted with very high concentration of acid.

Recently hydrolysis of protein has been utilized for tampering of milk, i.e. "leather milk".

Leather Milk

Milk is very important to mammals. Among human it is the only source of nutrition for infants, hence quality is important. Dishonest dairy producers can unfortunately increase their profit substantially by diluting milk with water as in the melamine scandal, and consumers suffer and potentially become exposed to harmful agents. With an economical motive in hand new cunning attempts are conducted.

An alternative tampering technique is to add protein hydrolysate to diluted milk. Unscrupulous Chinese dairy producers have, for years, been collecting the scraps left over from the leather softening process at local tanneries, and putting it into milk, thereby boosting the milk's protein content as measured with the standard Kjeldahl test. This toxic milk has been named "leather milk", and again the most vulnerable victims are infants.

The consumer risk factor is not acute but those who consume leather milk are at risk of developing osteoporosis and cancer after long-term exposure. In fact, as early as 2005, this was reported in Chinese media. Addition of leather hydrolyzed protein is more difficult to detect than nitrogen-rich compounds like melamine, because it is of protein origin itself. The Chinese Ministry of Agriculture has recently advised manufacturers to check milk for L-hydroxyproline (L-Hyp). This amino acid is a good marker of hydrolyzed animal collagen as it is formed from the hydrolysis of connective tissue protein (the content is about 13%) and is not present naturally in lactoprotein. Another possibility is to check for sodium and potassium dichromate; chemicals used for softening leather, which eventually end up in the milk via the collagen hydrolysis procedure, and where chromium exist in its most toxic form; hexavalent chromium ($CrVI^{+}$).

In this compilation, a new sensitive method is presented based on hydrophilic interaction liquid chromatographic separation and tandem mass spectrometric detection (HILIC-MS/MS) suitable for positive identification and quantitative analysis of L-Hydroxyproline in dairy products. A qualitative method based on HILIC and evaporative light scattering detection (ELSD) or single MS detection has also been developed. Herein, we present the new method and illustrate the effectiveness of having simultaneous qualitative and quantitative methods at hand for L-hydroxyproline to check milk quality.

Detection of L-Hydroxyproline in Milk using HILIC-MS/MS

Recommended column

SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 150×2.1 mm	(1.50454.0001)

Alternative column

SeQuant® ZIC®-HILIC (3.5 µm, 200Å) PEEK 100×2.1 mm	(1.50447.0001)

Recommended solvents and reagents

Acetonitrile:	Hypergrade for LC-MS LiChrosolv®	(1.00029)
Water:	Water for chromatography LiChrosolv® or freshly purified water from Milli-Q® water purification system	(1.15333)
Acetic acid:	(glacial) for analysis EMSURE® ACS,ISO,Reag. Ph Eur	(1.00063)
Ammonium acetate:	for analysis EMSURE® ACS,Reag. Ph Eur	(1.01116)
Hydrochloric acid:	32% for analysis EMSURE®	(1.00319)
Sodium hydroxide pellets:	EMSURE® ACS, Reag. Ph Eur	(1.06469)
L-Hydroxyproline:	for biochemistry	(1.04506)

Recommended filtration tools

Mobile phase filtration:

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

Sample filtration:

Millex-LCR Filter, 0.45 µm, PTFE, 13 mm, non-sterile	(SLCRT13NL)
Samplicity™ starter bundle with filter 0.45µm	(SAMPLCRBL)

Detection of L-Hydroxyproline in Milk using HILIC-MS/MS

Column: SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 150x2.1 mm (1.50454.0001)
Alt. Column: SeQuant® ZIC®-HILIC (3.5 µm, 200Å) PEEK 100x2.1 mm (1.50447.0001)

Mobile phase: A: 100 % ammonium acetate (50 mM, pH 5.6)
B: 100 % acetonitrile

Gradient profile:

Time (min)	Solution A (%)	Solution B (%)	Flow rate (mL/min)	Elution
0.0-10.0	15	85	0.800	isocratic
10.0-12.0	15→45	85→55	0.800	gradient
12.0-20.0	45	45	0.800	isocratic
20-25.0	15	85	0.800	equilibration

MS/MS parameters:

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (eV)
L-Hydroxyproline (L-Hyp)	132.1	86.0* 68.2	22 18
L-Leucine (L-Leu)	132.1	86.10 43.2	16 35
L-Proline (L-Pro)	116.0	70.0	25
L-Histidine (L-His)	156.0	109.9 93.0	25 35
L-Arginine (L-Arg)	175.2	116.0 70.3	21 34
L-Valine (L-Val)	115.2	72.2 55.2	19 33

Sample preparation

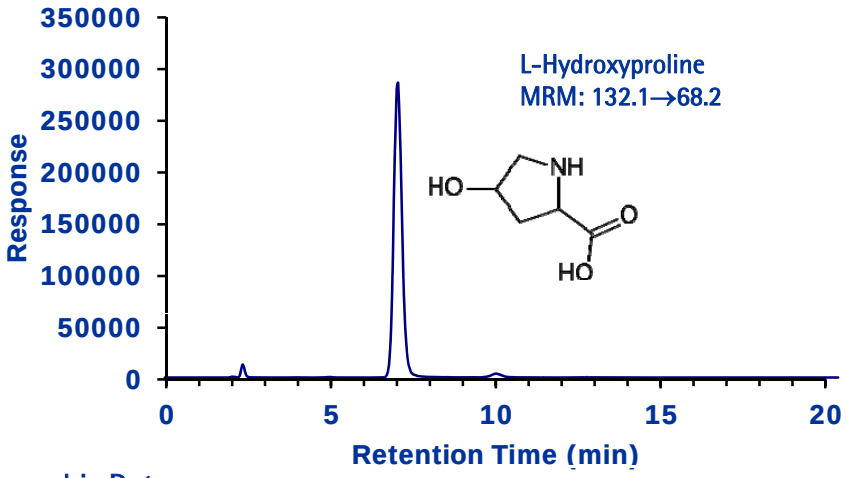
Local brand milk (2.0 g) was digested with a solution of 10 N HCl (6mL), placed under vacuum and sealed, then boiled for 12 hours. The mixture was evaporated to remove the solvent, followed by addition of 5ml of water and pH adjustment to 7 by NaOH solution (1N) before transfer to a volumetric flask and a final addition of water to bring the total volume to 25mL. The hydrolyzed mixture was centrifuged for 3min (8000 rpm) to give a clear solution. 1mL of the sample solution was transferred to a volumetric flask (10mL) and a solvent combination of ammonium acetate (50mM, pH5.6) and acetonitrile (20:80 v/v) was added to the scale. The diluted solution was filtered by 0.45 PTFE filter prior LC-MS/MS analysis

Detection of L-Hydroxyproline in Milk using HILIC-MS/MS

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 150x2.1 mm	1.50454.0001
Injection:	2 µL	
Detection:	Agilent 1200 LC system equipped with an Applied Biosystems API3200 MS/MS. Electrospray ionization was performed in positive ion mode, and multiple-reaction monitoring mode (MRM) for detection, see Table 2.	
Flow Rate:	0.8 mL/min.	
Mobile Phase (v/v):	A: 100% ammonium acetate (50 mM, pH 5.6) B: 100 % acetonitrile	
Gradient profile:	See gradient table on page 30.	
Temperature:	25 °C	
Diluent	Mobile phase	
Sample:	Local milk sample treated as per sample preparation method.	



Chromatographic Data

No.	Compound	Time (min)	Transition (m/z)
1	Void volume (t0)	0.6	
2	L-Leucine	3.3	132.1→86.1; 132.1→43.0
3	L-Valine	4.7	115.2→72.2; 115.2→55.2
4	L-Proline	4.8	116→70.0
5	L-Hydroxyproline	7.1	132.1→86.0; 132.1→68.2
6	L-Histidine	15.3	156.0→109.9; 156.0→93.0
7	L-Arginine	15.9	175.2→116.0; 175.2→70.3

Residues

Definition

Pesticides

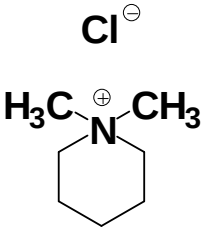
1. The designation pesticide applies to any substance or mixture of substances intended to prevent, destroy, or control any unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of pure articles. The designation includes substances intended for use as growth regulators, defoliants, or desiccants, and any substance applied to crops before or after harvest to protect the product from deterioration during storage and transport.
2. Pesticides are commonly used in agriculture. Pesticides may stay in small amounts (called residues) in or on fruits, vegetables, grains, and other foods. To make sure the food is safe for consumption, official bodies like the United States Environmental Protection Agency (EPA) regulates the amount of each pesticide that may remain in and on foods.
3. Pesticides are categorized into four main substituent chemicals: herbicides; fungicides; insecticides and bactericides.

Antibiotics

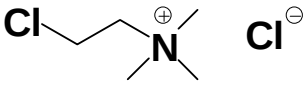
1. During their lifetime animals may have to be treated with different medicines for prevention or cure of diseases. In food producing animals such as cattle, pigs, poultry and fish this may lead to residues of the substances used for the treatment in the food products derived from these animals (e.g. meat, milk, eggs). The residues should however not be harmful to the consumer. To guarantee a high level of consumer protection, legislation requires that the toxicity of potential residues is evaluated before the use of a medicinal substance in food producing animals is authorized. If considered necessary, maximum residue limits (MRLs) are established and in some cases the use of the relevant substance is prohibited.

Further reading on pesticide and antibiotic residues:
http://www.fda.gov/Food/FoodSafety/FoodContaminantsAdulteration/Pesticides/default.htm
http://www.epa.gov/pesticides/index.htm
http://www.agf.gov.bc.ca/pesticides/
http://ec.europa.eu/food/food/index_en.htm
http://ec.europa.eu/food/plant/protection/pesticides/index_en.htm
http://ec.europa.eu/sanco_pesticides/public/index.cfm
http://en.wikipedia.org/wiki/Pesticide

Pesticides



Mepiquat



Chlormequat

Pesticides are biological (such as a virus, bacterium, antimicrobial or disinfectant) or chemical substances or mixture of substances intended for preventing, destroying, repelling or mitigating any pest. Target pests can include insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes (roundworms), and microbes that destroy property, cause nuisance, spread disease or are vectors for disease.

There are currently 507 pesticides that are listed with maximum residue limits by the European Union. Many of these are difficult to analyze using traditional methods. For example chlormequat and mepiquat are two very hydrophilic pesticides, they are widely used as plant growth regulators. They act by inhibition of vegetative growth and promotion of flowering in a wide range of fruits, vegetables, cereals and cotton. They are eliminated in soil through microbiological processes and the end-product is carbon dioxide, but can accumulate in plants, animals and humans. The US-EPA (Environmental Protection Agency) has listed the compounds and hence it requires to be measured.

In the following example, strategies for determination of mepiquat and chlormepiquat are presented.

Detection of Mepiquat and Chlormequat using HILIC–MS in Positive Mode

SeQuant® ZIC®-HILIC

Column:	SeQuant® ZIC®-HILIC (3.5 µm, 200Å) PEEK 100×2.1 mm	(1.50447.0001)

Recommended Solvents and Reagents

Acetonitrile:	isocratic grade for HPLC LiChrosolv®	(1.14291)
Water:	Water for chromatography LiChrosolv® or freshly purified water from Milli-Q® water purification system	(1.15333)
Ammonium acetate	for analysis EMSURE® ACS,Reag. Ph Eur	(1.01116)

Recommended filtration tools

Mobile phase filtration:

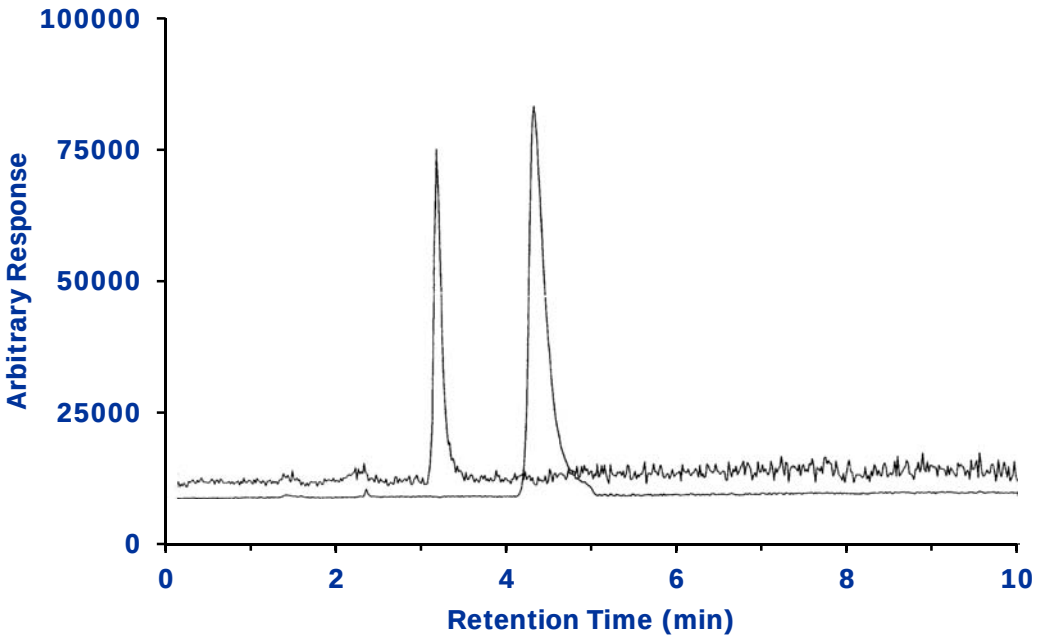
PTFE coated with funnel, base, stopper clamp	(XX1004720)
Omnipore PTFE membrane filter 0.45µm	(JHWP04700)

Detection of Mepiquat and Chlormequat using HILIC–MS in Positive Mode

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (3.5 µm, 200Å) PEEK 100x2.1 mm	1.50447.0001
Injection:	20 µL	
Detection:	Electrospray-MS in positive mode (ESI+). Single ion monitoring (SIM) at m/z 114 and 122	
Flow Rate:	0.2 mL/min.	
Mobile Phase (v/v):	Acetonitrile and 25 mM ammonium acetate (80:20)	
Temperature:	Ambient	
Diluent:	Mobile phase	
Sample:	Standard injection of chlormequat and mepiquat	



By courtesy of: Dr.-Ing. Ludmila Havlik, Chemisches Labor Dr. Wirts + Partner, Hannover, Germany, www.wirts.de

Chromatographic Data

No.	Compound	Time (min)	Retention Factor
	Void volume (t0)	1.6	-
1	Chlormequat	3.1	0.9
2	Mepiquat	4.3	1.7

Determination of Mepiquat and Chlormequat in seed by HILIC–MS/MS

Column: SeQuant® ZIC®-HILIC (3.5 µm, 200Å) PEEK 100x2.1 mm (1.50447.0001)

Mobile phase: Acetonitrile, ammonium acetate buffer (25 mM overall ionic strength), formic acid (80:19.5:0.5)
Flow rate: 0.4 mL/min
Injection: 1 µL
Detection: MS/MS, using an Agilent 1200 RRLC system and 6410 QQQ System.

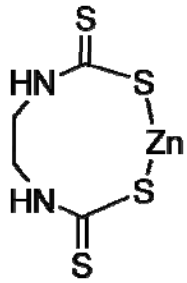
Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor	Collision Energy (eV)
Chlormequat-D4	126	58.1	140	35
Chlormequat	122	58.1	140	35
Mepiquat-D3	117.1	98.2 (Quant) 61.1 (Qual)	140 140	30 30
Mepiquat	114.1	98.2 (Quant) 58.1 (Qual)	140 140	30 140

Sample preparation

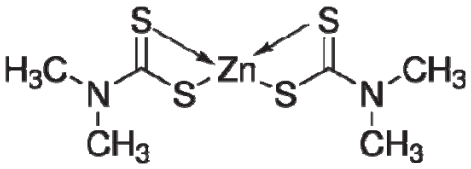
Briefly (for seed samples):
- Weigh 10 g of seed
- Grind and add 20 mL of Milli-Q® plus 40 mL ethanol with ISTD
- Take an aliquot of 5 mL of extraction solvent
- Add the appropriate amount of standard to the standard samples
- Filtration

For further details please refer to the presentation:
http://amcham.dk/dl/esac/ESAC08-5.pdf

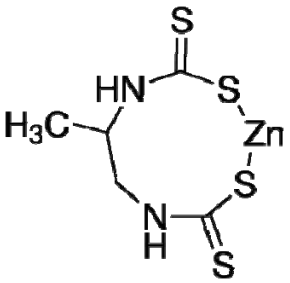
Dithiocarbamates



Zineb



Ziram



Propineb

Dithiocarbamates is an important class of pesticides. They are widely used as fungicides mainly for preservation of fruit and vegetables, but are also used by the paper and pulp industry as slimicides and as vulcanization accelerators in the rubber industry. It is the class of pesticides that most often exceed the maximum allowed residue limits (MRL) in food imported to Europe (European Commission (EC), SEC (2007) 1411, monitoring of pesticide residues in products of plant origin in the European Union, Norway, Iceland and Lichtenstein 2005, EC Brussels, Belgium 2007). Dithiocarbamates decompose instantly under acidic conditions forming carbon disulfide.
The method currently used for dithiocarbamate analysis is a cumbersome hot acid digestion of the sample. The released carbon disulfide is then analyzed either by titration or UV/Vis absorbance. This procedure makes determination of individual pesticides impossible and also leads to problems when analyzing crops rich in natural carbon disulfide like broccoli, cabbage, cauliflower or papaya.

Much effort has been spent trying to develop LC-MS methods for dithiocarbamate determination, and for this analysis a pH stable column is essential since the pH has to be high during the entire analysis. A reversed phase method based on methyl iodide derivatization has been proposed, but suffers from using a very carcinogenic derivatisation agent. A better approach is to use pH stable HILIC columns, e.g. SeQuant® ZIC®-pHILIC (pH range 2-12), where pre-column derivatization can be omitted for separation of these hydrophilic molecules.

A ZIC®-pHILIC LC-MS method was developed at the University of Hohenheim and later evaluated and validated by the German NRL for Pesticide Residues within the Federal Office of Consumer Protection and Food Safety. This method uses an eluent containing only acetonitrile and 10mM aqueous ammonia in a step gradient, and with tandem MS and MRM limits of quantitation (LOQs) are between 3 and 8 ppb.

Determination of Dithiocarbamates using HILIC–MS in Negative Mode

SeQuant® ZIC®-pHILIC

Column

SeQuant® ZIC®-pHILIC (5 µm, polymer) PEEK 150×4.6 mm	(1.50461.0001)

Recommended Solvents and Reagents

Acetonitrile	Hypergrade for LC-MS LiChrosolv®	(1.00029)
Water	Water for chromatography LiChrosolv® or freshly purified water from Milli-Q® water purification system	(1.15333)
Ammonia	25% solution for analysis EMSURE®	(1.05432)

Recommended filtration tools

Mobile phase filtration:

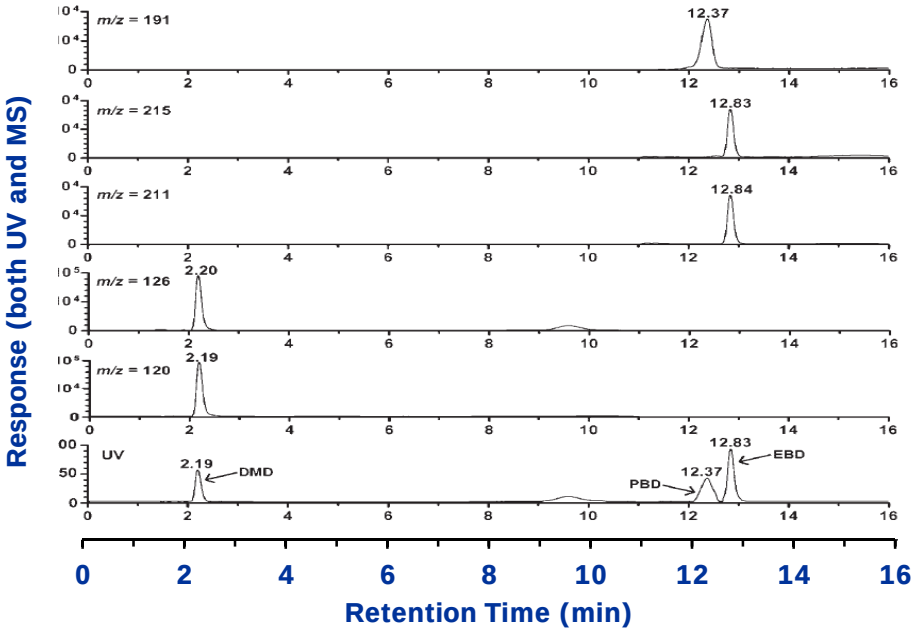
PTFE coated with funnel, base, stopper clamp	(XX1004720)
Omnipore PTFE membrane filter 0.45µm	(JHWP04700)

Determination of Dithiocarbamates using HILIC–MS in Negative Mode

SeQuant® ZIC®-pHILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-pHILIC (5 µm, polymer) PEEK 150x4.6 mm	1.50461.0001
Injection:	5 µL	
Detection:	Electrospray-MS in negative mode. Single ion monitoring (SIM) at m/z 120, 126, 211, 215 and 191	
Flow Rate:	0.7 mL/min	
Mobile Phase (v/v):	A: Acetonitrile, B: 10 mM ammonia Gradient 90:10 A/B 0-5 min, 60:40 A/B 5-13min 40:60 A/B 13-16 min	
Temperature:	Ambient	
Diluent:	10mM each of $NaHCO_3$ and DL-Penicillamine pH 12 with NaOH	
Sample:	DMD, d6-DMD, EBD, d4-EBD and PBD (20mg/L for UV and 0.4mg/L for MS)	



Chromatogram reprinted with permission from JOHN/WILEY & SONS LTD. Original article Rapid Commun. Mass Spectrom., 21 (2007) 4009-4016.

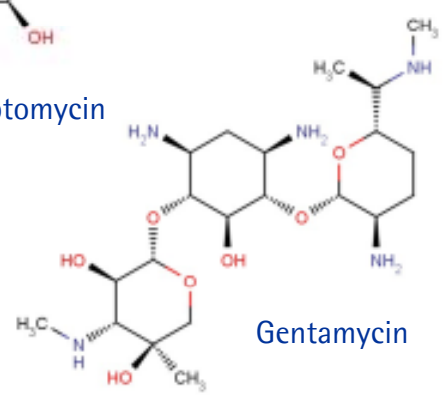
Chromatographic Data

No.	Compound	Time (min)	Retention Factor
	Void volume (t0)	2.0	-
1	DMD (Ziram)	2.2	0.1
2	PBD (Propineb)	12.4	5.2
3	EBD (Zineb)	12.8	5.4

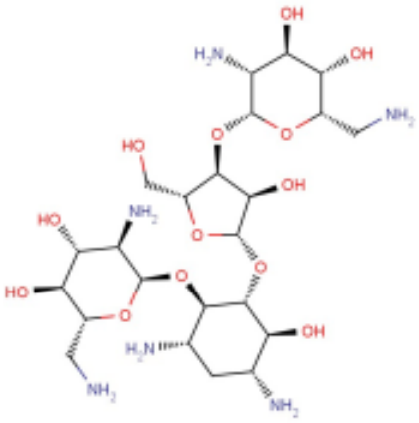
Merck Millipore is a division of Merck KGaA, Darmstadt, Germany
www.merckmillipore.com/chromatography
chromatography@merckgroup.com

Aminoglycosides

Streptomycin



Gentamycin



Neomycin

Aminoglycosides are bactericidal antibiotics which have amino-modified sugar in their molecules. This particular group of antibiotics is widely used as clinical and veterinary medicines to treat infections caused by gram-negative or some gram-positive bacteria, and are classified as bactericidal agents because of their interference with bacterial replication. However, these antibiotics can also cause varying degree of ototoxicity and nephrotoxicity.

Overuse of antibiotics and exposure from the animal food are the two major routes attributed to the antibiotic resistance. Therefore, it is important to develop sensitive and reliable analytical methods for determining and monitoring aminoglycosides residuals in different sample matrices. Aminoglycosides are normally very hydrophilic and carry several amino groups, which mean they are very positively charged at neutral pH condition.

In this compilation, an application for determination of streptomycin, gentamycin, and neomycin illustrate the benefit of combining HILIC with mass spectrometric detection.

Determination of Streptomycin, Gentamycin, and Neomycin Using HILIC-MS

SeQuant® ZIC®-HILIC

Column:	SeQuant® ZIC®-HILIC (3.5 µm, 100Å) PEEK 100×2.1 mm	(1.50441.0001)

Recommended solvents and reagents

Acetonitrile:	Hypergrade for LC-MS LiChrosolv®	(1.00029)
Water:	Water for chromatography LiChrosolv® or freshly purified water from Milli-Q® water purification system	(1.15333)
Ammonium acetate:	for analysis EMSURE® ACS,Reag. Ph Eur	(1.01116)
Formic acid:	98-100% for analysis EMSURE® ACS,Reag. Ph Eur	(1.00264)

Recommended filtration tools

Mobile phase filtration:

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

Determination of Streptomycin, Gentamycin, and Neomycin Using HILIC-MS

SeQuant® ZIC®-HILIC

Column: SeQuant® ZIC®-HILIC (3.5 µm, 100Å) PEEK 100x2.1 mm (1.50441.0001)

Mobile phase A: Acetonitrile and formic acid (99:1)
B: Ammonium acetate (100 mM) and 3% formic acid (100%)

Time (min)	Solution A (%)	Solution B (%)	Elution
0	50	50	isocratic
4-8	50→5	50→95	gradient
8-16	50	50	Equilibration

Flow rate: 0.4 mL/min

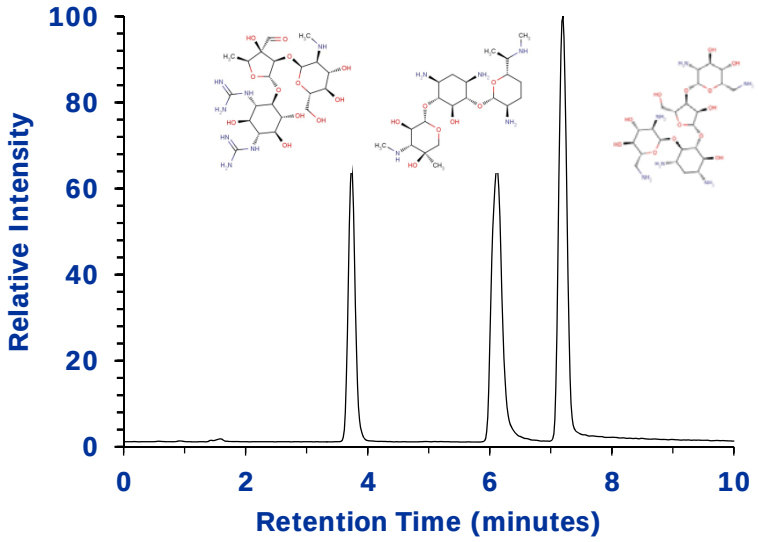
Detection: Shimadzu LCMS-2010EV; ESI in positive mode; Heat block and CDL temperature 250 ℃;
Spray gas: nitrogen at 1.5 L/min; Detector voltage: 2 kV.
SIM in positive mode: m/z 582 (Streptomycin), 464 (Gentamicin), and 615 (Neomycin)

Determination of Streptomycin, Gentamycin, and Neomycin Using HILIC-MS

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (3.5 µm, 100Å) PEEK 100x2.1 mm	1.50441.0001
Injection:	5 µL	
Detection:	Shimadzu LCMS-2010EV; ESI in positive mode; Heat block and CDL temperature 250 ℃; Spray gas: nitrogen at 1.5 L/min; Detector voltage: 2 kV. SIM in positive mode: m/z 582 (Streptomycin), 464 (Gentamycin), and 615 (Neomycin)	
Mobile Phase (v/v):	A: Acetonitrile and formic acid (99:1) B: Ammonium acetate (100 mM) and 3% formic acid (100%)	
Flow Rate:	0.4 mL/min.	
Gradient:	See Table.	
Temperature:	50 °C	
Diluent (v/v):	Acetonitrile/Milli-Q® water (30:70)	
Sample:	5 µg/mL STR, 25 µg/mL GEN and NEO in diluent	



Chromatographic Data

No.	Compound	Time (min)	Tailing Factor (USP)
1	Streptomycin (STR)	3.7	1.1
2	Gentamycin (GEN)	6.1	1.2
3	Neomycin (NEO)	7.2	1.1

Merck Millipore is a division of Merck KGaA, Darmstadt, Germany
www.merckmillipore.com/chromatography
chromatography@merckgroup.com

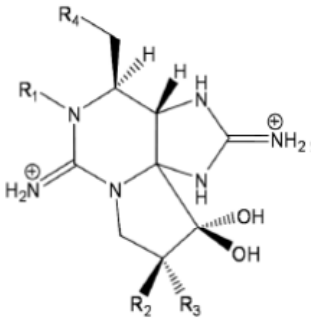
Toxins

Definition

1. A toxin is a poisonous substance produced within living cells or organisms; man-made substances created by artificial processes are thus excluded.

2. Toxins usually consist of an amino acid chain which can vary in molecular weight between a couple of hundred (peptides) and one hundred thousand (proteins). They may also be low-molecular organic compounds. Toxins are produced by numerous organisms, e.g., bacteria, fungi, algae and plants. Toxins vary greatly in their severity, ranging from usually minor and acute to almost immediately deadly. Many of them are extremely poisonous, with a toxicity that is several orders of magnitude greater than chemical warfare nerve agents.

Shellfish toxin analysis



With increasing sea temperatures the number of alagal blooms are increasing especially in over fertilized areas like the Baltic sea. These algae are consumed by shellfish filter feeders who, therefore, accumulate toxins produced by dinoflagellates , diatoms and cyanobacteria. Paralytic shellfish poisoning (PSP) toxins often referred to as Saxitoxins, can reach lethal levels in shellfish, and detection of saxitoxin in mussels, clams and scallops frequently leads to closures of commercial and recreational shellfish harvesting, especially in California, Oregon, Washington, and New England.

PSP toxins are powerful sodium channel blockers that can cause respiratory insufficiency and death. An AOAC mouse bioassay used to determine PSP's only determines the total toxicity of the sample. Chromatographic separation is used to identify individual toxins. Here an analytical method for the determination of paralytic shellfish toxins using hydrophilic interaction liquid chromatography is described. This method provides a sensitive and selective tool which can be employed with either a fluorescence detector or a mass spectrometric detector. The method can be used for various phytoplankton and the routine analysis of seafood.

There are a number of other toxins assosiated with algae and cyanobacteria like anatoxins and β-N-methylamino-L-alanine (BMAA) where methods using the ZIC®-HILIC column are available in the scientfic literature.

Determination of Paralytic Shellfish Toxins

SeQuant® ZIC®-HILIC

Column:	SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 250×4.6 mm	(1.50458.0001)

Recommended solvents and reagents

Acetonitrile	Hypergrade for LC-MS LiChrosolv®	(1.00029)
Water	Water for chromatography LiChrosolv® or freshly purified water from Milli-Q® water purification system	(1.15333)
Formic Acid	98-100% for analysis EMSURE® ACS,Reag. Ph Eur	(1.00264)
Ammonia	25% solution for analysis EMSURE®	(1.05432)
Periodic Acid	for analysis EMSURE®	(1.00524)
Nitric Acid	69% for analysis EMSURE® ACS,Reag. Ph Eur	(1.01799)
Hydrochloric acid:	32% for analysis EMSURE®	(1.00319)
Ammonium Formate	Use ACS grade or HPLC grade.	

Recommended filtration tools

Mobile phase filtration:

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

Determination of Paralytic Shellfish Toxins using Fluorescence Detection

SeQuant® ZIC®-HILIC

Column: SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 250x4.6 mm (1.50458.0001)

Mobile phase: A: 10 mM ammonium formate and 10 mM formic acid in Milli-Q® water (100%)
B: Acetonitrile and Milli-Q® water with I_{tot} 8mM with ammonium formate (80:20 v/v)

Gradient:

Time (min)	Solution A (%)	Solution B (%)	Elution
0-24.0	18	82	Isocratic
24.1-35.0	30	70	Isocratic
35.1-50.0	35	65	Isocratic
50.1-70.0	18	82	equilibration

Detection: Fluorescence detection (Ex=350nm, Em=395mn).
Post-column oxidation with 10 mM periodic acid and 550 mM ammonia in water (flow rate 0.3 mL/min).
Nitric acid (0.75 M; flow rate 0.3 mL/min) was used to lower the pH value to 2–3.

Sample preparation:

Extraction of PSP toxins from shellfish material:
The sample material (2 g) was homogenized with 3 mL of hydrochloric acid (0.2 M) using an ultra sonic probe. The extracts were heated for 15 min at 90 degrees Celsius and the suspension was centrifuged for 10 min (14000 rpm), afterwards the supernatant was passed through a 0.45 µm nylon filter.
The treatment with HCl induced a conversion of N-sulfocarbamoyl toxins to the corresponding carbamoyl toxins.

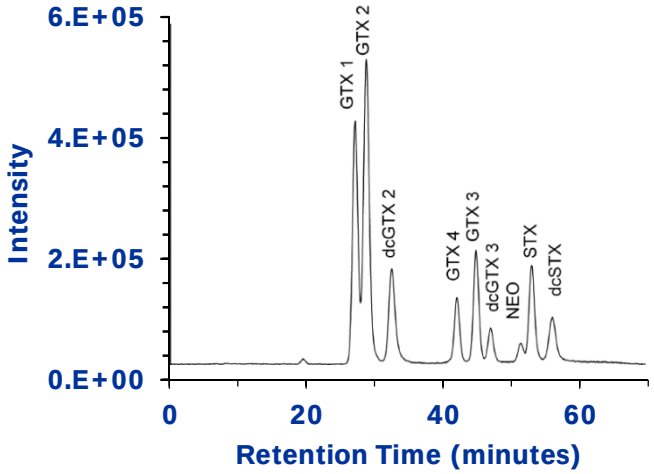
Extraction of the PSP toxins from Alexandrium catenella and Gymnodinium catenatum:
Aqueous acetic acid (2 mL, 0.03 M) was added to a suspension of algae cells (1 mL).
The suspension was then homogenized with an ultra sonic probe and centrifuged for 10 min (14000 rpm).
The supernatant was passed through a 0.45 µm nylon filter and the extracts obtained were used as a qualitative PSP standard solution during the development and optimization of the HILIC method.

Determination of Paralytic Shellfish Toxins

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 250x4.6 mm	(1.50458.0001)
Detection:	Fluorescence detection (Excitation=350nm, Emission=395nm)	
Mobile Phase (v/v):	A: 10 mM Ammonium formate and 10 mM formic acid in Milli-Q® water (100%) B: acetonitrile and Milli-Q® water with I_{tot} 8 mM Ammonium formate (80:20)	
Gradient:	See gradient table on page 47	
Flow Rate:	0.7 mL/min	
Sample:	GTX 1 (10.20 ng), GTX 2 (0.58 ng), dcGTX 2, (0.50 ng), GTX 4 (3.60 ng), GTX 3 (0.20 ng), dcGTX 3 (0.14 ng), NEO (2.05 ng), STX (0.72 ng), and dcSTX (0.79 ng).	
Temperature:	Ambient	



Chromatogram reprinted from J. Sep Sci. 2007, 30, 1821-1826, with permission from Wiley-VCH Verlag GmbH&Co., KGaA, Weinheim, Germany).

Chromatographic Data

No.	Compound	Time (min)
	Void volume (t0)	3.5
1	Gonyautoxin 1 (GTX 1)	27.1
2	Gonyautoxin 2 (GTX 2)	28.6
3	Decarbamoyl-gonyautoxin 2 (dcGTX 2)	32.3
4	Gonyautoxin 4 (GTX 4)	41.8
5	Gonyautoxin 3 (GTX 3)	44.7
6	Decarbamoyl-gonyautoxin 3 (dcGTX 3)	46.6
7	Neosaxitoxin (NEO)	51.4
8	Saxitoxin (STX)	53.0
9	Decarbamoyl-saxitoxin (dcSTX)	55.9

HILIC-MS/MS - Paralytic Shellfish Toxins

SeQuant® ZIC®-HILIC

Column: SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 250x4.6 mm (1.50458.0001)

Mobile phase: A: 10 mM ammonium formate and 10 mM formic acid in Milli-Q® water (100%)
B: Acetonitrile and Milli-Q® water with I_{tot} 8mM with ammonium formate (80:20 v/v)

Gradient:

Time (min)	Solution A (%)	Solution B (%)	Elution
0.0	20	80	Initial
5.0	35	65	Gradient
10.0	40	60	Gradient final
20.0	40	60	Isocratic
25.1	20	80	Equilibration
40.0	20	80	Equilibration

Sample preparation:

Extraction of PSP toxins from shellfish material:
The sample material (2 g) was homogenized with 3 mL of hydrochloric acid (0.2 M) using an ultra sonic probe. The extracts were heated for 15 min at 90 degrees Celsius and the suspension was centrifuged for 10 min (14000 rpm), afterwards the supernatant was passed through a 0.45 µm nylon filter.
The treatment with HCl induced a conversion of N-sulfocarbamoyl toxins to the corresponding carbamoyl toxins.

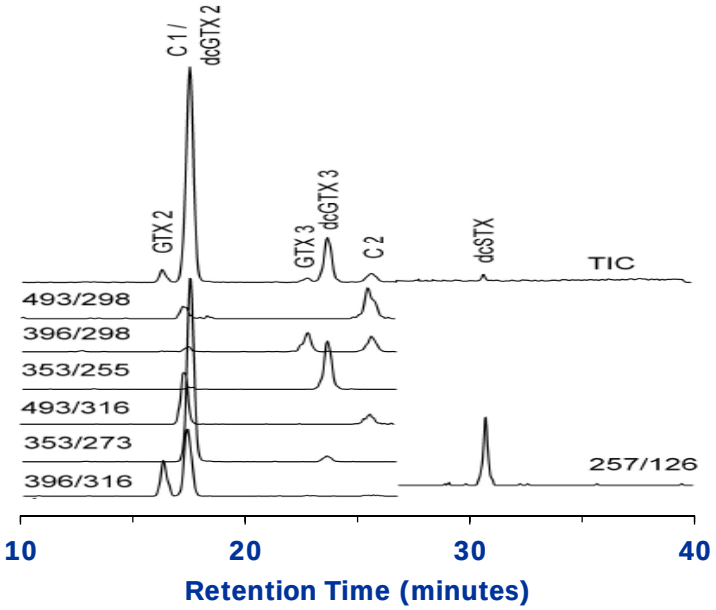
Extraction of the PSP toxins from Alexandrium catenella and Gymnodinium catenatum:
Aqueous acetic acid (2 mL, 0.03 M) was added to a suspension of algae cells (1 mL).
The suspension was then homogenized with an ultra sonic probe and centrifuged for 10 min (14000 rpm).
The supernatant was passed through a 0.45 µm nylon filter and the extracts obtained were used as a qualitative PSP standard solution during the development and optimization of the HILIC method.

HILIC-MS/MS - Paralytic Shellfish Toxins

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200Å) PEEK 250x4.6 mm	(1.50458.0001)
Detection:	ESI-MS/MS, details given in table below	
Flow Rate:	0.7 mL/min.	
Mobile Phase (v/v):	A: 10 mM Ammonium formate and 10 mM formic acid in water (100 %) B: 80% ACN and 20% water v/v I_{tot} 8 mM Ammonium formate.	
Gradient:	See gradient table on page 49	
Temperature:	Ambient	
Sample:	Gymnodinium catenatum extract, Baja California, Mexico	



Chromatogram reprinted from J. Sep Sci. 2007, 30, 1821-1826, with permission from Wiley-VCH Verlag GmbH&Co., KGaA, Weinheim, Germany).

Chromatographic Data

No.	Compound	Time (min)	Transition (m/z)
1	Void volume (t0)	3.5	
2	Gonyautoxin 2 (GTX 2)	16.7	396→316
3	Saxitoxin C1 (C1)	17.7	493→316
4	Decarbamoyl-gonyautoxin 2 (dcGTX 2)	17.9	353→373
5	Gonyautoxin 3 (GTX 3)	23.0	396→298
6	Decarbamoyl-gonyautoxin 3 (dcGTX 3)	24.0	353→255
7	Saxitoxin C2 (C2)	25.7	493→298
8	Decarbamoyl-saxitoxin (dcSTX)	31.3	257→126

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 Alimentarious 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 intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging transport or holding of such food results, or may be reasonably expected to result (directly or indirectly) in it or its by-products becoming a component or otherwise affecting the characteristics of such foods. The term 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.

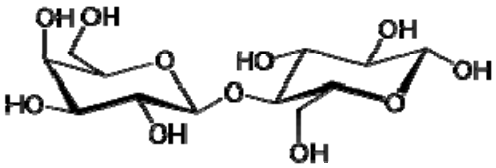
Food additives are classified on the basis of their functional use and are grouped as:

Colors	Preservatives	Acidity Regulators
Antioxidants	Ant caking agents	Antifoaming Agents
Artificial sweeteners	Enzymes	Emulsifiers
Emulsifying agents	Flavors	Flavor enhancers
Modified Starches	Phosphates	Stabilizers

Thickening and jellying agents.

Examples provided so far in this compilation have been focused on adulteration and potential threats. The following applications illustrate analysis of sugars, vitamins and organic acids, ie, typical constituents and additives in food.

Sugar analysis



Lactose

In chromatography the mutarotation of reducing sugars in solution causes these to elute as two peaks, one for each anomer. From 1975 before HILIC became known as HILIC it was extensively used for sugar separations using first silica but later aminopropyl silica columns. The amino columns catalyse the mutarotation of sugars effectively causing the retention time of the sugar to be the average of the two anomers, showing as only one peak in the chromatogram. These amino columns are however notoriously unstable as they catalyse their own degradation.

The use of an amine containing buffer component, like ammonium hydroxide (NH4OH) in the mobile phase also catalyzes the anomer interconversion. Silica based chromatography columns are not stable at this high pH but the polymeric ZIC®-pHILIC column can be used for several weeks and hundreds of injections using a 1% ammonium hydroxide eluent (pH~11). The chemical stability of the ZIC®-pHILIC columns allow for direct ESI-MS quantitation of simple and complex carbohydrates at basic pH and elevated temperatures. By using high pH mobile phase to collapse anomers of for example glucose and lactose into a single peak it is possible to, simplify identification of carbohydrates in different types of samples even with difficult matrices. Combined with simple sample preparation procedures like protein precipitation or liquid-liquid extraction, efficient and cost-efficient analytical work-schemes can be developed and used for monitoring of sugar, sugar alcohols and other carbohydrates in different type of formulations and matrices.

Lactose is a reducing sugar often needed to be quantified in milk products. Lactose free products are in most cases defined as those with a non measurable level of remaining lactose. In Scandinavia the measurement is performed by an enzymatic method having a LOQ of 100 ppm. This method is, however, not applicable if the lactose has been removed by enzymatic degradation. Then a chromatographic method has to be used.

On the following pages lactose determination using a simple sample preparation consisting of protein percipitation, centrifugation and filtration is presented. For more information on chromatography of carbohydrates please visit www.sequant.com/sugars

Determination of Lactose in Milk

SeQuant® ZIC®-pHILIC

Column:	SeQuant® ZIC®-pHILIC (5 µm, polymer) PEEK 100×2.1 mm	(1.50462.0001)

Recommended solvents and reagents

Acetonitrile :	Hypergrade for LC-MS LiChrosolv®	(1.00029)
Water:	Water for chromatography LiChrosolv® or freshly purified water from Milli-Q® water purification system	(1.15333)
Ammonia:	25% solution for analysis EMSURE®	(1.05432)

Recommended filtration tools

Mobile phase filtration:

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

Sample filtration:

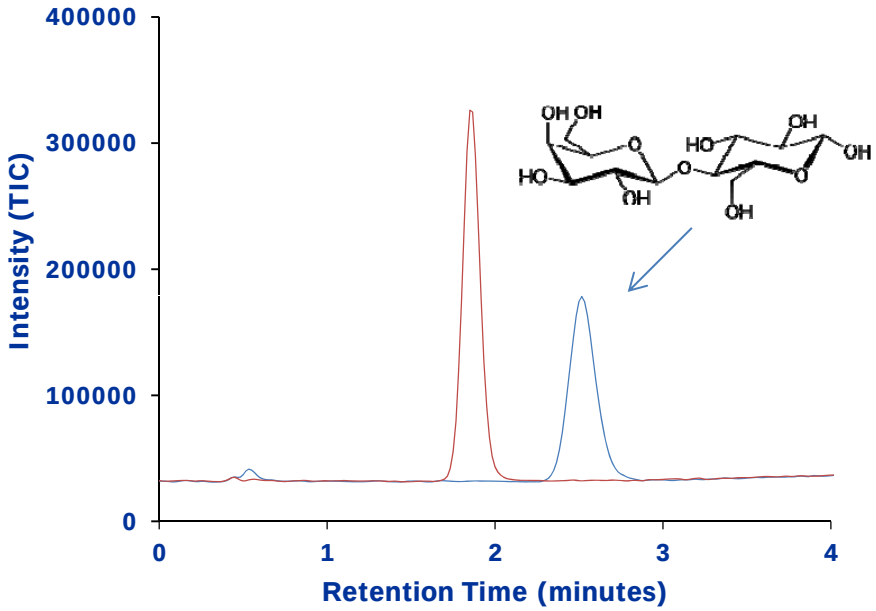
Millex-LCR Filter, 0.45 µm, PTFE, 13 mm, non-sterile	(SLCRT13NL)
Samplicity™ starter bundle with filter 0.45µm	(SAMPLCRBL)

Determination of Lactose in Milk

SeQuant® ZIC®-pHILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-pHILIC (5 µm, polymer) PEEK 100x2.1 mm	(1.50462.0001)
Injection:	2 µL	
Detection:	ESI MS SIM, negative mode	
Flow Rate:	0.35 mL/min.	
Mobile Phase (v/v):	Acetonitrile and 1% Ammonia Gradient 73:27 to 60:40 in 0 to 3 min.	
Temperature:	55 °C	
Diluent:	Mobile phase	
Sample:	Milk samples Valio Lactose free milk, Glucose/Galactose (red), Reduced fat milk Norrmejerier, Lactose (blue), Milk samples were diluted in water 5 times. Preciptation in 4 parts basic acetonitrile (1% ammonium hydroxide). Centrifugation: 6400rpm 5 min. Filtration: 0.45mm PTFE syringe filter. Dilution 100x.	

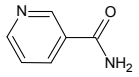


Chromatographic Data

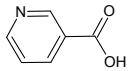
No.	Compound	Time (min)	Retention Factor
	Void volume (t0)	0.5	-
1	Glucose/Galactose	1.9	2.7
2	Lactose	2.5	4.1

Vitamins

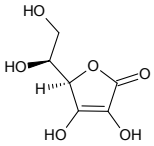
Nicotinamide
pKa 3.35 logP -0.37



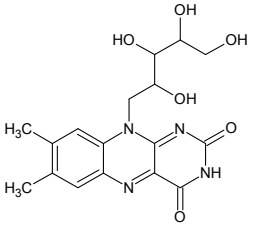
Nicotinic acid
pKa 3.0 4.7 logP 0.36



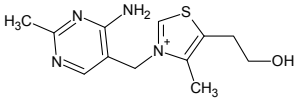
Ascorbic acid
pKa 4.1, 11.2 logP -1.85



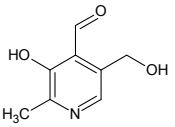
Riboflavin
pKa 10.2 logP -1.46



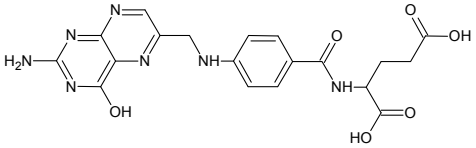
Thiamin
pKa 4.8, 9.0 logP -4.6



Pyridoxine
pKa 5.6 8.6 logP -0.77



Folic acid
pKa 2.7, 4.1, 8.9 logP -0.02



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 focus on the hydrophilic and polar compounds, e.g. the water-soluble vitamins, and two examples are presented.

Further reading:

http://www.nlm.nih.gov/medlineplus/ency/article/002399.htm
http://en.wikipedia.org/wiki/Vitamin

Determination of Water Soluble Vitamins

SeQuant® ZIC®-HILIC

Column:	SeQuant® ZIC®-HILIC (3.5 µm, 100 Å) PEEK 150×2.1 mm	(1.50442.0001)

Recommended solvents and reagents

Acetonitrile:	Hypergrade for LC-MS LiChrosolv®	(1.00029)
Water:	Water for chromatography LiChrosolv® or freshly purified water from Milli-Q® water purification system	(1.15333)
Formic acid:	98-100% for analysis EMSURE® ACS,Reag. Ph Eur	(1.00264)
Ammonium formate:	Use ACS grade or HPLC grade.	

Recommended filtration tools

Mobile phase filtration:

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

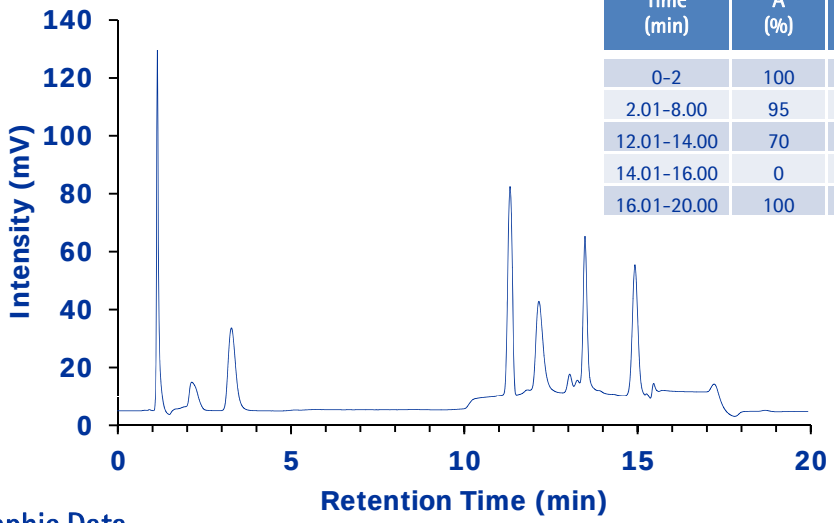
Determination of Water Soluble Vitamins

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (3.5 µm, 100Å) PEEK 150x2.1 mm	(1.50442.0001)
Injection:	5 µL	
Detection:	UV at 254 nm. Shimadzu LC-20 equipped with 2.5µL semi-micro flow-cell	
Flow Rate:	See Table	
Mobile Phase (v/v):	A: Acetonitrile and 100 mM Ammonium formate (50 mM), pH 3.5; 90:10 (v/v) B: Acetonitrile and 100 mM Ammonium formate (50 mM), pH 3.5; 50:50 (v/v)	
Gradient:	See Table.	
Temperature:	30 °C	
Diluent	Initial mobile phase	
Sample:	40 ppm of each vitamin in initial mobile phase composition	

Time (min)	A (%)	B (%)	Flow Rate (mL/min)
0-2	100	0	0.5
2.01-8.00	95	5	0.6
12.01-14.00	70	30	0.6
14.01-16.00	0	100	0.6
16.01-20.00	100	0	0.5



Chromatographic Data

No.	Compound	Time (min)	Tailing Factor (USP)
	Void volume (t0)	0.6	-
1	Nicotinamide	1.1	1.6
2	Pyridoxal	2.1	1.6
3	Nicotinic Acid	3.3	1.2
4	Ascorbic Acid	11.3	1.0
5	Thiamine	12.2	1.3
6	Riboflavin	13.5	1.1
7	Folic Acid	14.9	1.1

Determination of Ascorbic acid

SeQuant® ZIC®-HILIC

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) PEEK 150×4.6 mm	(1.50455.0001)

Recommended solvents and reagents

Acetonitrile:	Isocratic grade for HPLC LiChrosolv®	(1.14291)
Water:	Water for chromatography LiChrosolv® or freshly purified water from Milli-Q® water purification system	(1.15333)
Ammonium acetate:	for analysis EMSURE® ACS,Reag. Ph Eur	(1.01116)

Recommended filtration tools

Mobile phase filtration:

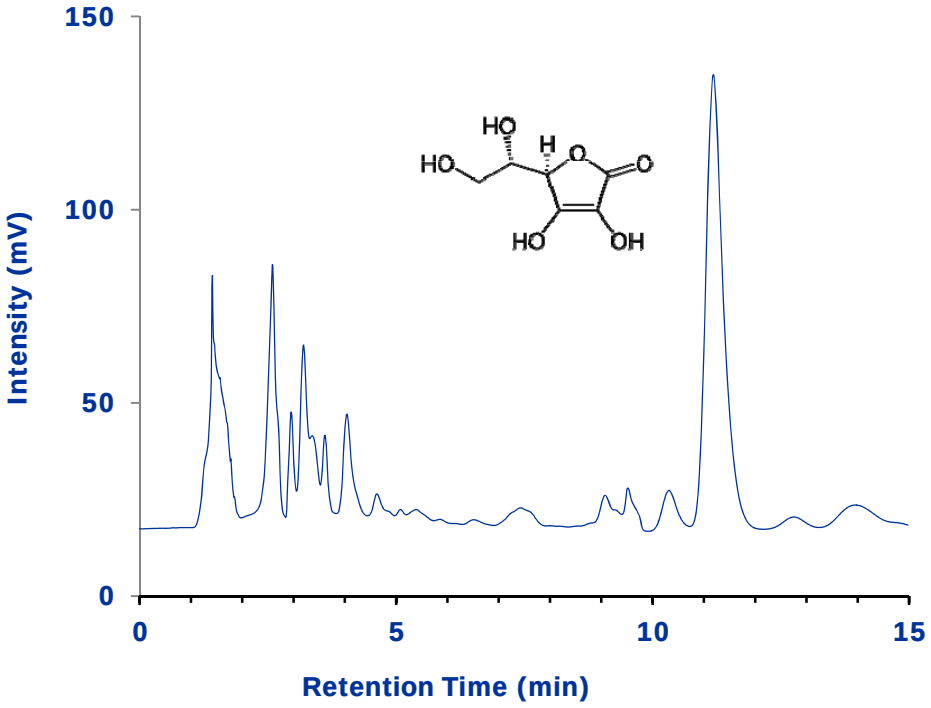
PTFE coated with funnel, base, stopper clamp	(XX1004720)
Omnipore PTFE membrane filter 0.45µm	(JHWP04700)

Determination of Ascorbic Acid in Wine

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5µm, 200Å) PEEK 150x4.6 mm	(1.50455.0001)
Injection:	250 µL	
Detection:	UV at 240 nm. Shimadzu LC-10Vp equipped with 2.5µL semi-micro flow-cell	
Flow Rate:	1.0 mL/min.	
Mobile Phase (v/v):	Acetonitrile and 200mM Ammonium acetate buffer, pH 6.8 (80:20)	
Pressure drop:	50 Bar (720 psi)	
Temperature:	Ambient	
Diluent	Mobile phase	
Sample:	Riesling wine diluted 1:3 (v/v) with acetonitrile	



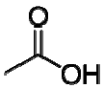
Chromatographic Data

No.	Compound	Time (min)	Retention Factor	Tailing Factor (USP)
	Void volume (t0)	1.5		
2	Ascorbic Acid	11.3	6.5	1.0

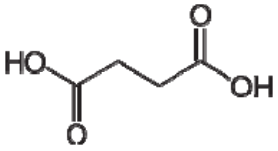
Merck Millipore is a division of Merck KGaA, Darmstadt, Germany
www.merckmillipore.com/chromatography
chromatography@merckgroup.com

Organic acids

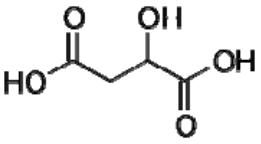
ZIC®-cHILIC coming soon



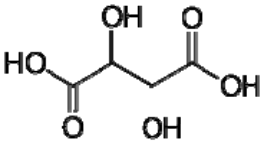
Acetic acid



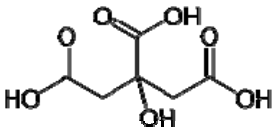
Succinic acid



Malic acid



Tartaric acid



Citric acid

Preserved by addition of Benzoic acid, lactic acid or pickled in acetic acid. Sushi, sauerkraut, balsamic vinegar and of course beer, vodka and it is crucial to get the right balance of organic acids in wine. Organic acids are present in every meal we eat. Separating organic acids with HILIC is orthogonal to using Reversed Phase Chromatography, the difficult hydrophilic acids citric and tartaric acid will be well retained in and well separated, there will be no co-elution of malic acid and succinic acid as is often the case in ion chromatography.

In grapes the predominant organic acids 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).

HILIC has so far mainly been used as an MS friendly technique using volatile acetate or formate buffers. For QC applications with UV detectors RP columns and phosphate buffer has so far been predominant. Using HILIC with low UV cut of buffers like phosphate is possible despite the limited solubility of potassium phosphate in high acetonitrile eluents. There are some rules to using phosphate buffer in HILIC, the same rules apply to RP when using a high proportion of acetonitrile in the eluent.

1. Always use premixed eluents, never use pure Acetonitrile as one mobile phase constituent.
2. Never use over 80% Acetonitrile, at low buffer strengths 85% is the absolute maximum.
3. If using gradients make the difference between mobile phase A and B as small as possible.
4. HILIC gradients should be shallower than in RP since changes in mobile phase has a larger effect in HILIC than in RP.

Determination of Organic Acids in wine

SeQuant® ZIC®-cHILIC

ZIC®-cHILIC coming soon

Column:	SeQuant® ZIC®-cHILIC (3 µm, 100Å) PEEK 150×2.1 mm	(1.50658.0001*)

Recommended solvents and reagents

Acetonitrile	Isocratic grade for HPLC LiChrosolv®	(1.14291)
Water	Water for chromatography LiChrosolv® or freshly purified water from Milli-Q® water purification system	(1.15333)
Potassium phosphate	for analysis EMSURE® ISO	(1.04873)

Recommended filtration tools

Mobile phase filtration:

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

*For more information on specifications and launch of SeQuant® ZIC®-cHILIC, please contact your Merck Millipore sales representative or visit www.merckmillipore.com/chromatography or www.sequant.com/zicchilic.

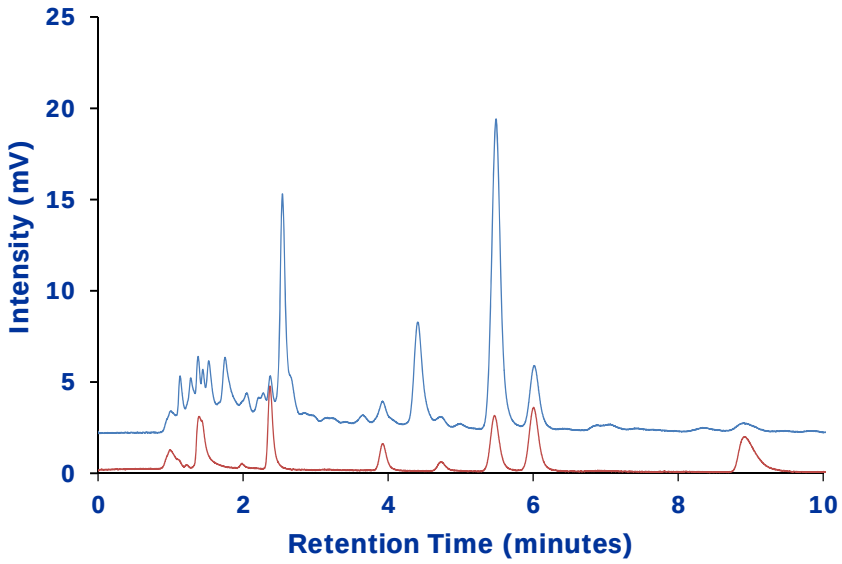
Determination of Organic Acids in wine

SeQuant® ZIC®-cHILIC

ZIC®-cHILIC coming soon

Chromatographic Conditions

Column:	SeQuant® ZIC®-cHILIC (3 µm, 100Å) PEEK 150x2.1 mm	(1.50658.0001*)
Injection:	5 µL	
Detection:	UV at 200 nm. Shimadzu LC-10Vp equipped with 2.5µL semi-micro flow-cell	
Flow Rate:	0.3 mL/min.	
Mobile Phase (v/v):	Acetonitrile and 25mM Potassium Phosphate buffer pH 6.0 (75:25)	
Temperature:	30 °C	
Diluent	Mobile phase	
Sample:	Riesling wine (blue), 10ppm mix of standards (red)	



Chromatographic Data

No.	Compound	Time (min)	Retention Factor
	Void volume (t0)	1	-
1	Acetic acid	2.4	1.4
2	Succinic acid	3.9	2.9
3	Malic acid	5.5	4.5
4	Tartaric acid	6.0	5.0
5	Citric acid	8.9	7.9

Merck Millipore is a division of Merck KGaA, Darmstadt, Germany
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chromatography@merckgroup.com