HPLC
for Food
Analysis

A Primer

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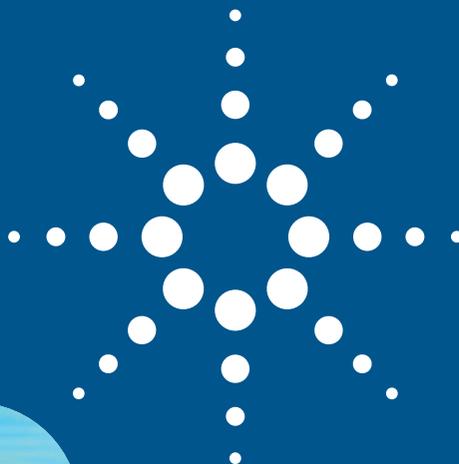
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HPLC
for Food
Analysis

A Primer

The fundamentals of an
alternative approach to
solving tomorrow's
measurement
challenges

Angelika
Gratzfeld-Hüsgen and
Rainer Schuster

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Mrs. Miller is an application
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Mr. Jaskowiak, who wrote chapter 7,
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chromatography products at
Agilent Technologies.

Preface

Modern agriculture and food processing often involve the use of chemicals. Some of these chemicals and their functions are listed below:

- Fertilizers: increase production of agricultural plants
- Pesticides: protect crops against weeds and pests
- Antibiotics: prevent bacteria growth in animals during breeding
- Hormones: accelerate animal growth
- Colorants: increase acceptability and appeal of food
- Preservatives and antioxidants: extend product life
- Natural and artificial sweeteners and flavors: improve the taste of food
- Natural and synthetic vitamins: increase the nutritive value of food
- Carbohydrates: act as food binders

Such chemicals improve productivity and thus increase competitiveness and profit margins. However, if the amounts consumed exceed certain limits, some of these chemicals may prove harmful to humans.

Most countries therefore have established official tolerance levels for chemical additives, residues and contaminants in food products. These regulations must be monitored carefully to ensure that the additives do not exceed the prescribed levels. To ensure compliance with these regulatory requirements, analytical methods have been developed to determine the nature and concentration of chemicals in food products. Monitoring of foodstuffs includes a check of both the raw materials and the end product. To protect consumers, public control agencies also analyze selected food samples.

High-performance liquid chromatography (HPLC) is used increasingly in the analysis of food samples to separate and detect additives and contaminants. This method breaks down complex mixtures into individual compounds, which in turn are identified and quantified by suitable detectors

and data handling systems. Because separation and detection occur at or slightly above ambient temperature, this method is ideally suited for compounds of limited thermal stability. The ability to inject large sample amounts (up to 1–2 ml per injection) makes HPLC a very sensitive analysis technique. HPLC and the nondestructive detection techniques also enable the collection of fractions for further analysis. In addition, modern sample preparation techniques such as solid-phase extraction and supercritical fluid extraction (SFE) permit high-sensitivity HPLC analysis in the ppt (parts per trillion) range. The different detection techniques enable not only highly sensitive but also highly selective analysis of compounds.
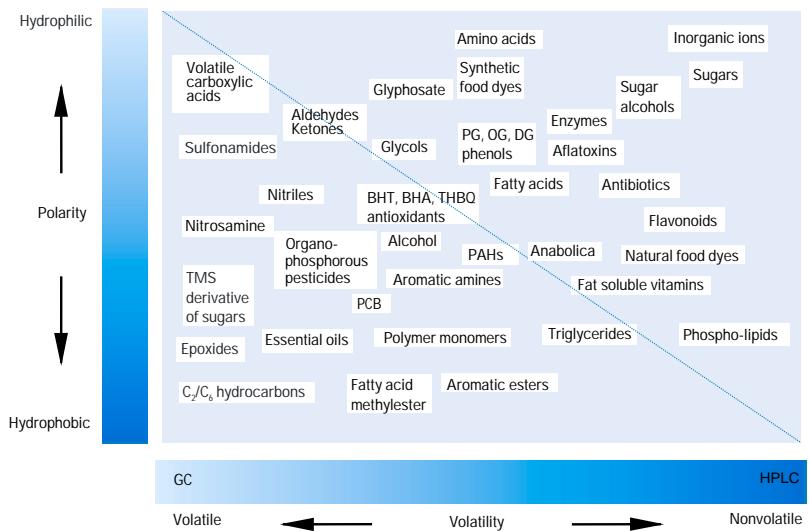
Figure 1
Match of analyte characteristics to carrier medium

Its selective detectors, together with its ability to connect a mass spectrometer (MS) for peak identification, make gas chromatography (GC) the most popular chromatographic method.

HPLC separates and detects at ambient temperatures. For this reason, agencies such as the U.S. Food and Drug Administration (FDA) have adopted and recommended HPLC for the analysis of thermally labile, nonvolatile, highly polar compounds.

Capillary electrophoresis (CE) is a relatively new but rapidly growing separation technique. It is not yet used in the routine analysis of food, however. Originally CE was applied primarily in the analysis of biological macromolecules, but it also has been used to separate amino acids, chiral drugs, vitamins, pesticides, inorganic ions, organic acids, dyes, and surfactants.[1, 2, 3]

Part 1 is a catalog of analyses of compounds in foods. Each section features individual chromatograms and suggests appropriate HPLC equipment. In addition, we list chromatographic parameters as well as the performance characteristics that you can expect using the methods shown. In part 2 we examine sample preparation and explain the principles behind the operation of each part of an HPLC system—sampling systems, pumps, and detectors—as well as instrument control and data evaluation stations. In the last of 11 chapters, we discuss the performance criteria for HPLC, which are critical for obtaining reliable and accurate results. Part 3 contains a bibliography and an index.

Contents

Chapter 8 Detectors

**The
HPLC
Approach**

Part One

**A demonstration
of liquid chromatographic
separations in
food analysis**

Chapter 1

Analytical examples
of food additives

Acidulants

Sorbic acid and citric acids are commonly used as acidulants[4] and/or as preservatives. Acetic, propionic, succinic, adipic, lactic, fumaric, malic, tartaric, and phosphoric acids can serve as acidulants as well. Acidulants are used for various purposes in modern food processing. For example, citric acid adds a fresh, acidic flavor, whereas succinic acid gives food a more salty, bitter taste. In addition to rendering foods more palatable and stimulating, acidulants act as

- flavoring agents to intensify certain tastes and mask undesirable aftertastes

- buffering agents to control the pH during food processing and of the finished products

- preservatives to prevent growth of microorganisms

- synergists to antioxidants to prevent rancidity and browning

- viscosity modifiers in baked goods

- melting modifiers in cheese spreads and hard candy

- meat curing agents to enhance color and flavor

Sample preparation

Sample preparation depends strongly on the matrix to be analyzed, but in general steam distillation and solid-phase extraction techniques can be used.

Chromatographic conditions

High-performance liquid chromatography (HPLC) with UV-visible diode-array detection (UV-DAD) has been applied in the analysis of citric acid in wine and in a vodka mixed drink. Retention time and spectral data were used as identification tools.

Water

Isocratic pump + vacuum degasser

Auto-sampler

Column compart-ment

Detector (VWD, DAD or refractive index)

Control and data evaluation
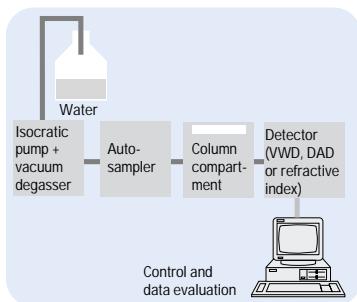
Sample preparation	filtration
Column	300 x 7.8 mm BioRad HPX 87-H, 9 µm
Mobile phase isocratic	0.0035 M H_2SO_4
Flow rate	0.6 ml/min
Column compartment	65 °C
Injection volume	10 µl
Detector	UV-VWD detection wavelength 192 nm or 210 nm
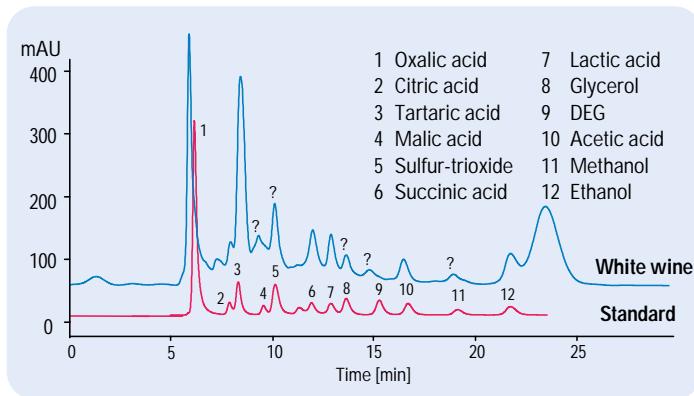
Figure 2
Analysis of acidulants in white wine

Conditions as above except	
Mobile phase isocratic	0.007 M H_2SO_4
Detector	UV-DAD

HPLC method performance

Limit of detection	100 ng injected amount, S/N = 2 equivalent to 2 ppm with 50 µl injected volume
Repeatability of RT over 10 runs	< 0.1 %
areas over 10 runs	< 3 %
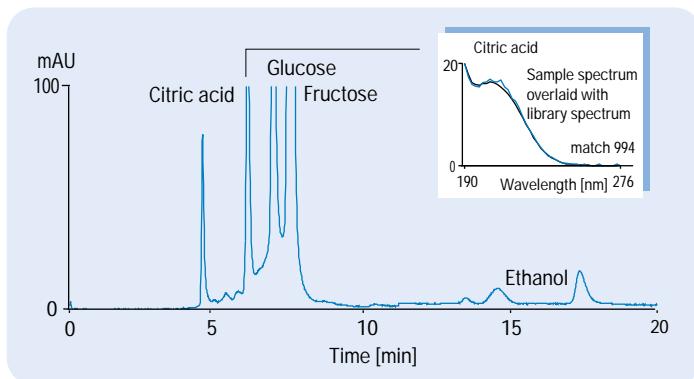
Figure 3
Analysis of citric acid in vodka

4. *Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants,* 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; Official Method AOAC 986.13: quinic, malic, citric acid in cranberry juice cocktail and apple juice.

Antioxidants

The following compounds are used as antioxidants in food products:[4]

Natural antioxidants:

- vitamin C
- vitamin E

Synthetic antioxidants:

- BHT   butylated hydroxytoluene
- BHA   butylated hydroxyanisole
- TBHQ   mono-tert-butylhydroquinone
- THBP   2,4,5-trihydroxybutyrophenone
- PG   propyl gallate
- OG   octyl gallate
- DG   dodecyl gallate
- Ionox-100   4-hydroxymethyl-2,6-di(tert-butyl)phenol
- NDGA   nordihydroguaiaretic acid
- TDPA   3,3'-thiodipropionic acid
- ACP   ascorbyl-palmitate

Antioxidants may be naturally present in food, or they may be formed by processes such as smoking. Examples of natural antioxidants include tocopherols (vitamin E) and acsorbic acid (vitamin C). A second category of antioxidants comprises the wholly synthetic antioxidants. When these antioxidants are added to foodstuffs, they retard the onset of rancidity by preventing the oxidative degradation of lipids. In most countries where antioxidants are permitted either singly or as combinations in foodstuffs, maximum levels for these compounds have been set.

Sample preparation

Sample preparation depends strongly on the matrix to be analyzed. For samples low in fat, liquid extraction with ultrasonic bath stimulation can be used. For samples with more complex matrices, solid-phase extraction, liquid/liquid extraction, or steam distillation may be necessary.

Chromatographic conditions

HPLC and UV-visible diode-array detection have been applied in the analysis of antioxidants in chewing gum. Spectral information and retention times were used for identification.

Sample preparation	ultrasonic liquid extraction with acetonitrile (ACN)
Column 1	100 x 4 mm BDS, 3 µm
Mobile phase	A = water + 0.2 ml H_2SO_4, pH = 2.54 B = ACN
Gradient	start with 10 % B at 3 min 60 % B at 4 min 80 % B at 11 min 90 % B
Flow rate	0.5 ml/min
Post time	4 min
Column compartment	30 °C
Injection volume	5 µl
Detector	UV-DAD detection wavelength 260/40 nm, reference wavelength 600/100 nm

HPLC method performance

Limit of detection	0.1–2 ng (injected amount), S/N = 2
Repeatability of RT over 10 runs	< 0.2 %
areas over 10 runs	< 1 %
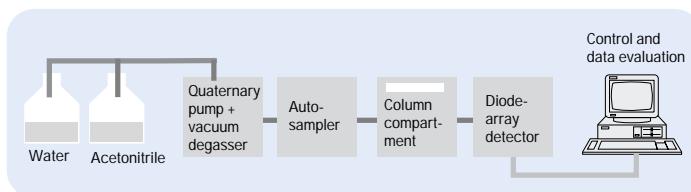
1 Vitamin C
2 PG
3 THBP
4 TBHQ
5 BHA
6 4-hydroxy
7 BHT
8 ACP

Chewing gum extract

Standard

Figure 4
Analysis of antioxidants in chewing gum

4. *Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants,* 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; AOAC Official Method 983.15: Antioxidants in oils and fats.

Preservatives

The following compounds are used as preservatives in food products:

• benzoic acid

• sorbic acid

• propionic acid

• methyl-, ethyl-, and propylesters of p-hydroxy benzoic acid (PHB-methyl, PHB-ethyl, and PHB-propyl, respectively)[4]

Preservatives inhibit microbial growth in foods and beverages. Various compound classes of preservatives are used, depending on the food product and the expected microorganism. PHBs are the most common preservatives in food products. In fruit juices, in addition to sulfur dioxide, sorbic and benzoic acid are used as preservatives, either individually or as a mixture.

Sample preparation

Sample preparation depends strongly on the matrix to be analyzed. For samples low in fat, liquid extraction with ultrasonic bath stimulation can be used. For samples with more complex matrices, solid-phase extraction, liquid/liquid extraction, or steam distillation may be necessary.
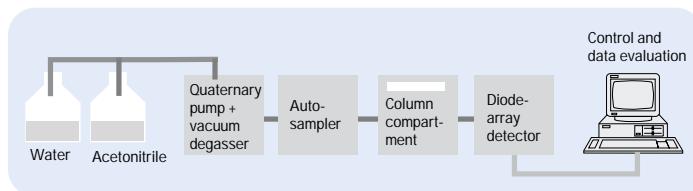
Chromatographic conditions

HPLC and UV-visible diode-array detection have been applied in the analysis of preservatives in white wine and salad dressing. Spectral information and retention times were used for identification.

Sample preparation	Carrez clearing and filtration for the salad dressing. None for white wine.
Column	125 x 4 mm Hypersil BDS, 5 µm
Mobile phase	A = water + 0.2 ml H_2SO_4, pH = 2.3 B = ACN
Gradient	start with 10 % B at 3 min 60 % B at 4 min 80 % B at 6 min 90 % B at 7 min 10 % B
Flow rate	2 ml/min
Post time	1 min
Column compartment	40 °C
Injection volume	2 µl
Detector	UV-DAD detection wavelength 260/40 nm

HPLC method performance

Limit of detection	10 ppm, S/N = 2
Repeatability of RT over 10 runs	< 0.1 %
areas over 10 runs	< 3 %
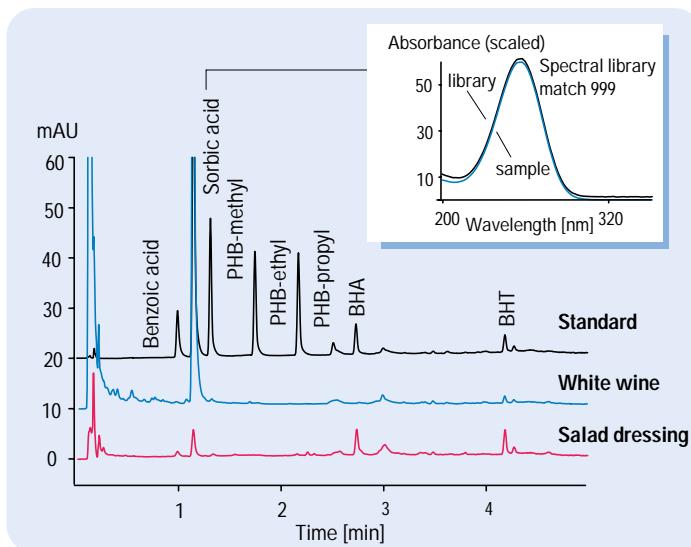
Figure 5
Analysis of preservatives in white wine and salad dressing

4. Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants, 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; AOAC Official Method 979.08: Benzoate, caffeine, saccharine in carbonated beverages.

Artificial sweeteners

The following compounds are used as artificial sweeteners in food products:

- acesulfam

- aspartame

- saccharin[4]

Nowadays, low-calorie sweeteners are widely used in foods and soft drinks. Investigations of the toxicity of these compounds have raised questions as to whether they are safe to consume. As a result, their concentration in foods and beverages is regulated through legislation in order to prevent excessive intake.

Sample preparation

Sample preparation depends strongly on the matrix to be analyzed. For sample low in fat, liquid extraction at low pH with ultrasonic bath stimulation can be used. For samples with more complex matrices, solid-phase extraction, liquid/liquid extraction, or steam distillation may be necessary.
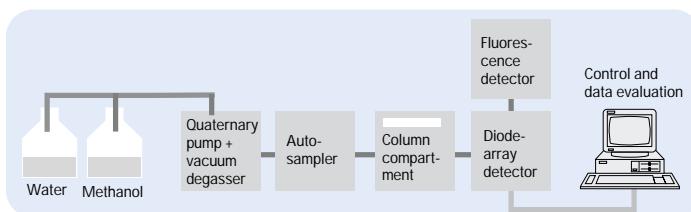
Chromatographic conditions

The HPLC method presented here for the analysis of aspartame is based on automated on-column derivatization and reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.[5]

Derivatization agent	o-phthalaldehyde (OPA) mercapto-propionic acid (MPA)
Column	100 x 2.1 mm Hypersil ODS, 5 µm
Mobile phase	A = 0.01 mM sodium acetate B = methanol
Gradient	start with 5 % B at 5 min 25 % B at 10 min 35 % B at 13 min 55 % B at 18 min 80 % B at 20 min 95 % B
Flow rate	0.35 ml/min
Post time	5 min
Column compartment	40 °C
Injection volume	1 µl
Injector program for online derivatization	
1. Draw 5.0 µl from vial 3 (borate buffer)	
2. Draw 0.0 µl from vial 0 (water)	
3. Draw 1.0 µl from vial 1 (OPA/MPA)	
4. Draw 0.0 µl from vial 0 (water)	
5. Draw 1.0 µl from sample	
6. Mix 7 µl (6 cycles)	
7. Inject	
Detectors	
UV-DAD:	detection wavelength 338/20 nm or
fluorescence:	excitation wavelength 230 nm, emission wavelength 445 nm
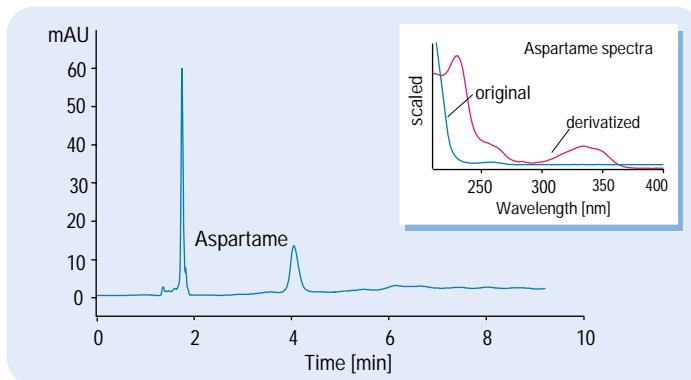
Figure 6
Chromatogram and spectra of derivatized and non derivatized aspartame

HPLC method performance

Limit of detection	
for fluorescence	200 pg (injected amount), S/N = 2
for DAD	1 ng (injected amount), S/N = 2
Repeatability	
of RT over 10 runs	< 0.1 %
of areas over 10 runs	< 5 %

5. A.M. Di Pietra et al., "HPLC analysis of aspartame and saccharin in pharmaceutical and dietary formulations"; *Chromatographia*, **1990**, *30*, 215–219.
4. *Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants*, 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; Official Method *AOAC 979.08: Benzoate, caffeine, saccharin in soda beverages.*

Colorants

We have selected the food color E104 Quinolin yellow and E131 Patent blue as application examples. Synthetic colors are widely used in the food processing, pharmaceutical, and chemical industries for the following purposes:[4]

• to mask decay

• to redye food

• to mask the effects of aging

The regulation of colors and the need for quality control requirements for traces of starting product and by-products have forced the development of analytical methods. Nowadays, HPLC methods used are based on either ion-pairing reversed-phase or ion-exchange chromatography.
UV absorption is the preferred detection method. The UV absorption maxima of colors are highly characteristic. Maxima start at approximately 400 nm for yellow colors, 500 nm for red colors, and 600–700 nm for green, blue, and black colors. For the analysis of all colors at maximum sensitivity and selectivity, the light output from the detector lamp should be high for the entire wavelength range. However, this analysis is not possible with conventional UV-visible detectors based on a one-lamp design. Therefore, we have chosen a dual-lamp design based on one deuterium and one tungsten lamp. This design ensures high light output for the entire wavelength range.

Sample preparation

Whereas turbid samples require filtration, solid samples must be treated with 0.1 % ammonia in a 50 % ethanol and water mixture, followed by centrifugation. Extraction is then performed using the so-called wool-fiber method. After desorption of the colors and filtration, the solution can be injected directly into the HPLC instrument.

Water   Acetonitrile

Quaternary pump + vacuum degasser — Auto-sampler — Column compartment — Diode-array detector

Control and data evaluation
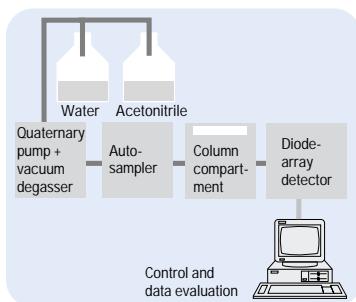
Chromatographic conditions

The HPLC method presented here for the analysis of dyes is based on ion-pairing reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.[6]

Sample preparation	injection without further preparation
Column	125 x 3 µm Hypersil BDS, 3 mm
Mobile phase	A = 0.01 M NaH_2PO_4 + 0.001 M tetrabutyl-ammoniumdihydrogen-phosphate, pH = 4.2 B = ACN
Gradient	start with 15 % in 10 min to 40 % in 14 min to 90 % until 19 min at 90 % in 20 min to 15 % ACN
Stop time	20 min
Post time	4 min
Flow rate	0.8 ml/min
Column compartment	40 °C
Injection volume	1 µl
Detector	UV-DAD signal A: 254/50 nm (for optimization of separation) signal B: 350/20 nm signal C: 465/30 nm signal D: 600/40 nm

HPLC method performance

Limit of detection for UV-DAD	2 ng (injected amount) S/N = 2
Repeatability	
of RT over 10 runs	< 0.2 %
of areas over 10 runs	< 3 %
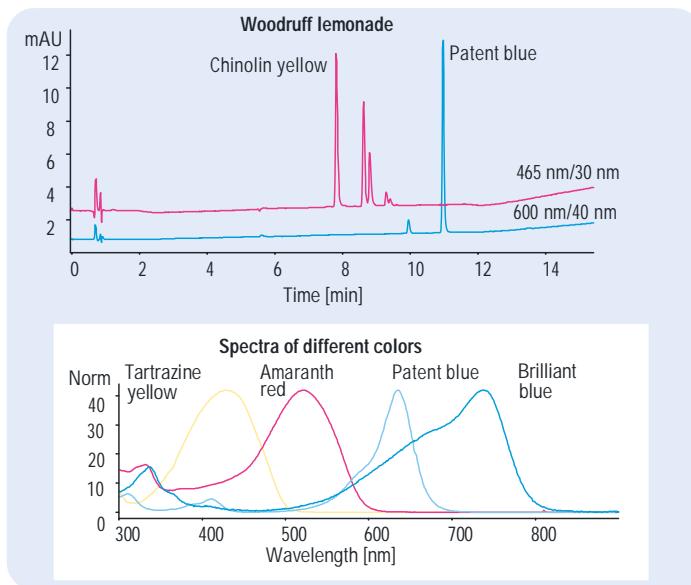
Figure 7
Analysis of synthetic colors in lemonade. Overlay of spectra of yellow, red, blue and "black" colors

4. *Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants,* 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; Official Method AOAC 981.13: Cresidine sulfonic acid in FD&C Red No. 40; Official Method AOAC 982.28: Intermediates and reaction by-products in FD&Y Yellow No. 5; Official Method AOAC 977.23: 44' (Diazoamino) dibenzene sulfonic acid (DAADBSA) in FD&C Yellow No. 6; Official Method AOAC 980.24: Sulfanilic acid in FD&C Yellow No. 6.
6. A.G. Huesgen, R.Schuster, "Sensitive analysis of synthetic colors using HPLC and diode-array detection at 190–950 nm", *Agilent Application Note 5964-3559E,* **1995**.

Flavors

The following compounds are examples of flavoring agents used in food products:

• lupulon and humulon (hop bittering compounds)

• vanillin

• naringenin and hesperidin (bittering compounds)

Three major classes of compounds are used as flavoring agents: essential oils, bitter compounds, and pungency compounds. Although the resolution afforded by gas chromatography (GC) for the separation of flavor compounds remains unsurpassed, HPLC is the method of choice if the compound to be analyzed is low volatile or thermally unstable.

Vanillin

Sample preparation

Turbid samples require filtration, whereas solid samples must be extracted with ethanol. After filtration, the solution can be injected directly into the HPLC instrument.

Control and
data evaluation

Quaternary
pump +
vacuum
degasser

Auto-
sampler

Column
compart-
ment

Diode-
array
detector

Water   Acetonitrile
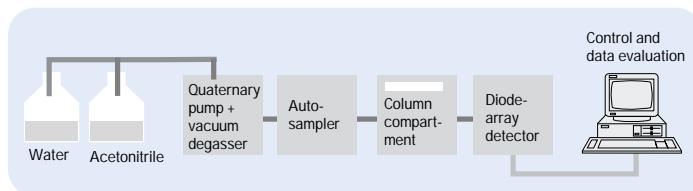
Sample preparation	injection without further preparation
Column	100 x 4 mm Hypersil BDS, 3 µm
Mobile phase	A = water + 0.15 ml H_2SO_4 (conc.), pH = 2.3 B = ACN
Gradient	start with 10 % B at 3 min 40 % B at 4 min 40 % B at 6 min 80 % B at 7 min 90 % B
Flow rate	0.8 ml/min
Post time	3 min
Column compartment	30 °C
Injection volume	5 µl
Detector	UV-DAD detection wavelength 280/80 nm, reference wavelength 360/100 nm

Chromatographic conditions

The HPLC method presented here for the analysis of vanillin is based on reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.[7]
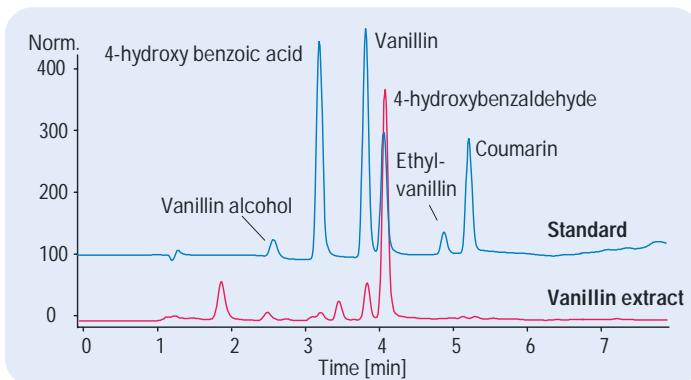
Figure 8
Determination of the quality of vanillin extract

Conditions as above, except	
Column	100 x 2.1 mm Hypersil ODS, 5 µm
Mobile phase	A = water + 5 mM NaH_2PO_4 B = methanol
Gradient	at 10 min 70 % B
Flow rate	0.4 ml/min

HPLC method performance	
Limit of detection	0.2–5 ng (injected amount) S/N = 2
Repeatability of RT over 10 runs	< 0.2 %
of areas over 10 runs	< 1 %
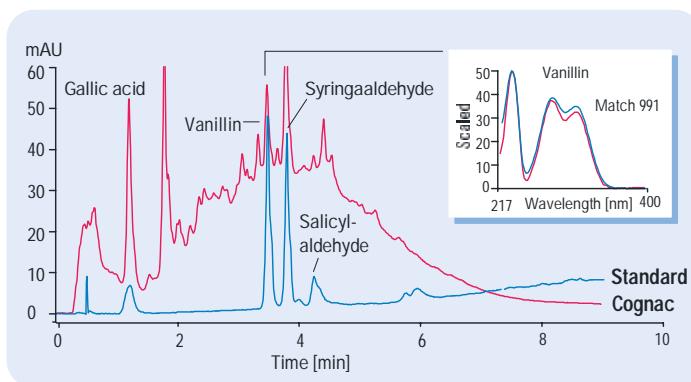
Figure 9
Analysis of vanillin in cognac. Identification of vanillin through spectra comparison

7. Herrmann, A, et al.;,"Rapid control of vanilla-containing products using HPLC"; *J. Chromatogr.*, **1982**, 246, 313–316.

Bitter compounds: hesperidin and naringenin

Sample preparation for bitter compounds in orange juice[8]

The samples were prepared according to Carrez 1 and 2. This method uses potassium ferrocyanide and zinc sulfate for protein precipitation.

Chromatographic conditions

The HPLC method presented here for the analysis of hesperidin and naringenin is based on reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.

Sample preparation	The orange juice was prepared according to Carrez 1 and 2.
Column	125 x 4 mm Hypersil BDS, 5 µm
Mobile phase	A = water + 0.15 ml/l H_2SO_4 (conc.), pH = 2.4 B = ACN
Gradient	start with 20 % B at 3 min 20 % B at 5 min 90 % B at 6 min 20 % B
Flow rate	2 ml/min
Post time	1 min
Column compartment	40 °C
Injection volume	1 µl
Detector	UV-DAD detection wavelength 260/80 nm, reference wavelength 380/80 nm

HPLC method performance

Limit of detection for DAD	1 ng (injected amount), S/N = 2
Repeatability	
of RT over 10 runs	< 0.2 %
of areas over 10 runs	< 1 %.
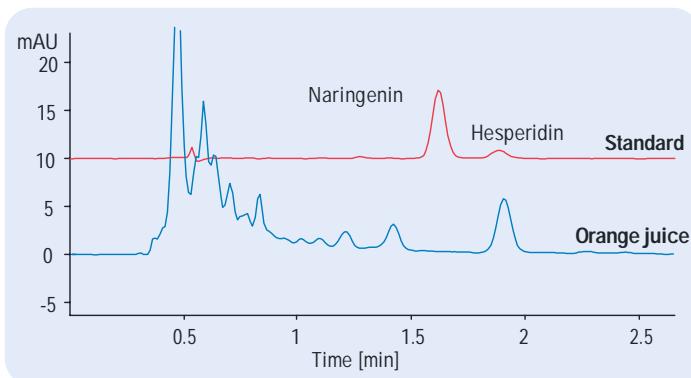
Figure 10
Analysis of bitter compounds in orange juice

8. *Official Methods of Analysis*; Horwitz, W., Ed.; 14th ed.; AOAC: Arlington, VA, **1984**; secs 12.018–12.021.

Chapter 2

**Analytical examples
of residues
and contaminants**
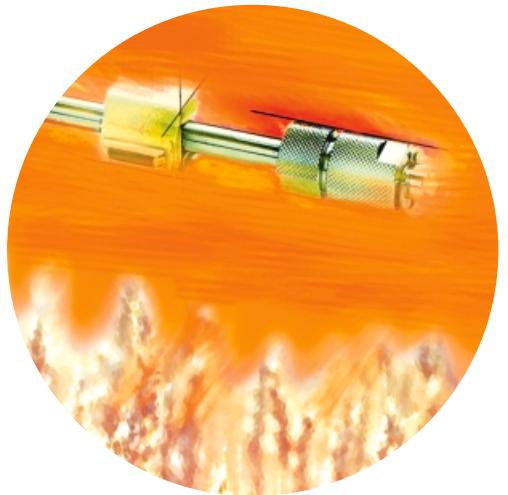
Residues of chemotherapeutics and antiparasitic drugs

In addition to several other drugs, nitrofurans and sulfonamides such as sulfapyridine, N-acetyl metabolite, ethopabat, chloramphenicol, meticlorpindol, metronidazol, ipronidazol, furazolidone, and nicarbazin are frequently fed to domestic cattle.

Modern intensive animal breeding demands permanent suppression of diseases caused by viruses, bacteria, protozoa, and/or fungi. A number of chemotherapeutics are available for the prevention and control of these diseases. After application, residues of these drugs can be found in foods of animal origin such as milk, eggs, and meat. These chemotherapeutics can cause resistancy of bacteria. Because of the toxic nature of chemotherapeutics, for example, choramphenical, government agencies in many countries, including the United States, Germany, and Japan, have set tolerance levels for residues of these drugs.

Simple and reliable analysis methods are necessary in order to detect and quantify residues of chemotherapeutic and antiparasitic drugs in food products. Malisch et al. have developed an HPLC method to determine 11 of these compounds.[9,10] The internal standard (ISTD) comprises benzothiazuron and pyrazon.

Sample preparation

After homogenization or mincing and pH adjustment, the samples were extracted using liquid/liquid extraction followed by degreasing, purification, and concentration.
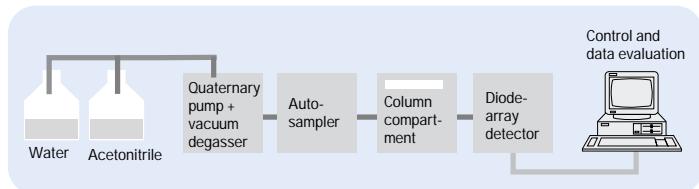
Sample preparation	Sample preparation was done according to reference[9]
Column	250 x 4.6 mm Spherisorb ODS-2, 5 µm
Mobile phase	A = sodium acetate buffer, 0.02 M, pH = 4.8 B = ACN/water (60:40)
Gradient	start with 8 % B at 5 min 8 % B at 7 min 20 % B at 14 min 23 % B at 16 min 33 % B at 19 min 40 % B at 21 min 50 % B at 26 min 60 % B at 30 min 80 % B at 33 min 90 % B at 43 min 90 % B at 55 min 8 % B
Flow rate	1.5 ml/min
Injection volume	20 µl
Detector	UV-DAD detection wavelengths 275/80 nm, 315/80 nm, and 360/80 nm, reference wavelength 500/100 nm

HPLC method performance

Limit of detection	0.001–0.05 mg/kg
Repeatability	
of RT over 10 runs	< 0.12 %
of areas over 10 runs	< 1.5 %

Chromatographic conditions

The HPLC method presented here for the analysis of residues of drugs in eggs, milk, and meat is based on reversed-phase chromatography and multisignal UV-visible diode-array detection (UV-DAD). UV spectra were evaluated as an additional identification tool.

1 metronidazol
2 meticlorpindol
3 sulfapyridine
4 furazolidone
5 pyrazon
6 ipronidazol
7 chloramphenicol
8 N-acetyl metabolite of 3
9 3-ethopabat
10 benzothiazuron
11 nicarbazin
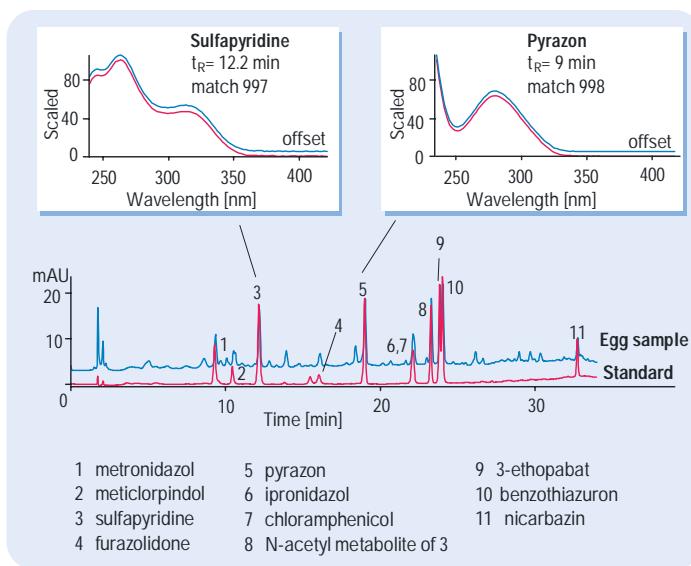
Figure 11
Analysis of residues in an egg sample. Identification through spectra comparison

9. H. Malisch, et al., "Determination of residues of chemotherapeutic and antiparasitic drugs in food stuffs of anomaly origin with HPLC and UV-Vis diode-array detection", *J. Liq. Chromatogr.*, **1988**, 11 (13), 2801–2827.14.
10. EC Guideline 86/428 EWG **1985**.

Tetracyclines

Tetracyclines are used worldwide as oral or parenteral medication in the form of additives in animal feed. In food-producing animals, these drugs exhibit a high degree of activity toward a wide range of bacteria.[9, 11]

Sample preparation

After homogenization or mincing and addition of mineral acids to dissociate tetracyclines from proteins, the samples were extracted using liquid/liquid extraction followed by degreasing and/or deproteinization, purification, and concentration.[12]

Chromatographic conditions

The HPLC method presented here for the analysis of meat is based on reversed-phase chromatography and UV-visible diode-array detection. UV spectra were evaluated as an additional identification tool.

Sample preparation	1 g sample was mixed with citric acid (100 mg).
	➜ add 1 ml nitric acid (30 %) or 0.1 m oxalic acid
	➜ add 4 ml methanol 5 min in the ultrasonic bath
	➜ add water up to 10 ml total volume
	➜ centrifuge
	➜ inject
Column	100 × 4 mm Hypersil BDS, 3 µm
Mobile phase	A = water, pH = 2.1 with sulfuric acid B = ACN
Gradient	start with 15 % B at 10 min 60 % B
Flow rate:	0.5 ml/min
Column compartment	25 ºC
Detector	UV-DAD detection wavelength 355 nm/20 nm, reference wavelength 600/100 nm

HPLC method performance

Limit of detection for UV-DAD	100 ppb
Repeatability	
of RT over 10 runs	< 0.2 %
of areas over 10 runs	< 2 %
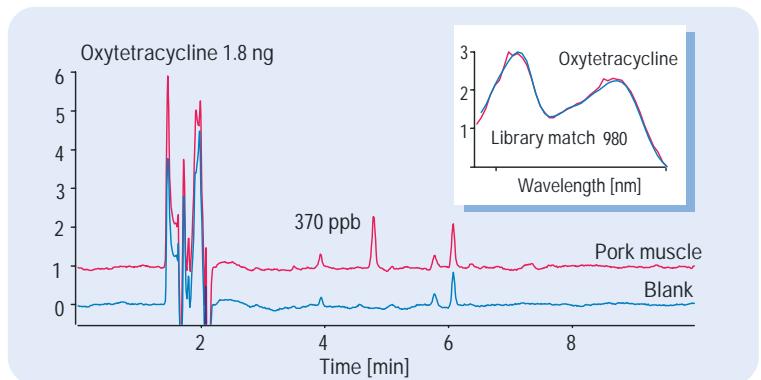
Figure 12
Trace analysis of tetracycline residues in meat. Identication of oxytetracycline through spectra comparison

9. H. Malisch et al., "Determination of residues of chemotherapeutic and antiparasitic drugs in food stuffs of anomaly origin with HPLC and UV-Vis diode-array detection" *J. Liq. Chromatogr.*, **1988**, 11 (13), 2801–2827.14.
11. M.H. Thomas, *J. Assoc. Off. Anal.;* **1989** , 72 (4) 564.
12. Farrington et. al., "Food Additives and Contaminants, **1991**, Vol. 8, No. 1, 55-64".

Fumonisins

Fumonisins are characterized by a 19-carbon aminopoly-hydroxyalkyl chain which is diesterified with propane-1,2,3-tricarboxylic acid. Analogues B 1-3 in figure 13 show a difference only in the number and position of the hydroxyl groups present on the molecule.

Fragmentation experiments using collision induced disso-ciation (CID) show no difference between fumonisins B2 and B3. Consequently, it was necessary to separate these compounds chromatographically for quantitative analysis. However, in crude corn extracts the CID-fragment ions provide important confirmatory information. In order to obtain spectra of the fragment ions as well as the pseudo-molecular ions in a single scan, operating at maximum sensitivity, the fragmentor voltage was set to 230 V while scanning from 150 amu to 680 amu and then to 100 V when scanning from 690 amu to 800 amu.

Sample preparation

Extraction according to § 35, LMBG.[13]

Chromatographic conditions

The Agilent 1100 Series LC/MSD proved to be capable of detecting and quantifying fumonisins at 250 picograms per component regardless of their chemical structure and without the need for derivatization during the sample preparation procedure. The Agilent 1100 Series LC/MSD provided optimum sensitivity in the selected ion monitor-ing mode. Even when operating in scan mode (150 amu to 800 amu), the Agilent 1100 Series LC/MSD still provided sensitivity more than a factor of 10 better than reported for a fluorescence detector.

LC/MS conditions

Colum	Zorbax Eclipse XDB-C18, 2.1 mm x 150 mm, 5 µm
Mobile phase A	5 mM ammonium acetate pH3
Mobile phase B	acetonitrile
Gradient	0 min 33% B
	8 min 60% B
	9 min 33% B
Flow rate	250 µl/min
Injection vollume	5 µl
Column compartment	40°C
Ionization mode	API-ES positive or APCI negative
Nebulizer pressure	30 psig
Dryng gas temp.	350°C
Drying gas flow	6 l/min
Vcap.	4000 volts
Fragmentor	100 volts
Scan range	m/z 120 –820
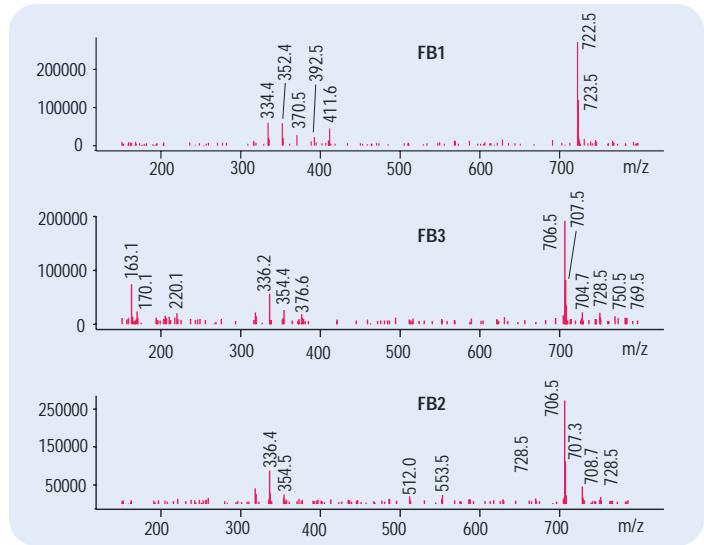
Figure 13
Mass spectra of Fumonisins B 1,2,3 when the fragmentor is ramped from 230 to 100V
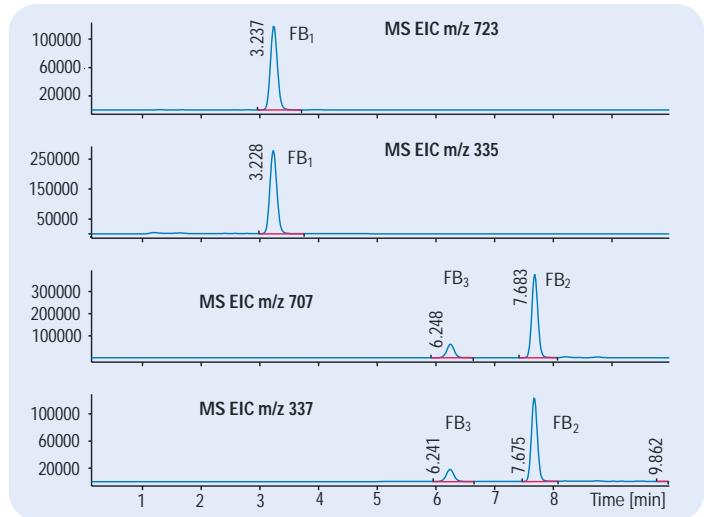
Figure 14
Identification of different Fumonisin species in corn extract by retention time with further confirmation through fragment ion

13. Lebensmittel- und Bedarfsgegenständegesetz, Paragraph 35, Germany.

Mycotoxins

The following mycotoxins have been analyzed: aflatoxins G_2, G_1, B_2, B_1, M_2, and M_1; ochratoxin A; zearalenone; and patuline.

Mycotoxins are highly toxic compounds produced by fungi. They can contaminate food products when storage conditions are favorable to fungal growth. These toxins are of relatively high molecular weight and contain one or more oxygenated alicyclic rings. The analysis of individual mycotoxins and their metabolites is difficult because more than 100 such compounds are known, and any individual toxin is likely to be present in minute concentration in a highly complex organic matrix. Most mycotoxins are assayed with thin-layer chromatography (TLC). However, the higher separation power and shorter analysis time of HPLC has resulted in the increased use of this method. The required detection in the low parts per billion (ppb) range [4,13] can be performed using suitable sample enrichment and sensitive detection.

Sample preparation

Samples were prepared according to official methods.[13] Different sample preparation and HPLC separation conditions must be used for the different classes of compounds. The table on the next page gives an overview of the conditions for the analysis of mycotoxins in foodstuffs.

Chromatographic conditions

The HPLC method presented here for the analysis of myc-otoxins in nuts, spices, animal feed, milk, cereals, flour, figs, and apples is based on reversed-phase chromatography, multisignal UV-visible diode-array detection, and fluores-cence detection. UV spectra were evaluated as an additional identification tool.

Column class	Matrix	Sample preparation	Chromatographic conditions
Aflatoxins G_2, G_1, B_2, B_1, M_2, M_1	nuts, spices, animal feed, milk, dairy products	⇨ extraction according to Para. 35, LMBG*[8,12]	Hypersil ODS, 100×2.1 mm id, 3-μm particles water/methanol/ACN (63:26:11) as isocratic mixture** flow rate: 0.3 ml/min at 25 °C DAD: 365/20 nm Fluorescence detector (FLD): excitation wavelength 365 nm, emission wavelength 455 nm
Ochratoxin A	cereals, flour, figs	⇨ extraction according to Para. 35, LMBG ⇨ acidify with HCl ⇨ extract with toluene ⇨ SiO_2 cleanup elute toluene/acetic acid (9:1)	Lichrospher 100 RP18, 125×4 mm id, 5-μm particles water with 2 % acetic acid/ACN (1:1)* flow rate: 1 ml/min at 40 °C FLD: excitation wavelength 347 nm, emission wavelength 480 nm
Zearalenone	cereals	⇨ extract with toluene ⇨ Sep-pak cleanup ⇨ elute toluene/acetone (95:5) ⇨ AOAC 985.18:4 α-zearalenol and zearalenone in corn	Hypersil ODS, 100×2.1 mm id, 3 μm particles water/methanol/ACN (5:4:1) isocratic mixture* flow rate: 0.45 ml/min at 45 °C DAD: 236/20 nm FLD: excitation wavelength 236 nm, emission wavelength 464 nm
Patuline	apple products	⇨ cleanup on Extrelut ⇨ silica gel cleanup ⇨ elute toluene/ethylacetate (3:1)	Superspher RP18, 125×4 mm id, 4-μm particles water 5 %–95 % ACN flow rate: 0.6 ml/min at 40 °C DAD: 270/20 nm or Lichrospher diol, 125×4 mm id, 5-μm particles hexane/isopropanol (95:5) as isocratic mixture flow rate: 0.6 ml/min at 30 °C DAD: 270/20 nm

* Lebensmittel- und Bedarfsgegenständegesetz, Germany
** 100 % B is recommended for cleaning the column

HPLC method performance

Limit of detection	1–5 µg/kg
Repeatability	
of RT over 10 runs	< 0.12 %
of areas over 10 runs	< 1.5 %
Linearity	
of UV-visible DAD	1–500 ng
of fluorescence	30 pg to 2 ng

FLD: λ_{em} 365 nm λ_{ex} 455 nm

DAD: 365 nm

M$_2$ 5 ng   G$_1$ 5 ng   B$_1$ 5 ng
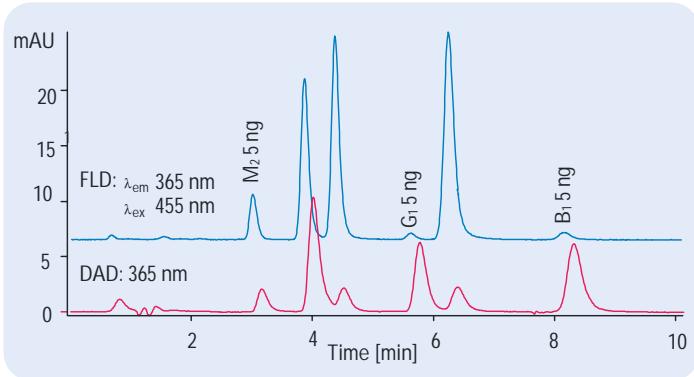
Figure 15
Analysis of aflatoxins with UV and fluorescence detection

Pistachio nut

FLD

DAD
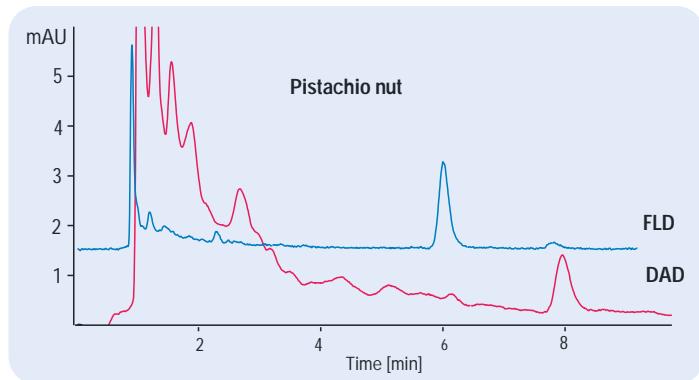
Figure 16
Analysis of aflatoxins in pistachio nuts with UV and fluorescence detection

Water   Methanol

Quaternary pump + vacuum degasser

Auto-sampler

Column compart-ment

Diode-array detector

Fluores-cence detector

Control and data evaluation
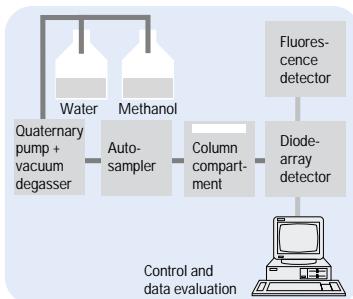
13. Lebensmittel- und Bedarfsgegenständegesetz, Paragraph 35, Germany.
4. *Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants*, 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; AOAC Official Method 980.20: aflatoxins in cotton seed products; AOAC Official Method 986.16: Aflatoxins M$_1$, M$_2$ in fluid milk; AOAC Official Method 985.18: α-zearalenol.

Bisphenol A diglycidyl-ether (BADGE)

Bisphenol A diglycidyl-ether (BADGE) is present in the three most common coatings (epoxy lacquer, organosol lacquer and polyester lacquer) used to protect the inside surfaces of cans used for food packaging. In canned foods containing a high proportion of fat, BADGE tends to migrate into the fatty phase where it remains stable, whereas in water it is hydrolyzed.

BADGE was originally determined to be mutagenic during in vitro tests but a later re-assessment, using in vivo tests, led to a different conclusion. While further tests are being performed, a maximum concentration of 1 mg BADGE per kg of food has been agreed.

Sample preparation

Extracted with water/alcohol 50/50 or n-heptane at reflux temperature for six hours.

Chromatographic conditions

A fast separation was developed by using the enhanced specificity provided by the Agilent 1100 Series LC/MSD in CID (collision induced dissociation) mode allowing the detection of BADGE via the molecular ion combined with confirmation using the most abundant fragment ion.

LC/MS conditions

Colum	Zorbax Eclipse XDB-C8, 2.1 mm x 50 mm, 5 μ
Mobile phase A	5 mM ammonium acetate in water, pH3
Mobile phase B	acetonitrile
Gradient	0 min 25% B
	5 min 50 % B
Flow rate	300 μl/min
Injection volume	1 μl
Column compartment	40 °C
Detector	UV-DAD
	210 nm/6 nm, ref. 360/60 nm
	254 nm/6 nm, ref. 360/60 nm

Ionization mode	API-ES positive
Nebulizer pressure	50 psig
Dryng gas temp.	350 °C
Vcap.	3500 volts
Fragmentor	70 volts
Scan range	m/z 250 –400
Scan speed	2 s/scan
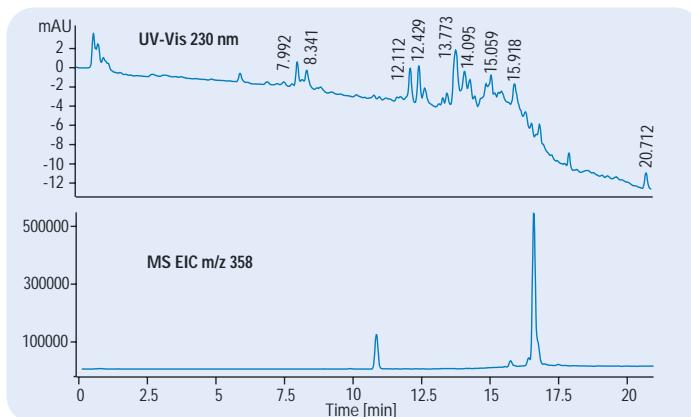
Figure 17
Extract from tuna 0.2 ppm, 1 μl injected
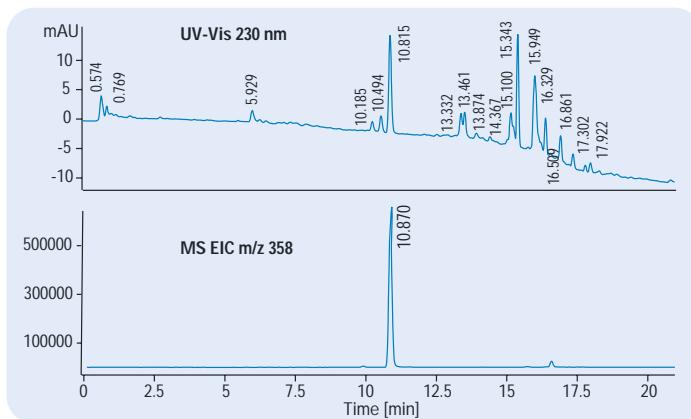
Figure 18
Extract from sardine 20 ppm, 1 μl injected

Pesticides

The following compound classes of pesticides have been analyzed: triazines, phenylurea-herbicides, methabenzthiazuron, diquat, paraquat, and mercaptobenzothiazol. Carbamates and glyphosate also have been analyzed but with different equipment. In most countries, growing concern about the residues of pesticides in food products is evident. Therefore, regulations limiting the concentration of pesticides in foodstuffs have been introduced to protect consumers from contaminated food products. Several methods are used to control these limits. HPLC is recommended for the analysis of low volatile compounds and for compounds that are unstable when heated.

Sample preparation

Sample preparation and enrichment depend strongly on the matrix. Drinking water samples, for example, must be extracted using solid-phase extraction, whereas vegetables are extracted with liquid/liquid extraction after homogenization, followed by additional cleaning and sample enrichment.
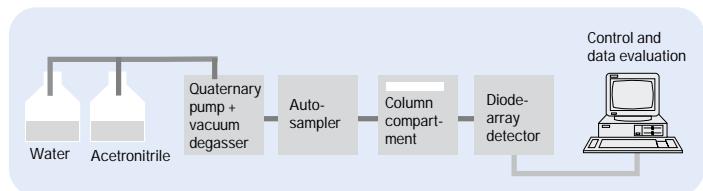
14. Specht, W. "Organochlor- und Organophosphor-Verbindungen sowie stickstoffhaltige sowie andere Pflanzenschutzmittel", DFG-Methodensammlung, **1982**, 19.

Chromatographic conditions

The HPLC method presented here was used for the analysis of pesticides in salad samples and spices.

Sample preparation	Salad was homogenized and then extracted with liquid/liquid extraction. The extract was cleaned with gel permeation chromatography using cyclohexane/ethyl acetate. Spices were prepared according to Specht[14] with gel permeation chromatography.
Column	100 × 3 mm Hypersil BDS, 3 µm
Mobile phase	water/ACN (95:5)
Gradient	at 10 min 25 % ACN at 26 min 42 % ACN at 34 min 60 % ACN
Flushing time	10 min at 100 % ACN
Post time	6 min
Flow rate	0.5 ml/min
Oven temperature	42 °C
Injection volume	3–10 µl
Detector	UV-DAD detection wavelengths 214/15 nm, 230/20 nm, and 245/20 nm reference wavelength 400/80 nm

HPLC method performance

Limit of detection	0.01 µg/l
Repeatability	
of RT over 10 runs	< 0.2 %
of areas over 10 runs	< 1 %
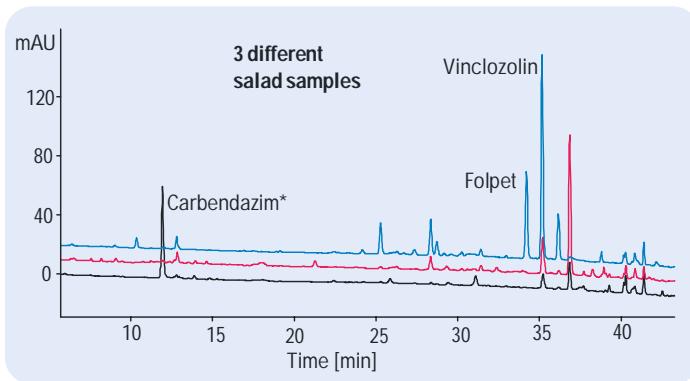
Figure 19

Analysis of pesticide residues in three different salad samples
* Carbendazim has a low recovery rate of only approximately 40 %
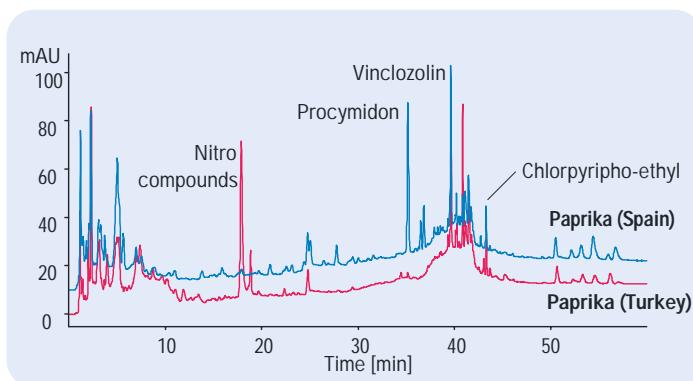
Figure 20

Analysis of pesticide residues in two paprika samples

Carbamates

Chromatographic conditions

The HPLC method presented here was used for the direct analysis of carbamates in water with postcolumn derivatization.[15] Fruits and vegetables must be extracted at neutral pH with water prior to HPLC analysis.

Sample preparation	none
Column	250 x 4 mm C18 phase from Pickering, 5 µm
Mobile phase	water/methanol (MeOH, 88:12)
Gradient	at 2 min 12 % MeOH
	at 42 min 66 % MeOH
	at 46 min 66 % MeOH
	at 46.1 min 100 % MeOH
	at 49 min 100 % MeOH
Flow rate	0.8 ml/min
Column compartment	37 °C
Injection volume	10 µl standard
Fluorescence detector	
Excitation wavelength: 230 nm or 330 nm	
Emission wavelength: 425 nm	
Photomultiplier gain: 12	
Response time: 4 s	
Derivatization reagent pump	
flow rate for hydrolization agent: 0.3 ml/min (NaOH)	
flow rate for derivatization agent: 0.3 ml/min (OPA)	

HPLC method performance

Limit of detection	100 ppt, S/N = 2
Repeatability	
of RT over 10 runs	< 0.1 %
of areas over 10 runs	< 0.5–5 %

Sample A

1	butocarboxim sulfoxide	9	ethiofencarb sulfone
2	aldicarb sulfoxide	14	butocarboxim
3	butoncarboxim sulfone	15	aldicarb
4	aldicarb sulfone	17	propoxur
6	methomyl	19	carbaryl
7	ethiofencarb sulfoxide	20	ethiofencarb

Sample B

5	oxamyl	16	3-ketocarbofuran
8	thiofanox sulfoxide	18	carbofuran
10	thiofanox sulfone	21	1-naphthol
11	3-hydroxycarbofuran	22	thiofanox
12	methiocarb sulfoxide	23	methiocarb
13	methiocarb sulfone		

Figure 21
Analysis of two different carbamate standards

15. "A new approach to lower limits of detection and easy spectral analysis" *Agilent Primer 5968-9346E*, **2000**

Glyphosate

Chromatographic conditions

The HPLC method presented here was used for the direct analysis of glyphosate in water with postcolumn derivatization.[16]

Sample preparation	none
Column	150 x 4 mm cation exchange, K + form from Pickering, 8 µm
Mobile phase	A = 5 mM KH_2PO_4, pH = 2.0
	B = 5 mM KOH
Flow rate	0.4 ml/min
Gradient	at 15 min 0 % B
	at 17 min 100 % B
Column compartment	55 °C
Injection volume	50 µl standard
Fluorescence detector	
Excitation wavelength: 230 nm or 330 nm	
Emission wavelength: 425 nm	
Photomultiplier gain: 12	
Response time: 4 s	
Derivatization reagent pump	
flow rate for hydrolization agent: 0.3 ml/min (OCl*)	
flow rate for derivatization agent: 0.3 ml/min (OPA)	

HPLC method performance

Limit of detection	500 ppt
Repeatability	
of RT over 10 runs	< 0.8 %
of areas over 10 runs	< 2.2 %
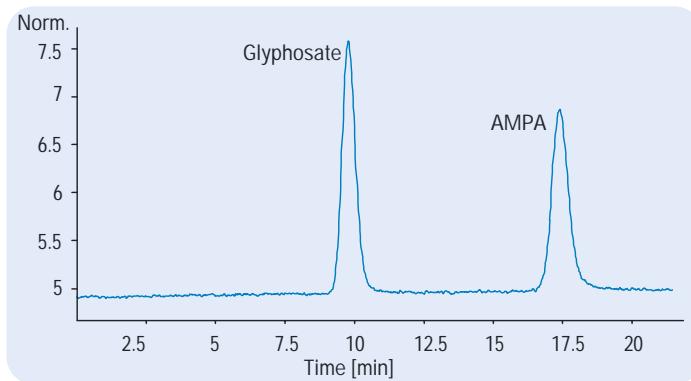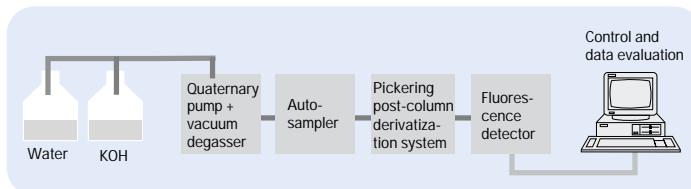
Figure 22
Analysis of glyphosate standard

16. R. Schuster, "A comparison of pre- and post-column sample treatment for the analysis of glyphosate", *Agilent Application Note 5091-3621E*, **1992**.

Chapter 3

**Analytical
examples of
natural
components**

Inorganic anions

Anions containing halogen, nitrogen, and sulfur are used as additives in food industries. For example, nitrites act as preservatives in smoked sausage. Nowadays, dedicated instrumentation such as special columns and electro-conductivity detectors are used in the analysis of inorganic anions. Because specialized equipment has a very limited application range, a method was developed for analyzing anionsusing reversed-phase chromatography and indirect UV detection. Another, more selective and sensitive approach for the analysis of selected anions is electro-chemical detection.

Sample preparation

Excepting filtration, sample preparation normally is unnecessary if the sample is aqueous. Other matrices can be extracted with hot water, followed by filtration.
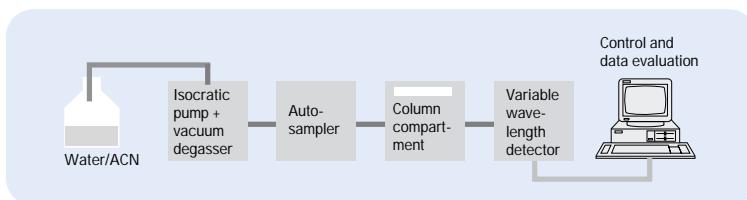
Water/ACN → Isocratic pump + vacuum degasser → Auto-sampler → Column compartment → Variable wavelength detector → Control and data evaluation

Chromatographic conditions

The HPLC method presented here was used for the analysis of anions in drinking water.

Sample preparation	filtration
Column	HP-IC (modifiers for the mobile phase are included)
Mobile phase	water/acetonitrile (ACN) (86:14), adjusted to pH = 8.6 with carbonate-free NaOH
Flow rate	1.5 ml/min
Oven temperature	40 °C
Injection volume	25 µl
Detector	UV-VWD detection wavelength 266 nm

HPLC method performance

Limit of detection for UV-VWD	0.1–1 ppb with S/N = 2 and 25 µl injected volume
Repeatability of	
RT over 10 runs	< 0.8 %
areas over 10 runs	< 1 %
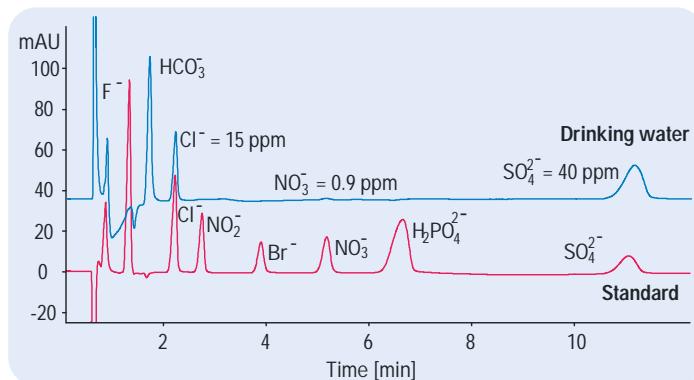
Figure 23
Analysis of anions in drinking water with indirect UV-detection

Chromographic conditions for electrochemical detection

The HPLC method presented here was used for the analysis of iodide in table salt.[17]

Figure 24
Analysis of iodide in table salt

Sample preparation	Table salt was dissolved in water.
Column	200 x 4 mm Sperisorb ODS2, 5 µm
Mobile phase	water with 5.2 g/l K_2HPO_4 + 3 g/l tetrabutylammoniumdi-hydrogenphosphat/ACN (85:15)
Flow rate	1 ml/min
Oven temperature	ambient 24 ºC
Injection volume	0.1 µl
Detector	electrochemical (ECD)
Electrode:	glassy carbon,
Working potential:	1 V
Operation mode:	amperometry

HPLC method performance

Limit of detection for ECD	40 µg/l
Repeatability of RT over 10 runs	< 0.1 %
areas over 10 runs	3 %
Linearity	min 50 pg to 150 ng
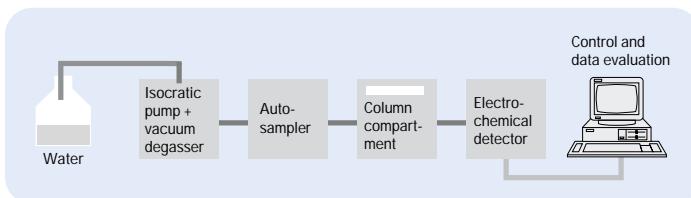
17. A.G. Huesgen, R. Schuster, "Analysis of selected anions with HPLC and electrochemical detection", *Agilent Application Note* 5091-1815E, **1991**.

Lipids

Both saturated and unsaturated triglycerides have been analyzed. Fats and oils are complex mixtures of triglycerides, sterols, and vitamins. The composition of triglycerides is of great interest in food processing and dietary control. Owing to the low stability of triglycerides containing unsaturated fatty acids, reactions with light and oxygen form hydroperoxides, which strongly influence the taste and quality of fats and oils. Adulteration with foreign fats and the use of triglycerides that have been modified by a hardening process also can be detected through triglyceride analysis.

Triglycerides and hydroperoxides in oils

The HPLC method presented here was used to analyze triglycerides, hydroperoxides, sterols, and vitamins with UV-visible diode-array detection (UV-DAD). Spectra were evaluated in order to trace hydroperoxides and to differentiate saturated from unsaturated triglycerides. Unsaturated triglycerides in olive oil have a very distinctive pattern. Other fats and oils are also complex mixtures of triglycerides but exhibit an entirely different pattern. Adulteration with foreign fats and the use of refined triglycerides in olive oil also can be detected through triglyceride analysis.

Sample preparation

Triglycerides can be extracted from homogenized samples with petrol ether. Fats and oils can be dissolved in tetrahydrofuran.[17]
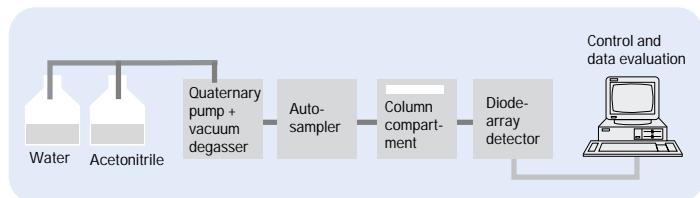
Sample preparation	Samples were dissolved in tetrahydrofuran (THF).
Column	200 x 2.1 mm Hypersil MOS, 5 µm
Mobile phase	A = water B = ACN/methyl-tert.butylether (9:1)
Gradient	at 0 min 87 % B at 25 min 100 % B
Post time	4 min
Flow rate	0.8 ml/min
Column compartment	60 ºC
Injection volume	1 µl standard
UV absorbance	

200 nm and 215 nm to detect triglycerides

240 nm to detect hydroperoxides

280 nm to detect tocopherols and decom-posed triglycerides (fatty acids with three conjugated double bonds)

Figure 25
Triglyceride pattern of aged sunflower oil. The increased response at 240 nm indicates hydroperoxides

HPLC method performance

Limit of detection

for saturated triglycerides   > 10 µg

for unsaturated triglycerides

fatty acids with 1 double bond >150 ng

fatty acids with 2 double bonds > 25 ng

fatty acids with 3 double bonds < 10 ng

Repeatability of

RT over 10 runs   < 0.7 %

areas over 10 runs   < 6 %
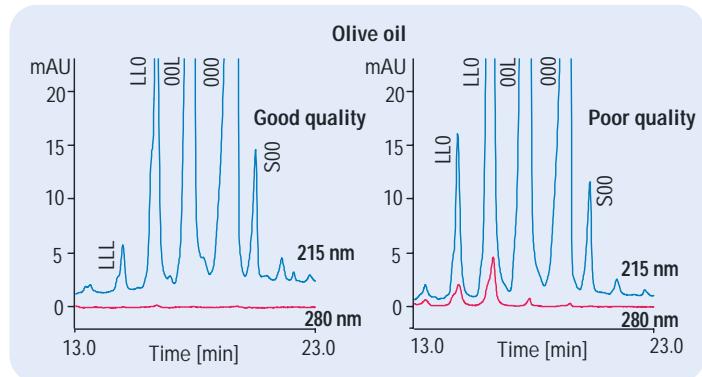
Figure 26
Analysis of olive oil. The response at 280 nm indicates a conjugated double bond and therefore poor oil quality

Triglycerides in olive oil

Unsaturated triglycerides in olive oil have very characteristic patterns. Other fats and oils are also complex mixtures of triglycerides but with different patterns.

Sample preparation information

Triglycerides can be extracted from homogenized samples with petrol ether. Fats and oils can be dissolved in tetrahydrofurane.

Chromatographic conditions

The presented HPLC method was used to analyze the unsaturated triglycerides, LnLnLn, LLL, and OOO.[18]

Sample preparation	Samples were dissolved in tetrahydrofurane.
Column	200 × 2.1 mm Hypersil MOS, 5 µm
Mobile phase	acetone/ACN (30:70)
Flow rate	0.5 ml/min
Column compartment	30 °C
Injection volume	2 µl
Detector	refractive index

HPLC method performance

Limit of detection for ECD	50 µg/l with S/N = 2
Repeatability of RT over 10 runs	< 0.3 %
areas over 10 runs	5 %
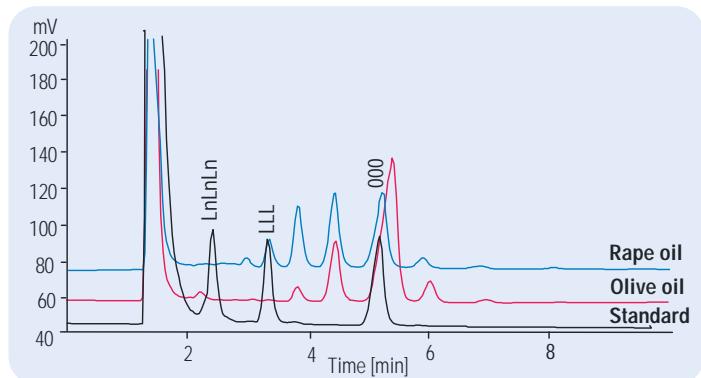
Figure 27
Analysis of the triglyceride pattern of olive and rape oil
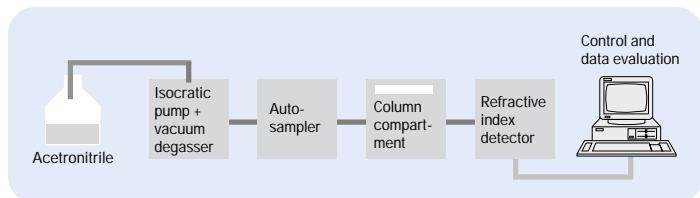
18. "Determination of triglycerides in vegetable oils", EC Regulation No. L248, 28ff.

Fatty acids

Saturated and unsaturated fatty acids from C_4 through C_{22} have been analyzed. Fatty acids are the primary components of oils and fats and form a distinctive pattern in each of these compounds. For example, butter and margarines can be differentiated by the percentage of butyric acid in the triglycerides. To determine the fatty acid pattern of a fat or oil, free fatty acids first are obtained through hydrolysis. Derivatization is then performed to introduce a chromophore, which enables analysis of the fatty acids using HPLC and UV-visible detection.

Sample preparation

The triglycerides were hydrolyzed using hot methanol and KOH, followed by derivatization.

Chromatographic conditions

The HPLC method presented here was used in the analysis of the fatty acid pattern of dietary fat. The method involves hydrolysis with hot KOH/methanol and online derivatization with bromophenacyl bromide.
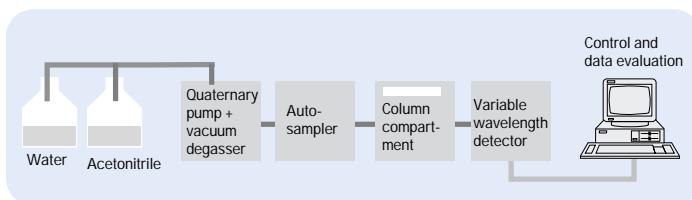
Sample preparation

0.215 g fat was hydrolyzed with 500 µl MEOH/ KOH at 80 ºC for 40 min in a thermomixer. After cooling 1.5 ml ACN/THF (1:1) was added, and the mixture was shaken for 5 min. The mixture was then filtered through a 0.45-µm Minisart RNML from Satorius.

Column	200 x 2.1 mm, MOS, 5 µm
Mobile phase	A = water (70 %)
	B = (ACN + 1 % THF) (30 %)
Gradient	at 5 min 30 % B
	at 15 min 70 % B
	at 17 min 70 % B
	at 25 min 98 % B
Flow rate	0.3 ml/min
Column compartment	50 °C
Detector	variable wavelength, 258 nm
Derivatization	60 mg/ml bromophenacyl bromide was dissolved in ACN.

Injector program for online derivatization

1. Draw 2.0 µl from vial 2 (ACN)
2. Draw 1.0 µl from air
3. Draw 1.0 µl from vial 3 (derivatization agent)
4. Draw 0.0 µl from vial 4 (wash bottle) (ACN/THF, 50:50)
5. Draw 1.0 µl from sample
6. Draw 0.0 µl from vial 4 (wash bottle)
7. Draw 1.0 µl from vial 3 (derivatization agent)
8. Draw 0.0 µl from vial 4 (wash bottle)
9. Draw 1.0 µl from vial 5 (acetonitrile + 5 % TEA)
10. Draw 0.0 µl from vial 4 (wash bottle)
11. Mix 9 µl in air, 30 µl/min speed, 10 times
12. Wait 2.0 min
13. Inject

HPLC method performance

Limit of detection	200 pg injected amount, S/N = 2
Repeatability of RT over 10 runs	< 0.1 %
areas over 10 runs	5 %
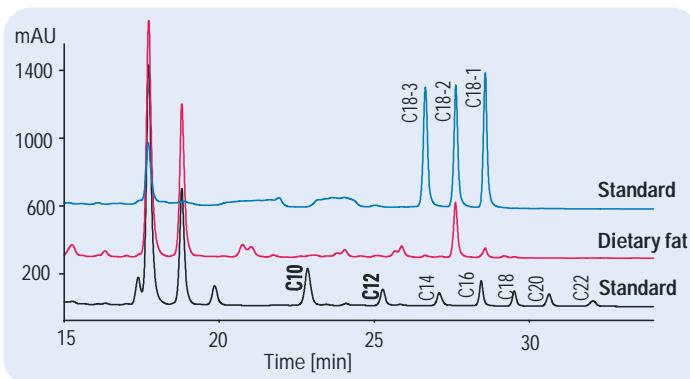
Figure 28
Analysis of a dietary fat triglyceride pattern. Overlay of one sample and two standard chromatograms
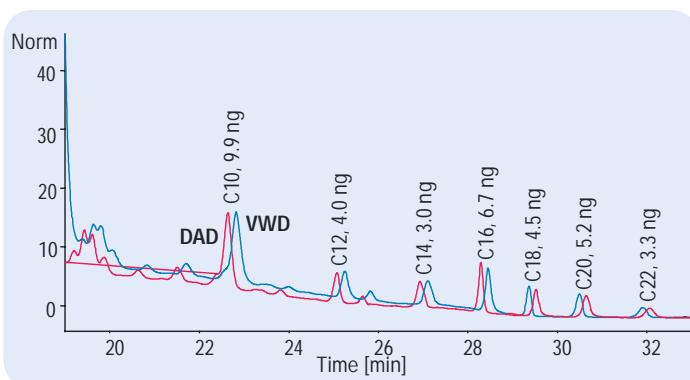
Figure 29
Trace analysis of triglycerides with a diode-array and a variable wavelength detector in series

3

Carbohydrates

The following carbohydrates have been analyzed: glucose, galactose, raffinose, fructose, mannitol, sorbitol, lactose, maltose, cellobiose, and sucrose. Food carbohydrates are characterized by a wide range of chemical reactivity and molecular size. Because carbohydrates do not possess chromophores or fluorophores, they cannot be detected with UV-visible or fluorescence techniques. Nowadays, however, refractive index detection can be used to detect concentrations in the low parts per million (ppm) range and above, whereas electrochemical detection is used in the analysis of sugars in the low parts per billion (ppb) range.

Sample preparation

Degassed drinks can be injected directly after filtration. More complex samples require more extensive treatment, such as fat extraction and deproteination. Sample cleanup to remove less polar impurities can be done through solid-phase extraction on C18 columns.
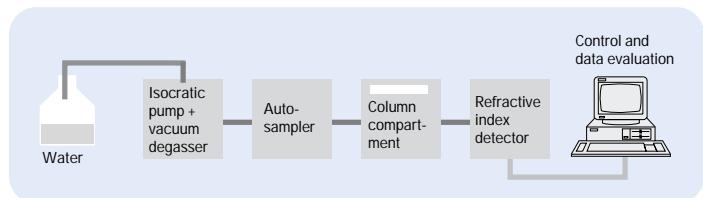
Chromatographic conditions

The HPLC method presented here was used to analyze mono-, di-, and trisaccharides as well as sugar alcohols.

Sample preparation	Samples were directly injected.
Column	300 x 7.8 mm Bio-Rad HPXP, 9 μm
Mobile phase	water
Column compartment	80 ºC
Flow rate	0.7 ml/min
Detector	refractive index

HPLC method performance

Limit of detection	< 10 ng with S/N = 2
Repeatability of RT over 10 runs	< 0.05 %
areas over 10 runs	2 %
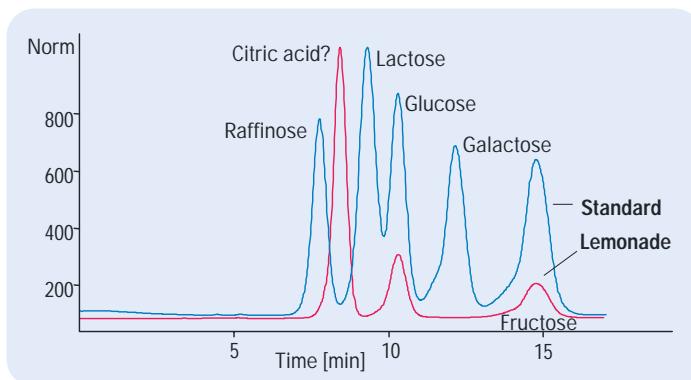
Figure 30
Analysis of carbohydrates in lemonade
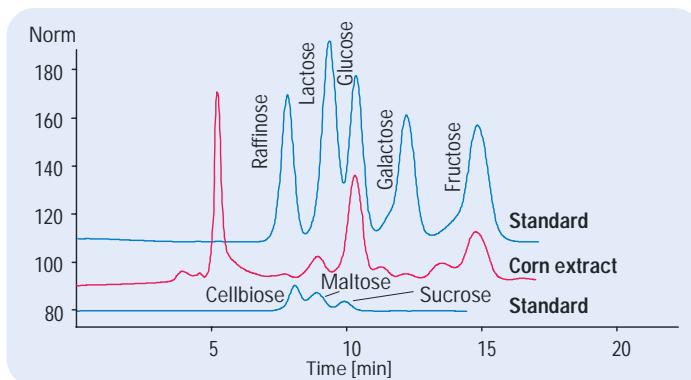
Figure 31
Analysis of carbohydrates in corn extract

4. *Official Methods of Analysis, Food Compositions; Additives, Natuaral Contaminants*, 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2; AOAC Official Method 980.13: Fructose, glucose, lactose, maltose, sucrose in milk chocolate; AOAC Official Method 982.14: Glucose, fructose, sucrose, and maltose in presweetened cereals; AOAC Official Method 977.20: Separation of sugars in honey; AOAC Official Method 979.23: Saccharides (major) in corn syrup; AOAC Official Method 983.22: Saccharides (minor) in corn syrup; AOAC Official Method 984.14: Sugars in licorice extracts.

3

Vitamins

Fat-soluble vitamins, such as vitamins E, D, and A, and water-soluble vitamins, such as vitamins C, B_6, B_2, B_1, and B_{12}, have been analyzed.

Vitamins are biologically active compounds that act as controlling agents for an organism's normal health and growth. The level of vitamins in food may be as low as a few micrograms per 100 g. Vitamins often are accompanied by an excess of compounds with similar chemical properties. Thus not only quantification but also identification is mandatory for the detection of vitamins in food. Vitamins generally are labile compounds that should not exposed to high temperatures, light, or oxygen. HPLC separates and detects these compounds at room temperature and blocks oxygen and light.[19] Through the use of spectral information, UV-visible diode-array detection yields qualitative as well as quantitative data. Another highly sensitive and selective HPLC method for detecting vitamins is electrochemical detection.

Water-soluble vitamins

Sample preparation

Different food matrices require different extraction procedures.[19] For simple matrices, such as vitamin tablets, water-soluble vitamins can be extracted with water in an ultrasonic bath after homogenization of the food sample.
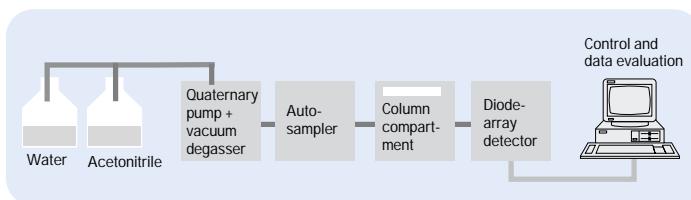
Chromatographic conditions for UV detection

The HPLC method presented here was used to analysis vitamins in a vitamin drink.

Sample preparation	filtration
Column	100 x 4 mm
	Hypersil BDS, 3 µm
Mobile phase	A= water with pH = 2.1
	$(H_2SO_4) = 99 \%$
	B = ACN 1 %
Gradient	at 3.5 min 1 % B
	at 11 min 25 % B
	at 19 min 90 % B
Post time	6 min
Flow rate	0.5 ml/min
Column compartment	30 ºC
Injection volume	2–5 µl
Detector	UV-DAD
	detection wavelength
	220/30 nm,
	reference wavelength
	400/100 nm

HPLC method performance

Limit of detection	< 500 pg (injected amount), S/N = 2
Repeatability of	
RT over 10 runs	< 0.2 %
areas over 10 runs	< 2 %
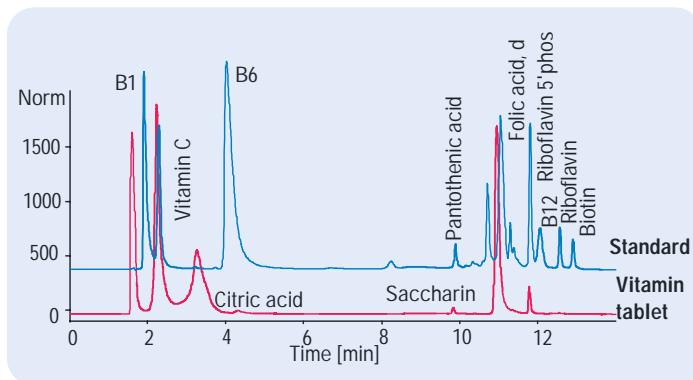
Figure 32
Analysis of water-soluble vitamins in a vitamin tablet
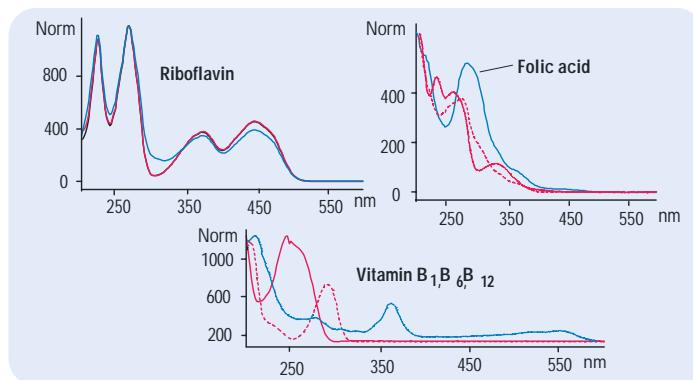
Figure 33
Spectra of water-soluble vitamins

19. L.M. Nollet, " *Food Analysis by HPLC*", New York, **1992**.

Chromatographic conditions for electrochemical detection

The HPLC method presented here was used in the analysis of vitamins in animal feed.[20]

Sample preparation	Vitamin preparation was diluted with water 1:100
Column	125 x 4 mm, Lichrospher RP 18, 5 µm
Mobile phase	water + 0.02 M KH_2PO_4 + 0.03 M tetrabutylammoniumhydrogensulfat + 0.03 M heptanesulfonic acid + 2 % ACN
Stop time	15 min
Flow rate	0.8 ml/min
Column compartment	30 ºC
Injection volume	1 µl standard 0.5 µl sample
Detector	electrochemical
Working electrode:	glassy carbon
Operation mode:	amperometry
Working potential:	1.2 V
Range:	0.5 µA
Reference electrode:	AgCl/KCl
Response time:	1 s

HPLC method performance

Limit of detection	30 pg (injected amount) S/N = 2
Repeatability of RT over 10 runs	< 0.5 %
areas over 10 runs	< 5 %
Linearity	30 pg to 1 ng
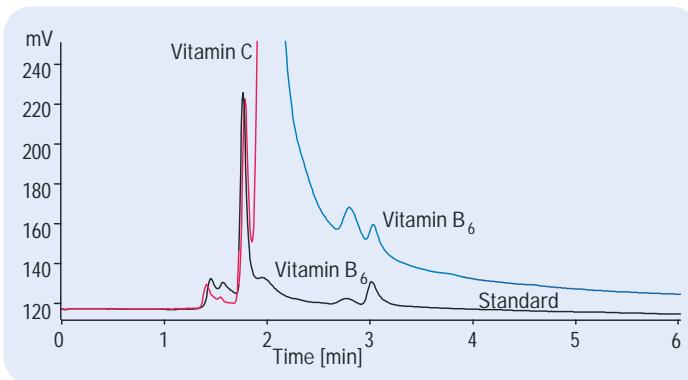
Figure 34
Analysis of vitamin B_6 in a vitamin preparation
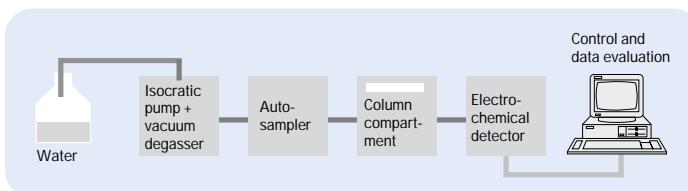
20. A.G. Huesgen, R. Schuster, "Analysis of selected vitamins with HPLC and electrochemical detection", *Agilent Application Note 5091-3194E* , **1992**.

Fat-soluble vitamins

Column	100 x 2.1 mm
	Hypersil MOS, 5 µm
Mobile phase	A = water
	B = ACN (70 %)
Gradient	at 15 min 90 % B
	at 16 min 95 % B
Post time	3 min
Flow rate	0.5 ml/min
Column compartment	40 ºC
Injection volume	2–5 µl
Detector	UV-DAD
	detection wavelengths
	230/30 nm, 400/100 nm;
	reference wavelengths
	280/40 nm, 550/100 nm

HPLC method performance

Limit of detection	1 ppb with S/N = 2
Repeatability of	
RT over 10 runs	< 0.82 %
areas over 10 runs	< 2.2 %
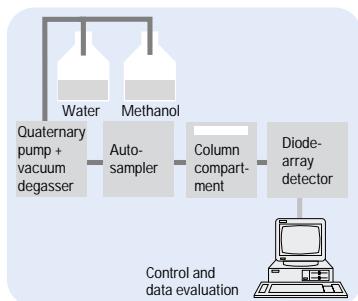
Sample preparation

Different food matrices require different extraction procedures. These procedures include alkaline hydrolysis, enzymatic hydrolysis, alcoholysis, direct solvent extraction, and supercritical fluid extraction of the total lipid content.

Chromatographic conditions for UV detection

The HPLC method presented here was used in the analysis of a vitamin standard.
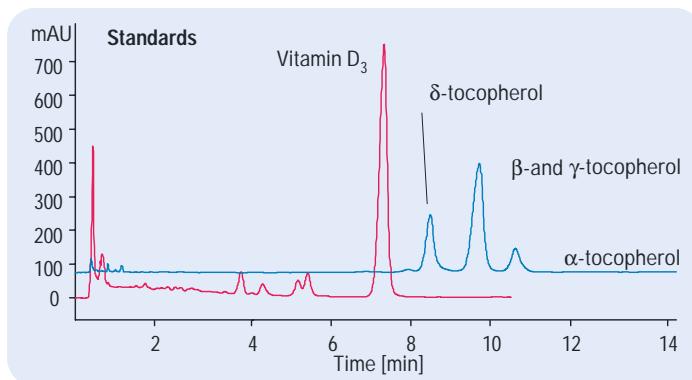
Figure 35
Analysis of fat-soluble vitamins with UV detection

3

Chromatographic conditions for electrochemical detection

The HPLC method presented here was used in the analysis of a vitamin standard.[20]

Column	125 x 4 mm
	Lichrospher RP18, 5 µm
Mobile phase	methanol + 5 g/l
	lithiumperchlorate +
	1 g/l acetic acid
Stop time	20 min
Flow rate	1 ml/min
Oven temperature	30 ºC
Injection volume	1 µl standard
Detector	electrochemical
Working electrode:	glassy carbon
Operation mode:	amperometry
Working potential:	0.9 V
Range:	0.5 µA
Reference	
electrode:	AgCl/KCl
Response time:	8 s

HPLC method performance

Limit of detection	80 pg (injected amount),
	S/N = 2
Repeatability of	
RT over 10 runs	< 0.5 %
areas over 10 runs	< 5 %
Linearity	30 pg to 1 ng
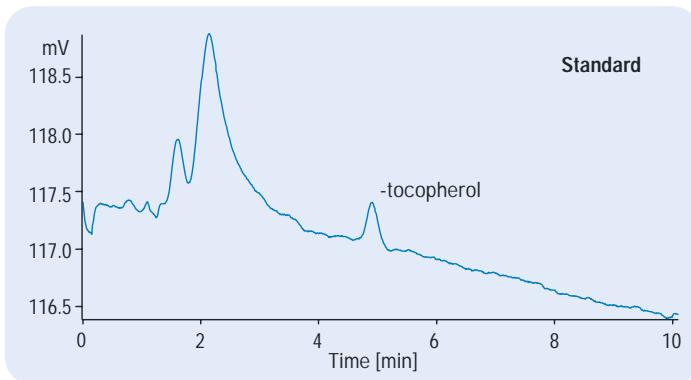
Figure 36
Analysis of a fat-soluble vitamin with electrochemical detection
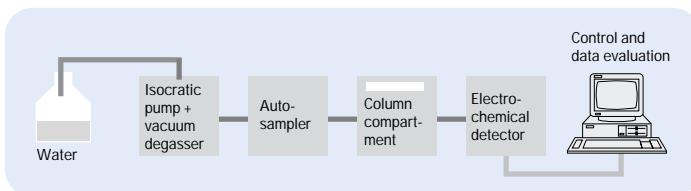
Analysis of tocopherols on normal-phase column

Tocopherols cannot be separated completely using reversed-phase chromatography. However, normal-phase chromatography can separate isocratically all eight tocopherols (T) and tocotrienols (T$_3$) naturally occurring in fats, oils, and other foodstuffs. Fluorescence detection is recommended for the analysis of total lipid extraction because UV absorbance detection is not selective enough to prevent detection of coeluting peaks.

Chromatographic conditions for analysis of tocopherols on normal-phase column

The HPLC method presented here was used in the analysis of margarine.

Sample preparation	20 g sample dissolved in 15 ml hexane
Column	100 x 2.1 mm Hypersil SI 100, 5 µm
Mobile phase	hexane + 2 % isopropanol
Stop time	8 min
Flow rate	0.3 ml/min
Column compartment	25 ºC
Injection volume	0.5 µl
Detector	
UV-DAD	295/80 nm
Fluorescence	excitation wavelength 295 nm, emission wavelength 330 nm

HPLC method performance

Limit of detection for diode-array	10–20 ng, S/N = 2
Limit of detection for fluorescence	0.5–2 ng S/N = 2
Repeatability of RT over 10 runs	< 2 %
areas over 10 runs	< 2 %

Hexane

Isocratic pump + vacuum degasser — Auto-sampler — Column compart-ment — Diode-array detector — Fluores-cence detector

Control and data evaluation
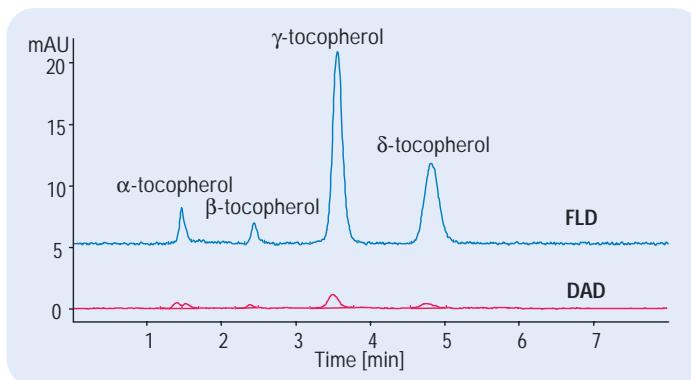
Figure 37
Analysis of tocopherols on normal phase using UV and fluorescence detection
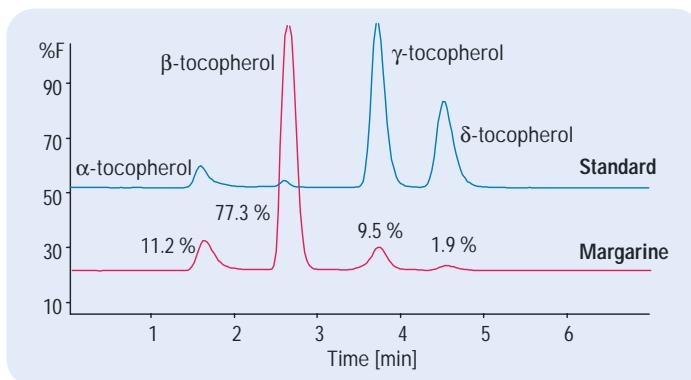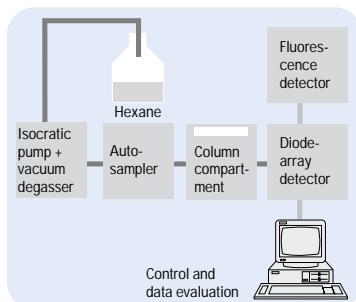
Figure 38
Analysis of tocopherol concentration in margarine fat extract with fluorescence detection

Biogenic amines

The following amines were analyzed: ammonia, amylamine, 1-butylamine, 1,4-diaminobutane, 1,5-diaminopentane, diethylamine, ethanolamine, ethylamine, hexylamine, histamine, isobutylamine, isopropylamine, methylamine, 3-methylbutylamine, morpholine, phenethylamine, propylamine, pyrrolidine, and tryptamine.

Free amines are present in various food products and beverages, including fish, cheese, wine, and beer.

High concentrations of specific amines can have toxic properties. As a result, several countries have set maximum tolerance levels for these compounds in foodstuffs. HPLC is now preferred for the analysis of amines in food matrices because of its shorter analysis time and relatively simple sample preparation.

Sample preparation

Amines can be extracted from different matrixes using liquid/liquid extraction or solid-phase extraction followed by derivatization.
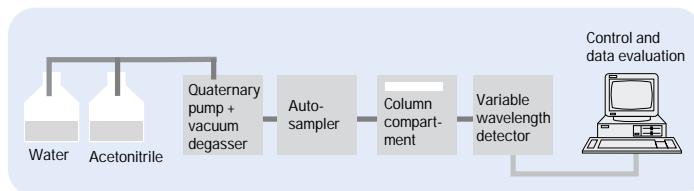
Chromatographic conditions for UV detection

The HPLC method presented here was used to analyze amines in wine.[21]

Sample preparation

25 ml wine was decolored with polyvinylpyrrolidoine. After filtration, the amines (5 ml sample, pH = 10.5) were derivatized with 2 ml dansyl chloride solution (1 %). The reaction solution was cleaned with solid-phase extraction using C18 cartridges (500 mg). After elution with 2 ml ACN, the solution was concentrated to 100 µl.

Column	250 x 4.6 mm Spherisorb ODS2, 5 µm
Mobile phase	A = water + 5 % ACN = 75 % B = ACN (25 %)
Gradient	at 5 min 45 % B at 30 min 45 % B at 50 min 60 % B at 55 min 80 % B at 60 min 80 % B
Stop time	60 min
Post time	4 min
Flow rate	1 ml/min
Column compartment	60 ºC
Detector	UV-VWD 250 nm

HPLC method performance

Recovery rate	> 85 %
Limit of detection	50–150 µg/l
Method repeatability for 5 red wine analyses	< 5 %
Linearity 500 µg/l to	20 mg/l

1	ethanolamine	8	pyrrolidine	15	amylamine
2	ammonia	9	i-butylamine	16	1,4-diaminobutane
3	methylamine	10	1-butylamine	17	1,5-diaminopentane
4	ethylamine	11	tryptamine	18	hexylamine
5	morpholine	12	diethylamine	19	histamine
6	i-propylamine	13	phenethylamine	20	heptylamine
7	propylamine	14	3-methylbutylamine		(internal standard)
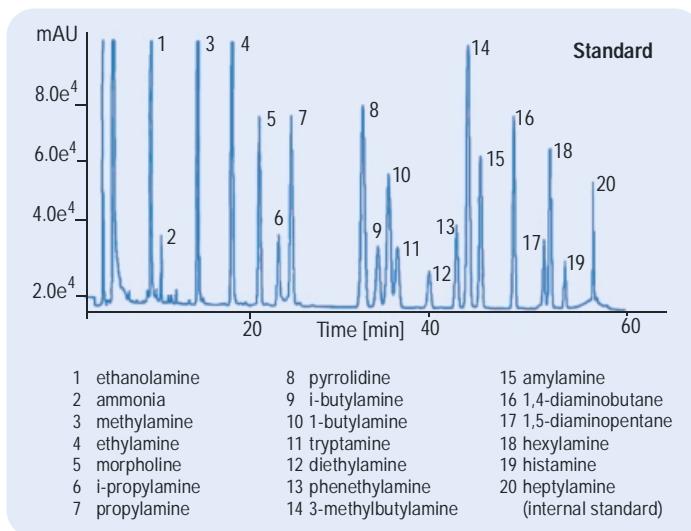
Figure 39
Analysis of amine standard with UV detection after derivatization

21. O. Busto, et al., "Solid phase extraction applied to the determination of biogenic amines in wines by HPLC", *Chromatographia*, **1994**, 38(9/10), 571–578.

Amino acids

Both primary and secondary amino acids were analyzed in one run.

The amino acid composition of proteins can be used to determine the origin of meat products and thus to detect adulteration of foodstuffs. Detection of potentially toxic amino acids is also possible through such analysis. Through the use of chiral stationary phases as column material, D and L forms of amino acids can be separated and quantified.

HPLC in combination with automated online derivatization is now a well-accepted method for detecting amino acids owing to its short analysis time and relatively simple sample preparation.

Sample preparation

Hydrolyzation with HCl or enzymatic hydrolysis is used to break protein bonds.

Chromatographic conditions

The HPLC method presented here was used in the analysis of secondary and primary amino acids in beer with precolumn derivatization and fluorescence detection.[22, 23]
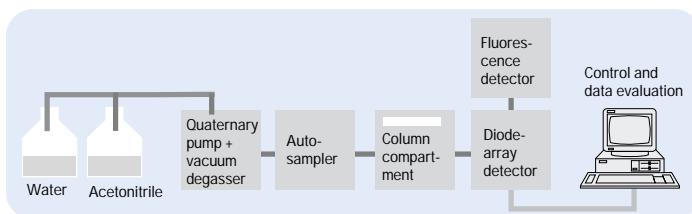
Sample preparation   filtration
Column   200 x 2.1 mm
                     Hypersil ODS, 5 µm
Mobile phase   A = 0.03 M sodium acetate
                     pH = 7.2 + 0.5% THF
                     B = 0.1 M sodium acetate/
                     ACN (1:4)
Gradient
  at 0 min 0 % B at 0.45 ml/min flow rate
  at 9 min 30 % B
  at 11 min 50 % B at 0.8 ml/min flow rate
  at 13 min 50 % B
  at 14 min 100 % B at 0.45 ml/min flow rate
  at 14.1 min at 0.45 ml/min flow rate
  at 14.2 min at 0.8 ml/min flow rate
  at 17.9 min at 0.8 ml/min flow rate
  at 18 .0 min at 0.45ml/min flow rate
  at 18 min 100 % B
  at 19 min 0 % B
Post time   4 min
Flow rate   0.45 ml/min
Column compartment   40 ºC
Injection volume   1 µl standard
Detector
  UV -DAD   338 nm and 266 nm
  Fluorescence
  Excitation wavelength: 230 nm
  Emission wavelength: 450 nm
  at 11.5 min
  Excitation wavelength: 266 nm
  Emission wavelength: 310 nm
  Photomultiplier gain: 12
  Response time: 4 s

HPLC method performance

Limit of detection	DAD < 5 pmol
	FLD < 100 fmol
Repeatability of	
RT over 6 runs	< 1 %
areas over 6 runs	< 5 %
Linearity DAD	1 pmol to 4 nmol
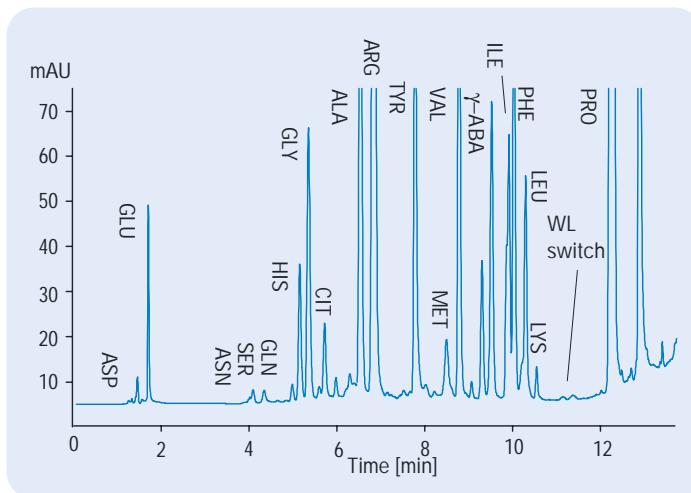
Figure 40
Analysis of amino acids in beer after online derivatization

Injector program for online derivatization
  1. Draw 3.0 µl from vial 2 (borate buffer)
  2. Draw 1.0 µl from vial 0 (OPA reagent)
  3. Draw 0.0 µl from vial 100 (water)
  4. Draw 1.0 µl from sample
  5. Draw 0.0 µl from vial 100 (water)
  6. Mix 7.0 µl (6 cycles)
  7. Draw 1.0 from vial 1 FMOC reagent
  8. Draw 0.0 µl from vial 100 (water)
  9. Mix 8.0 µl (3 cycles)
  10. Inject

22. "Sensitive and reliable amino acid analysis in protein hydrolysates using the Agilent 1100 Series", *Agilent Technical Note* 5968-5658E, **1999**
23. R. Schuster, "Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and HPLC", *J. Chromatogr.*, **1988**, 431, 271–284.

Peptides

Peptide mapping of phytochrome from dark grown oat seedlings using capillary liquid chromatography

The analyzed phytochrome is a photoreceptor protein that controls light-dependent morphogenesis in plants. For example, potato clod forms pale long sprouts if it germinates in a dark cellar. However, if this process takes place in the light, a normal plant with green leaves grows and photosynthesis occurs. Phytochrome proteins are present in very low concentrations in potato clod, and sample volume and concentration of these proteins is rather low following sample preparation. In this case, columns or capillaries with a small internal diameter are preferred because sensitivity increases with decreasing internal diameter of the column. The use of capillaries with an internal diameter of 100–300 μm enables flow rates as low as 0.5–4.0 μl/min, which reduces solvent consumption. Such flow rates are well-suited to liquid chromatography-mass spectroscopy (LC/MS) electrospray ionization.

In our experience, the appropriate conversion of standard HPLC equipment to a capillary HPLC system is cost-effective and yields the highest performance for running capillary columns. For conversion, a flow stream-split device, a 35-nL capillary flow cell for the detector, and capillary connections between system modules are required. System delay volume should be as low as possible. To meet the demands of such a system, the Agilent 1100 Series binary pump, which has inherently low delay volume, was selected as a pumping system. The flow splitter, the capillary flow cell for the detector, and the column were purchased from LC Packings in Amsterdam.[24]

With this design, a standard flow rate (for example, 100 or 50 μl/min) can be set for the pump. This flow then can be reduced by calibrated splitters between 0.5 and 4 μl/min, for example. This flow rate is optimal for capillary columns with an internal diameter of 300 μm.

Chromatographic conditions

Capillary HPLC with UV and MS detection has been used in the analysis of phytochrome protein from dark grown oat seedlings. Figures 41, 42 and 43 show the UV and total ion chromatogram together with two mass spectra of selected fragments. The Agilent 1100 Series LC system was used without mixer. All tubings were as short as possible, with an internal diameter of 75–120 µm id.

Sample preparation

The extracted protein was reduced and alkylated prior to digestion with trypsin.

Sample	tryptic digest of phytochrome from oat seedlings, 7 pmol/µl
Capillary column	300 µm x 25 cm, C18
Mobile phase	A = 0.025 % TFA in water
	B = 0.02 % TFA in ACN
Gradient	0.35 % B/min
Flow rate	100 µl/min split to
	4 µl/min
Column compartment	25 ºC
Injection volume	2.5 µl
Detector	UV-VWD
	wavelength 206 nm with a 35-nl, 8-mm flow cell

HPLC method performance

Limit of detection	1 pmol
Repeatability of	
RT over 10 runs	< 0.7 %
areas over 6 runs	< 1 %
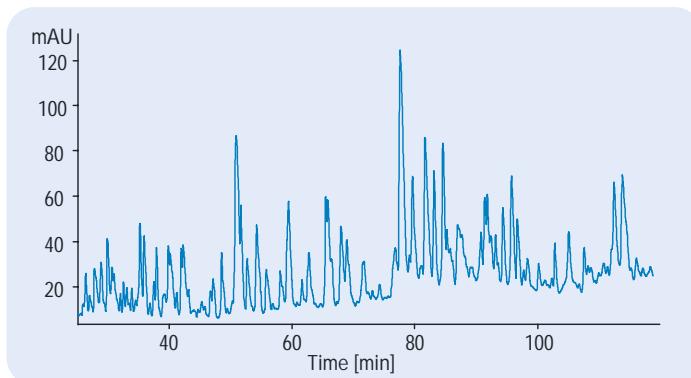
Figure 41

Capillary LC-MS of a phytochrome tryptic digest (17.5 pmol)—UV trace
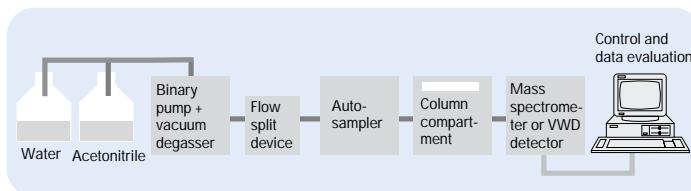
MS data was used for further evaluation. Some of the tryptic mass fragments of the phytochrome are signed. As an example, figure 42 shows two mass spectra.

Voltages	Vcyl -5500, Vend -3500, Vcap -4000, CapEx 150
Scan	400–1800 m/z
Threshold	150
Sampling	1
Stepsize	0.15 amu
Drying gas	nitrogen, 150 °C
Nebulizer	gas nitrogen, < 20 psi

The Agilent 5989B MS engine was equipped with an Iris™ Hexapole Ion Guide
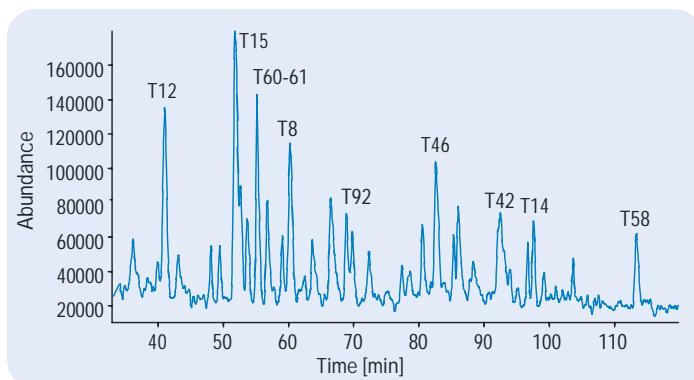
Figure 42
Capillary LC-MS of a phytochrome tryptic digest (17.5 pmol)—total ion chromatography (TIC)
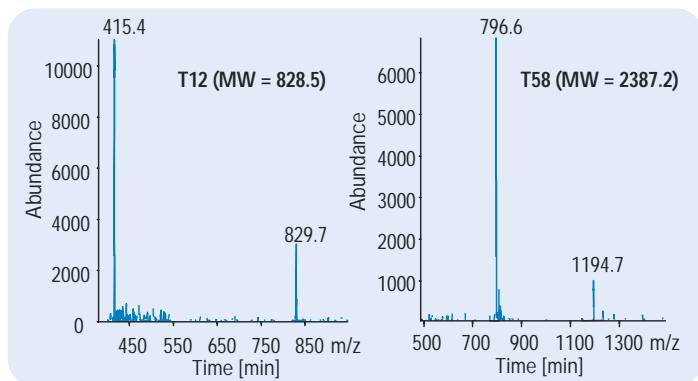
Figure 43
Mass spectra of T12 and T58

24. "Capillary Liquid Chromatography with the Agilent 1100 Series Modules and Systems for HPLC", *Agilent Technical Note 5965-1351E* , **1996**.

**The
Equipment
Basics**

Part Two

An overview of the
hardware and the software
components needed
for successful HPLC,
and an introduction to
the analytical techniques
that have become
routine in food analysis

Chapter 4

**Separation in
the liquid phase**

4

Liquid chromatography offers a wide variety of
separation modes and mobile phases for optimizing
your separation system.

Separation mechanisms

Stationary phases can be classified according to the
mechanism by which they separate molecules:

• partition phases

• adsorption phases

• ion-exchange phases

• size-exclusion phases

Nowadays the most popular column material is reversed
phase, in which separation is achieved through partition
and through adsorption by unprotected silanol groups. In
reversed-phase chromatography, the stationary phase is
nonpolar (or less polar than in the mobile phase) and the
analytes are retained until eluted with a sufficiently polar
solvent or solvent mixture (in the case of a mobile-phase
gradient).

Reversed-phase materials

Reversed-phase materials have wide application and a long
lifetime. Moreover, these media have good batch-to-batch
reproducibility, low equilibration times, high mechanical
stability, and predictable elution times and elution order.
Reversed-phase chromatography is frequently used in food
analysis, as shown in part one of this primer.

Ion-exchange materials

Compared with reversed-phase media, ion-exchange
materials have a shorter lifetime, are less mechanically
stable, and take longer to equilibrate. These columns have
limited application in food analysis and are used primarily
for inorganic cations and anions or for glyphosate.

Size-exclusion chromatography is used for sample cleanup and fractionation and is described in more detail in chapter 5 ("Sample preparation").

Adsorption chromatography is used for sampling and cleanup. For example, flavonoids from plant material can be cleaned, fractionated, and enriched on alumina. Other examples are given in chapter 5.

The advent of narrow-bore columns

Discussions of HPLC methods often revolve around the internal diameter (id), or bore, of the column to be used. Standard-bore columns have an internal diameter of approximately 4 or 5 mm, whereas narrow-bore columns have an internal diameter of approximately 2 mm. When packed with the same materials as the standard-bore column, the narrow-bore column can achieve the same resolving power with less solvent because the analytes can be eluted at a lower flow rate (< 0.5 ml/min) than the 2–3 ml/min required for standard-bore columns. In addition, narrow-bore columns are 4–6 times more sensitive using the injection volume required for a standard-bore column (see figure 44).

Narrow-bore columns nonetheless place higher demands on the equipment used than standard-bore columns. First, the HPLC pump must yield these low flow rates in a way that is both reproducible and precise. Second, all capillary connections, that is, from injector to column and from column to detector, must be kept to a minimum. Third, because column frits block more often, guard columns are recommended. An HPLC system designed for narrow-bore columns (low dead volume and high-performance pumping system) can achieve solvent economies of more than 60 % as well as improve detection limits with the same injection volume. Moreover, under the same conditions, a standard-bore column may have higher resolving power.

Figure 44
Effect of bore dimensions on separation

Influence of column temperature on separation

Many separations depend not only on the column material and mobile phases but also on the column temperature. In such cases, column temperature stability is the dominating factor for the elution order. A thermostatted column compartment using Peltier control with good ambient temperature rejection ensures stable chromatographic conditions. Periodic fluctuations in room temperature during 24-hour use influence these conditions. Figure 45 shows the advantage of Peltier control over conventional air cooling.
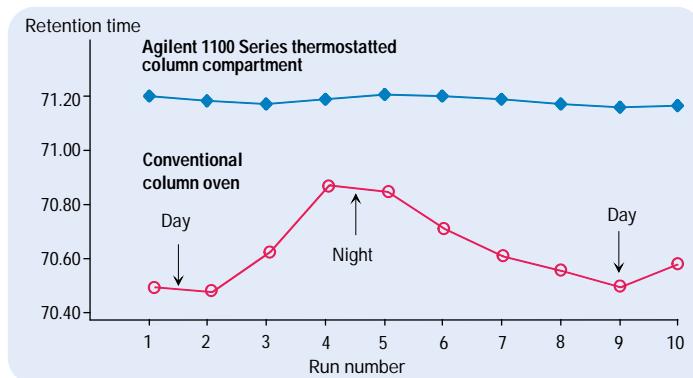
Figure 45
Comparison of Peltier and conventional cooling as demonstrated using retention time fluctuations of a peptide peak over a sequence of 10 consecutive tryptic peptide maps

In brief…

Reversed-phase stationary phases are the most popular LC media for the resolution of food mixtures. The use of narrow-bore columns can result in gains in sensitivity and reduced solvent consumption. For example, these columns have been applied successfully in the analysis of aflatoxins and fatty acids.

Chapter 5

Sample preparation

5
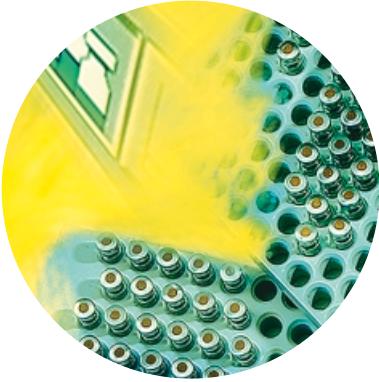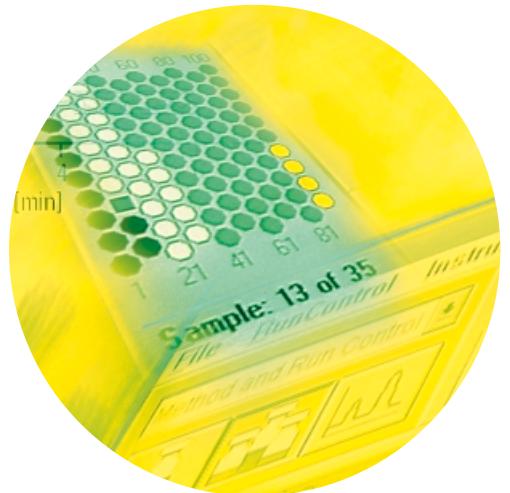
The isolation of analytes from other matrix
constituents is often a prerequisite for successful
food analysis. The broad selection of cleanup and
enrichment techniques takes into account the many
matrices and compound classes under study.

Sample preparation steps

Sample preparation for HPLC can be broken down into the
following main steps:

1. Sampling
Collection
Storage

2. Cleanup/enrichment offline
Homogenization, centrifugation, precipitation,
hydrolyzation, liquid/liquid extraction, solid-phase
extraction, ultrasonic bath liquid extraction,
supercritical fluid extraction, concentration

3. Cleanup/enrichment online
Guard columns
Online solid-phase extraction
Gel-permeation chromatography (GPC)

4. Chemical derivatization
Precolumn, online, or offline
(see also discussion of postcolumn derivatization,
chapter 9)

Automation
Manual extraction, cleaning, and concentration of the sample
prior to transfer to the HPLC instrument is time-consuming
and can drain resources. Sample preparation therefore
should be automated where possible. Nowadays the sample
can be fractionated and/or derivatized automatically.

Supercritical fluid extraction (SFE) systems and automated solid-phase extraction equipment also have been interfaced directly to liquid chromatographs. Equipment used to automate preparation of HPLC samples includes:

- Valves—Valves are used to switch to guard columns and online solid-phase extraction techniques.[25] Switching valves are common in HPLC, and some instruments even have built-in column compartment valves. With a six-port valve, for example, the eluant stream can be switched from one column to another to cut out a peak. This peak is then analyzed on the second column.

- SFE interfaces—This technique is rather new, and online systems are under development.[26] An offline procedure has been used successfully in the analysis of vitamin K in infant formula.[27]

- Precolumn derivatization—This well-accepted and commercially available technique[28] has been applied in the analysis of amino acids in beer (see page 50 ff.). Reagents also can be used postcolumn (see page 28).

- Automated solid-phase extraction—This relatively new technique is used to analyze bittering compounds in beer.[29]

Solids

Solid samples, for example chocolate or meat, should be homogenized before such techniques as steam distillation, SFE, or ultrasonic-stimulated liquid extraction are applied.[30]

Ultrasonic bath liquid extraction

Ultrasonic bath liquid extraction is a very simple extraction method. Selectivity is achieved through the use of appropriate solvents. Antioxidants and preservatives can be extracted with this technique if the matrix is low in fat.

✔	✘
Uses relatively small quantities of organic solvents, thereby reducing costs and facilitating disposal. Extraction times are in minutes rather than hours.	Samples high in fat cannot be extracted.

Steam distillation

Steam distillation is only used to extract volatile compounds from solid homogenized matrices. For example, biphenyl and 2-phenylphenol pesticides can be extracted from citrus fruits with this technique.[31]

✔	✘
Enables selective extraction of volatile compounds.	Extraction times are long and offline. Narrow range of use.

Supercritical fluid extraction

Until now, supercritical fluid extraction (SFE) was rarely used in food analysis. However, the input of modern SFE instruments can be automated with sampling devices. This method is used primarily for GC,[26, 32] although LC coupling also has been performed with SFE.[33]

✔	✘
Uses small quantities of organic solvents, thereby reducing costs and facilitating disposal. Extraction times are in minutes rather than hours. Can be automated.	Weak solvating power limits range of analytes. Ultrapure fluids for trace analysis are not always available.

Liquids

Liquid-liquid extraction, on- and offline solid-phase extraction, and GPC are used in the analysis of liquid samples or extracts from solid samples.

Liquid-liquid extraction

Liquid-liquid extraction is the most common extraction method. It requires an appropriate solvent and a separating funnel, or a continuous or counter-current distribution apparatus

✔	✘
Simple, with highly selective modifiers (pH, salts, or ion-pairing reagents).	Requires large amounts of toxic solvents, can emulsify, and is difficult to automate.

Solid-phase extraction

Suitable for cleaning clear liquids such as filtered beverages, solid-phase extraction (SFE) is simple in principle. The sample is first sucked through a preconditioned cartridge or disk filled with adsorbents. The solid then traps the compounds of interest, which can be extracted later with a small amount of an organic eluant. A variety of materials provides a choice of selectivities for use as a fractioning tool. Two or more separate cartridges filled with specific adsorption materials can trap individual fractions of the sample.

SPE is one of the fastest-growing sample preparation and cleanup techniques.[34] Attempts have been made to automate both the procedure and its interface with the chromatograph. Systems based on robotics and valves are available. Pumping a certain volume of water sample through one or more precolumns filled with extraction materials will extract and concentrate the compounds of interest. After desorption with a suitable solvent, the analytes can be introduced into a liquid or gas chromatograph for identification and quantification. The precolumns are

exchanged automatically between analyses to prevent clogging and memory effects.[35] So far this system has been used only to extract pesticides and polynuclear aromatics in river water. A different online solid-phase extraction system has been used to extract and analyze iso-a-acids in beer.[36, 40]

✔	✘
Uses small amounts of organic solvents, can run several samples at once, and can be automated.	Differing batch-to-batch efficiencies can reduce reproducibility. Risk of irreversible adsorption. Degradation by surface catalysis can occur.

Gel permeation chromatography

Also known as size-exclusion chromatography, gel permeation chromatography (GPC) has become a standard technique for isolating compounds of low molecular weight from samples that contain compounds of high molecular weight, such as oil or fats. The separation is based on differences in size, with higher molecular weight compounds retained less than smaller ones. GPC has been used successfully to separate vitamins A, D, and E from glycerides in infant formula and clean-up of pesticides in spices (see chapter 2, page 22 ff).[37]

✔	✘
Highly reproducible, good automation possibilities.	Large amounts of solvents needed, separation efficiency may differ from batch to batch.

Guard columns

A guard column is connected in front of the analytical column to prevent contamination of the analytical column by the matrix. Either the guard column can be included in analytical column design, or both columns can be interconnected by a valve that, when switched, transfers fractions from the precolumn to the analytical column. The latter technique is more flexible and can be used for sample cleanup and enrichment. Alternatively, a backflush valve can be used to enrich the sample on a precolumn. Reversing the direction of flow transfers compounds concentrated from the precolumn to the analytical column.

✔	✘
Highly reproducible, good automation possibilities.	More complex, and more expensive if a valve is used.

In brief…

Many food analyses are governed by officially recognized methods, which often include details on sample preparation. Recent trends toward automated sample preparation increase precision by eliminating operator variances. Should you adopt a newly developed sample preparation technique, however, please be aware that the method must comply with existing good laboratory practice (GLP) regulations and with accreditation standards.[38]
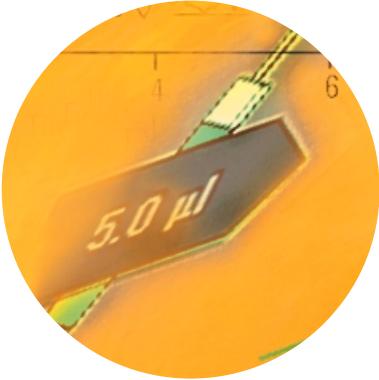
Chapter 6

Injection techniques

6

After the sample has been prepared for introduction onto the LC column, analysis can begin. Judgements based on analyte concentration require a reliable quantity of sample volume. The process of introducing the sample onto the column with precision syringes can be automated for increased throughput.[39, 40]

Characteristics of a good sample introduction device
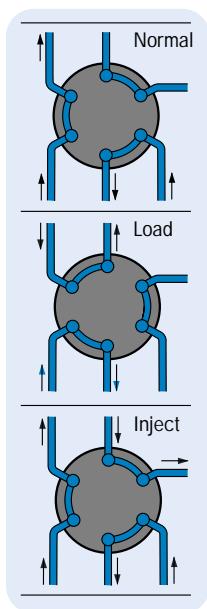
Figure 46
A typical 6-port
injection valve

The main requirements for any sampling device are good precision of injection volumes, low memory effects (carry-over of material from one injection to another), and the ability to draw viscous samples and inject variable volumes. Modern sampling systems can further increase productivity with features such as online precolumn derivatization for selective detection, heating and cooling for improved stability, and microsampling of material in low supply. Some analyses may require corrosive solvents or mobile-phase additives such as 0.1 N HCl or 60 % formic acid. Some vendors supply devices of corrosion-resistant titanium to solve this problem.

Injection systems often are based on a six-port valve, which is put through several steps for each injection, as illustrated in figure 46. In the first step, denoted here as *load*, the sample is either aspired by a vacuum (in automated systems) or expressed by a syringe plunger (in manual systems) into a sample loop, where it rests until the valve is switched to *inject*. This second step connects the pump and the mobile phase with the column. The contents of the sample loop then move into the solvent flow path and onto the analytical column. Because all parts of the system are constantly flushed during analysis, the remnants of a previous injection are removed before the next injection occurs.

The quality of the separation on the column depends on the quality of the injection—a short, sharp injection increases the likelihood of short, sharp peaks. The use of a minimum number of fittings between the injector and the column reduces the diffusion of the contents of the sample loop into the mobile-phase fronts in front of and behind the column. So-called low dead volume fittings with the minimum required internal capacity are available. These fittings have no "dead ends" or unnecessary spaces where solvent and sample can mix.

Manual injectors

Simple manual injectors remain popular in some laboratories because they are inexpensive and because their operation requires little previous experience, which is important if the equipment is used infrequently. With a precision syringe, the operator can fill the sample loop at atmospheric pressure by injecting the contents into the injection port. A switch of the rotor attached to the valve realigns the valve ports to the *inject* position. Solvent from the pump then flushes the contents of the sample loop onto the column. Continual flushing during the run keeps the injection port and valve clear of remnants of previous samples.

✔	✘
Inexpensive.	No automation and no provision for online derivatization. Syringe must be cleaned manually, offline.

Automated injectors

Automated injectors contain a mechanically driven version of the same six-port valve found in manual injectors. Pneumatic or electrical actuators control the valve as it switches between steps in the injection cycle. A metering device can handle injection volumes of 0.1–1500 µl. With sample loops of larger capacity, such a device can inject up to 5 ml. Vials designed to hold microliter volumes can be used to inject as little as 1 µl of a 5-µl sample. Even the way the needle enters the vial can be controlled with computer software: deep down to aspire from the denser of two layers, or a shallow dip into the supernatant phase. With this technique, even viscous samples can be analyzed if the right equipment is used. The time required to extract the syringe plunger is simply protracted, permitting meniscus motion of higher reproducibility.

✔	✘
Highly reproducible. Can be fully automated. Flow maintained over all parts in contact with sample, preventing inaccuracies from intermingling between runs.	Equipment can be costly.

Autosampler with sample pretreatment capabilities

Autosamplers can provide online precolumn derivatization, dilute small volumes of sample, and add internal standards. You may need to protect unstable species by keeping the sample chilled with a cooling device connected to a flow of refrigerated water or, more conveniently, by Peltier elements. Alternatively, you might want to induce reactions using heat applied within the injection device of the autosampler. Commercially available autosamplers offer all these features.

Derivatization

As discussed later in chapter 8, derivatization may be required if the analytes lack chromophores and if detection is not sensitive enough. In this process, a chromophore group is added using a derivatization reagent. Derivatization can occur either in front of or behind the analytical column and is used to improve sensitivity and/or selectivity. Precolumn derivatization is preferable because it requires no additional reagent pump and because reagents can be apportioned to each sample rather than pumped through continuously. Automated precolumn derivatization yields excellent precision. Moreover, it can handle volumes in the microliter range, which is especially important when sample volume is limited. The principles involved are illustrated in figure 47.
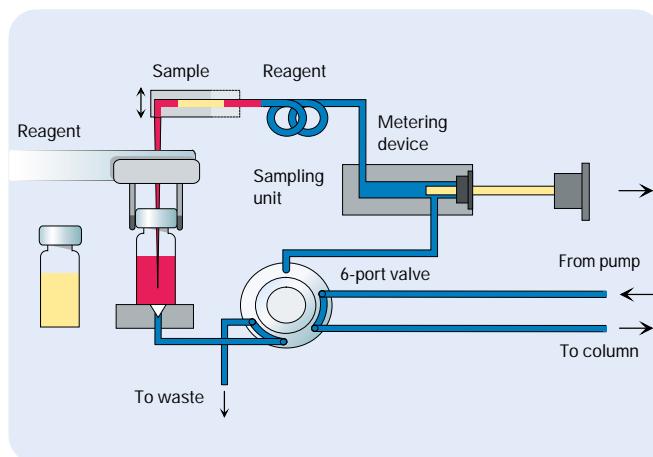
Figure 47
Automated precolumn derivatization

The robotic arm of the autosampler transports, in turn, a sample vial and several reaction vials under the injection needle. The needle is extended by a length of capillary at the point at which the derivatization reaction takes place.

The injector draws distinct plugs of sample and derivatization reagent into the capillary. The back-and-forth movement of the plunger mixes the plugs. With the right software, the autoinjector can be paused for a specified length of time to allow the reaction to proceed to completion. If the reaction requires several reagents, the autosampler must be programmable, that is, it must be able to draw sequentially from different reagent vials into one capillary.
In this complex sample manipulation, the needle must be cleaned between vials, for example by dipping into wash vials of distilled water.

In brief…

Automated sampling systems offer significant advantages over manual injectors, the most important of which is higher reproducibility of the injection volume. Sample throughput also can be increased dramatically. Modern autosamplers are designed for online sample preparation and derivatization. For food analysis, an automated injection system is the best choice.

Chapter 7

**Mobile phase
pumps and
degassers**
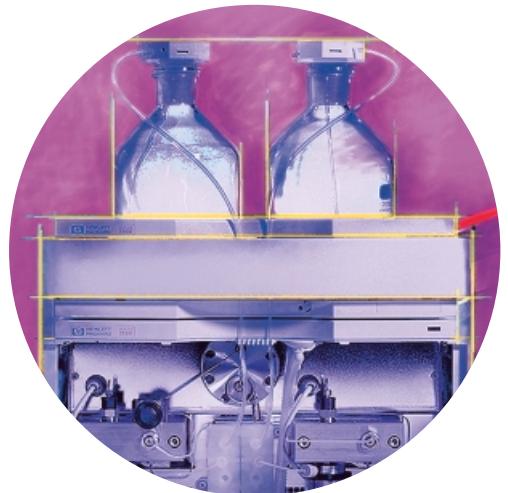
The pump is the most critical piece of equipment for successful HPLC. Performance depends strongly on the flow behavior of the solvent mixture used as mobile phase—varying solvent flow rates result in varying retention times and areas. Conclusions from a calibration run for peak identification or quantification depend on reproducible data. In this chapter we discuss multiple aspects of pump operation, including solvent pretreatment and its effect on performance.

Characteristics of a modern HPLC pump

A modern HPLC pump must have pulse-free flow, high precision of the flow rates set, a wide flow rate range, and low dead volume. In addition, it must exhibit control of a maximum operating pressure and of at least two solvent sources for mobile-phase gradients, as well as precision and accuracy in mixing composition for these gradients.

Flow ranges

We discuss two gradient pump types: that constructed for flow rates between 0.2 and 10 ml/min (low-pressure gradient formation), and that designed for flow rates between 0.05 and 5 ml/min (high-pressure gradient formation).

Gradient elution

In separating the multiple constituents of a typical food sample, HPLC column selectivity with a particular mobile phase is not sufficient to resolve every peak. Changing the eluant strength over the course of the elution by mixing increasing proportions of a second or third solvent in the flow path above the column improves peak resolution in two

ways. First, resolution is improved without extending the elution period, which prevents long retention times (peaks that have been retained on the column for a longer period of time tend to broaden and flatten through diffusion, lowering the S/N and therefore detection levels). Second, gradient elution sharpens peak widths and shortens run time, enabling more samples to be analyzed within a given time frame. The solvents that form the gradient in front of the column can be mixed either after the pump has applied high pressure or before, at low pressure.

Gradient formation at high pressure

If mixing takes place after pressure has been applied, a high-pressure gradient system results (this is most often achieved by combining the output of two isocratic pumps, each dedicated to one solvent).

✔	✘
Ability to form sharp gradient profiles and to change solvents rapidly (100% A to 100% B), without degassing, for standard applications.	Expensive. An additional mixer for lowest mixing noise at flow rates below 200 µl is needed for mobile-phase compositions.

Gradient formation at low pressure

At low pressure, mixing of the gradient solvents occurs early in the flow path before the pump applies pressure, as in the two examples below.

✔	✘
Less expensive than gradient elution. Can mix more than two channels. Low mixing noise without a dedicated mixer.	Degassing is necessary for highest reproducibility.

Pump designs for gradient operation

Low-pressure gradient Agilent 1100 Series pump
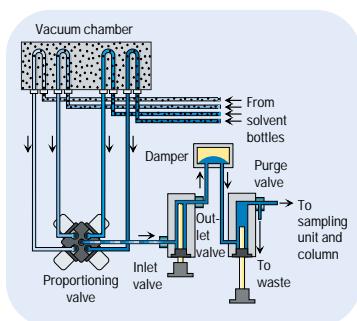
Figure 48
Low pressure gradient pump

In food analysis, pump performance is critical. In the examples, we describe a low-pressure gradient system and a high-pressure gradient system, both of which perform according to food analytical requirements. The former has a single dual-piston mechanism for low-pressure gradient formation, whereas the latter has a double dual-piston mechanism for high-pressure gradient formation. After passing the online vacuum degasser, the mobile phase enters the first pump chamber through an electronically activated inlet valve (see figure 48). Active valves resolve the problem of contaminated or sticky ball valves by making the pump easy to prime. Output from the first piston chamber flows through a second valve and through a low-volume pulse dampener (with pressure transducer) into a second piston chamber. Output from the second chamber flows onto the sampling unit and column. The pistons in the pump chambers are motor driven and operate with a fixed-phase
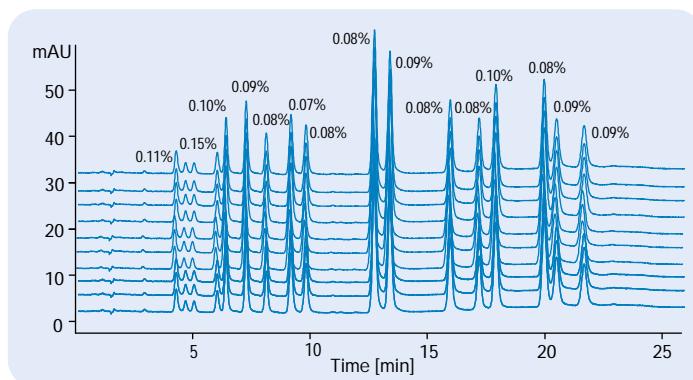
Figure 49
Retention time precision (% RSD) of 10 injections of a polycyclic aromatic hydrocarbon (PNA) standard sample

difference of 180°, so that as one delivers mobile phase, the other is refilling. The volume displaced in each stroke can be reduced to optimize flow and composition precision at low flow rates. With solvent compressibility, compensation, and a low-volume pulse dampener, pulse ripple is minimal, resulting in highly reproducible data for retention times and areas (see figure 49). A wide flow range of up to 10 ml/min and a delay volume of 800–1100 µl support narrow-bore, standard-bore, and semipreparative applications. Four solvents can be degassed simultaneously with high efficiency.
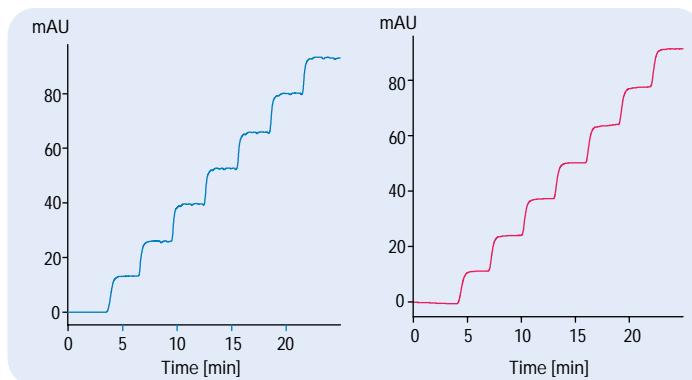
Figure 50
Results of a step-gradient composition (0–7%) of a high-pressure pump (left) and of a low-pressure pump (right)

Performance of low-pressure pump design	
Flow precision	< 0.3 % (typically < 0.15 %) based on retention times of 0.5 and 2.5 ml/min
Flow range	0.2–9.999 ml/min
Delay volume	ca. 800–1100 µl
Pressure pulse	< 2 % amplitude (typically < 1 %), 1 ml/min propanol, at all pressures
Composition precision	± 0.2 % SD at 0.2 and 1 ml/min

In this design, gradients are formed by a high-speed proportioning valve that can mix up to four solvents on the low-pressure side. The valve is synchronized with piston movement and mixes the solvents during the intake stroke of the pump. The solvents enter at the bottom of each chamber and flow up between the piston and the chamber wall, creating turbulences. Compared with conventional multisolvent pumps with fixed stroke volumes, pumps with variable stroke volumes generate highly precise gradients, even at low flow rates (see figure 50).

**High-pressure gradient
Agilent 1100 Series pump**

The Agilent 1100 Series high-pressure gradient pump is based on a double dual-piston mechanism in which two pumps are connected in series in one housing. This configuration takes up minimal bench space and enables very short internal and external capillary connections. Both pistons of both individual pumps are servocontrolled in order to meet chromatographic requirements in gradient formation (see figure 51).

Three factors ensure gradients with high precision at low flow rates: a delay volume as low as 180–480 µl internal volume (without mixer), maximum composition stability and retention time precision, and a flow range typically beginning at 50 µl/min.

The same tracer gradient used to determine composition precision and accuracy also was used to determine the ripple of the binary pump (see figures 50 and 52). The delay volume was measured by running a tracer gradient. Large delay volumes reduce the sharpness of the gradient and therefore the selectivity of an analysis. They also increase the run-time cycle, especially at low flow rates.

Performance of high-pressure pump design	
Flow precision	< 0.3 %
Flow range	0.05–5 ml/min
Delay volume	180–480 µl (600–900 µl with mixer)
Pressure pulse	< 2 % amplitude (typically, 1 %), 1 ml/min isopropanol, at all pressure > 1MPa
Composition precision	< 0.2 % at 0.1 and 1.0 ml/min
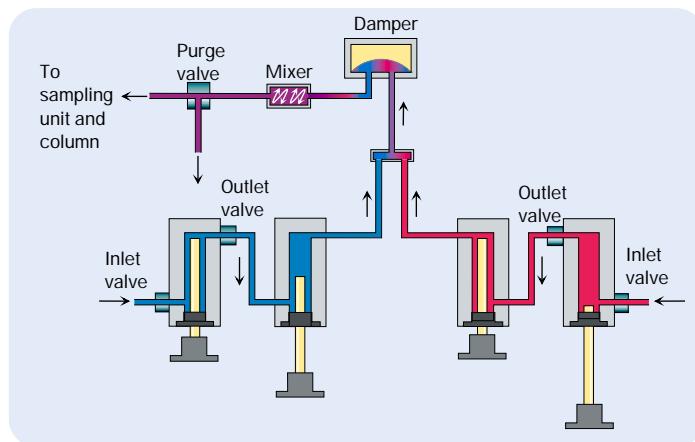
Figure 51
Schematics of the high-pressure gradient Agilent 1100 Series pump

When working at the lowest detection limits, it is important to use a mixer to reduce mixing noise, especially at 210–220 nm and with mobile phases containing solvents such as tetrahydrofuran (THF). Peptide mapping on 1-mm columns places stringent demands on the pump because small changes in solvent composition can result in sizeable changes in retention times. Under gradient conditions at a flow rate of 50 µl/min, the solvent delivery system must deliver precisely 1 µl/min per channel. A smooth baseline and nondistorted gradient profiles depend on good mixing and a low delay volume. Figure 53 shows six repetitive runs of a tryptic digest of myoglobin with a retention time precision of 0.07–0.5% RSD.
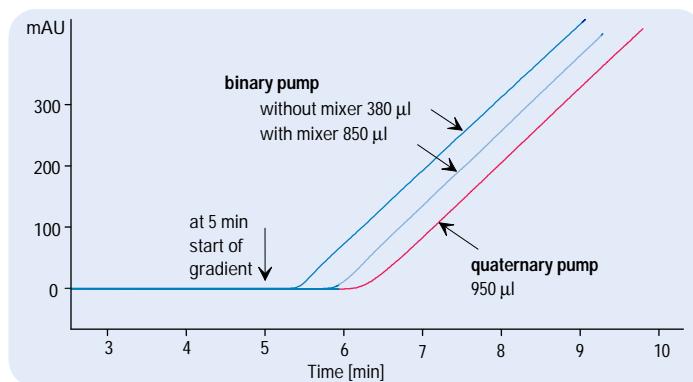
Figure 52
Delay volume of high- and low-pressure gradient pumps
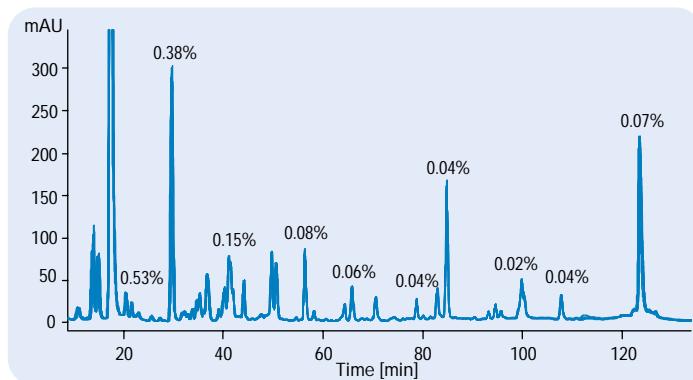
Figure 53
Overlay of six repetitive runs of a tryptic digest of myoglobin in RSD of
RT is as low as 0.07–0.5 %

Degassing

Degassing removes dissolved gases from the mobile phase before they are pumped over the column. This process prevents the formation of bubbles in the flow path and eliminates volumetric displacement and gradient mixing, which can hinder performance. Instable flow causes retention on the column and may increase noise and drift on some flow-sensitive detectors. Most solvents can partially dissolve gases such as oxygen and thereby harm detectors. Detrimental effects include additional noise and drift in UV detectors, quenching effects in fluorescence detectors, and high background noise from the reduction of dissolved oxygen in electrochemical detectors used in reduction mode (in oxidation mode, the effect is less dramatic).

Fluorescence

Signal heights
for selected PNAs

Time [min]

■ Agilent on-line degassing
■ Helium degassing
■ No degassing
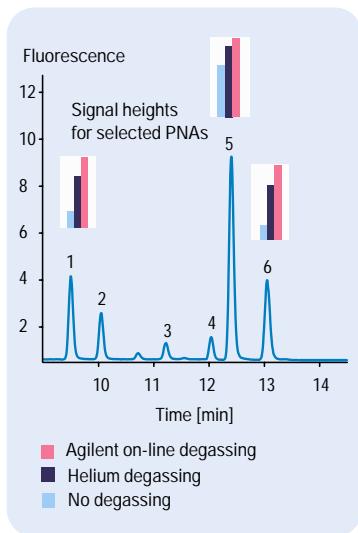
Figure 54
The loss of response due to
quenching can be recovered with
either helium or vacuum degassing.

The oxygen effect is most apparent in the analysis of polycyclic aromatic hydrocarbons (PNAs) with fluorescence detection, as shown in figure 50. The less oxygen present in the mobile phase, the less quenching occurs and the more sensitive the analysis.

In general, one of three degassing techniques is used: on- or offline vacuum degassing, offline ultrasonic degassing, or online helium degassing. Online degassing is preferable since no solvent preparation is required and the gas concentration is held at a constant, minimal level over a long period of time. Online helium and online vacuum degassing are the most popular methods.

Helium degassing

In helium degassing, gas is constantly bubbled through the mobile-phase reservoir. This process saturates the solvent and forces other gases to pass into the headspace above.

✔	✘
Requires only a simple regulator. Several channels can be purged simultaneously without additional dead volume.	Expensive. Evaporation of the more volatile components can change composition over time. Oxygen is better purged by vacuum degassing.

Vacuum degassing

In vacuum degassing, the solvent is passed through a membranous tube made of a special polymer that is permeable to gas but not to liquids under vacuum. The pressure differences between the inside and outside of the membrane cause continuous degassing of the solvent. New online degassers with low internal volume (< 1 ml) allow fast changeover of mobile phases.

✔	✘
Less expensive to use and maintain than helium degassing. The composition of premixed solvents is unaffected, and removal of oxygen is highly efficient. Several channels can be degassed simultaneously.	Increases dead volume and may result in ghost peaks, depending on the type of tubing and type of solvent used.

In brief…

The choice of pump depends on both elution mode (isocratic or gradient) and column diameter (narrow bore or standard bore). Although an isocratic system often is sufficient, gradient systems are more flexible. Moreover, their short analysis times make gradient systems ideal for complex samples, sharp peaks, resolution of multiple species, and automatic system cleansing with additional online solvent channel. Agilent 1100 Series pumps are best suited for flow ranges from 0.05 ml/min up to 10 ml/min and can therefore be used with columns that have an inner diameter of 1 mm to 8 mm. Although many officially recognized methods are based on standard columns and flow rates, the trend is toward narrow-bore columns. These consume less solvent, which also reduces waste disposal, thus lowering operating costs.

Chapter 8

Detectors
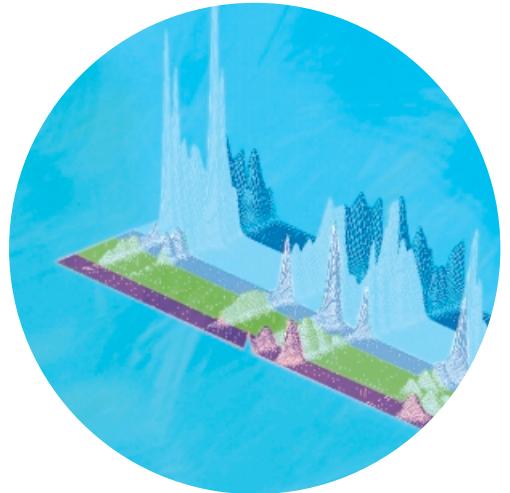
Most detectors currently used in HPLC also can be applied in the analysis of food analytes. Each technique has its advantages and disadvantages. For example, diode array UV-absorbance detectors and mass spectrometers provide additional spectral confirmation, but this factor must be weighed against cost per analysis when deciding whether to use a detector routinely.

The ability to use UV spectra to confirm the presence of certain food analytes and their metabolites and derivatives makes UV absorbance the most popular detection technique. However, for analytical problems requiring high sensitivity and selectivity, fluorescence detection is the method of choice. Although electrochemical detectors are also highly sensitive and selective, they are rarely used in food analysis. Conductivity detectors, on the other hand, are well-suited for the sensitive and selective analysis of cations and anions, and thermal energy detectors are used for high-sensitivity determination of nitrosamines down to 10 parts per trillion (ppt). Refractive index (RI) detectors are appropriate only if the above-mentioned detectors are not applicable or if the concentration of analytes is high, or both.

Analytical parameters

The most important parameters for food analysis are:

- limit of detection (LOD) and limit of quantification (LOQ)
- linearity
- selectivity
- qualitative information

Limit of detection and limit of quantification

The LOD and LOQ of an analytical system depend on the noise and drift of the detection equipment. Absolute detector LOD can be determined by injecting a sample directly into the detector. It is often expressed as minimum detectable level, which is sometimes defined as equal to the noise level. However, the LOD depends not only on the detector but may also depend on the oxygen content of the mobile phase, the injection system, peak broadening on the column, and temperature differences among system components. Taking these factors into account, the LOD is defined as 2 to 3 times the noise level. The LOQ is defined as 10 to 20 times the noise level. A UV detection system can be used to measure quantitative amounts down to 500 pg per injection. The LOD can be as low as 100 pg for food compounds such as antioxidants if detection wavelengths have been optimized to match the extinction coefficients of as many compounds as possible. Fluorescence and electrochemical detectors operate in the very low picogram range. The LOD of a mass spectrometer connected to HPLC equipment depends on the type of interface used. Instruments with electrospray interfaces can detect down to the picogram range. Refractive index detectors normally are appropriate above 500 ng.

Selectivity

We define the selectivity of a detection system as the ability to select only those compounds of interest in a complex matrix using specific compound properties. A detector is selective if it does not respond to coeluting compounds that

could interfere with analyte quantification. A UV absorbance detector can be made selective by setting an appropriate wavelength with a narrow bandwidth for the compound of interest. However, the selectivity of detectors based on such a universal feature is low compared with the selectivity of detectors based on fluorescence and electrochemistry. Response characteristics are very selective, shown by a limited number of compounds. Mass spectrometers can be applied selectively or universally (in total scan mode), depending on the analysis to be performed. RI detectors are universal by definition.

Linearity Detector response can be expressed both as dynamic range and as linear dynamic range. Dynamic range is the ratio of the maximum and the minimum concentration over which the measured property (absorbance, current, and so on) can be recorded. However, in practice, linear dynamic range— the range of solute concentration over which detector response is linear—is more commonly used. Plotting the response of injections of different analyte concentration against their concentrations should give a straight line over part of the concentration range. Response often is linear for only one tenth of the full dynamic range. UV detectors are linear over a range of a maximum of five orders of magnitude, whereas fluorescence and electrochemical detectors are linear over a range of two orders of magnitude. Mass spectrometers are usually linear over three orders of magnitude, and RI detectors are linear over a maximum of four orders of magnitude.

Qualitative information A classical identification tool in chromatography is the mass spectrogram, which is recorded by a mass spectrometer. Its appeal in HPLC, however, is limited owing to the cost of interfacing the mass spectrometer equipment. If the spectra of the analytes differ markedly, UV absorbance spectra can be used for identification using diode array technology. Fluorescence and electrochemical detectors can be used only to identify compounds based on their retention times.

UV detectors

Figure 55 shows the optical path of a conventional variable wavelength detector. Polychromatic light from a deuterium lamp is focused onto the entrance slit of a monochromator using spherical and planar mirrors. The monochromator selectively transmits a narrow band of light to the exit slit. The light beam from the exit slit passes through the flow cell and is partially absorbed by the solution in the flow cell. The absorbance of the sample is determined by measuring the intensity of the light reaching the photodiode without the sample (a blank reference) and comparing it with the intensity of light reaching the detector after passing through the sample.
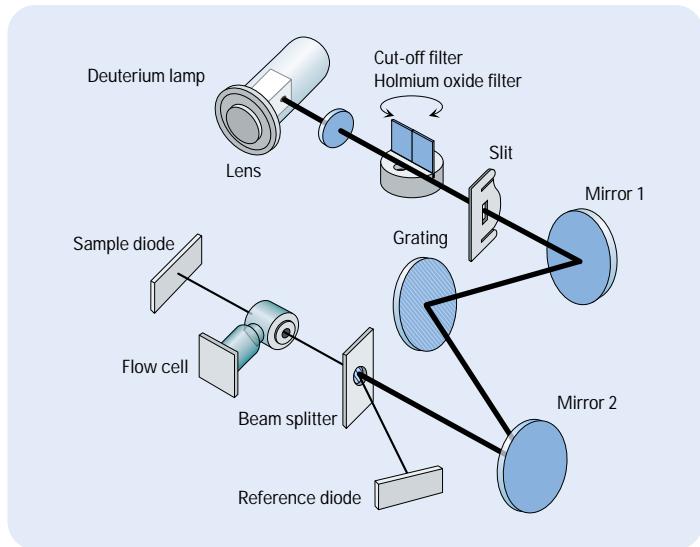
Figure 55
Conventional variable wavelength detector

Most variable wavelength detectors split off part of the light to a second photodiode on the reference side. The reference beam and the reference photodiode are used to compensate for light fluctuations from the lamp. For optimum sensitivity,

the UV detector can be programmed for each peak within a chromatographic run, which changes the wavelength automatically. The variable wavelength detector is designed to record absorbance at a single point in the spectrum at any given point in time. However, in practice, different wavelengths often must be measured simultaneously, for example when two compounds cannot be separated chromatographically but have different absorbance maxima. If the entire spectrum of a compound is to be measured, the solvent flow must be stopped in order for a variable wavelength detector to scan the entire range, since scanning takes longer than elution.

✔	✘
Sensitive; can be tuned to the wavelength maxima of individual peaks. Some instruments are equipped with scanning mechanisms with stopped-flow operation.	Single-wavelength measurement is not always sufficient. Without spectra, peaks cannot be identified.

Diode array detectors

Figure 52 shows a schematic diagram of a photodiode array detector (DAD). An achromatic lens system focuses poly-
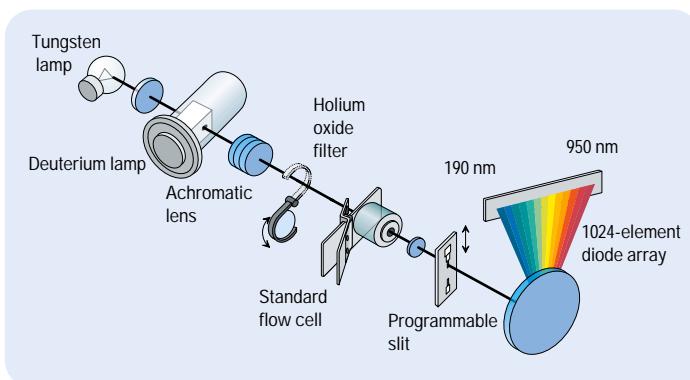
Figure 56
Diode array detector optics
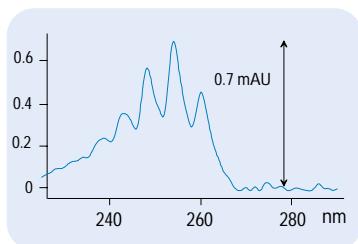
Figure 57
High-resolution spectrum for benzene in the low absorbance range

	Conventional	DAD
Signal acquisition	1	8
Spectra acquisition	stop flow	on-line

Three dimensions of data

chromatic light from the deuterium and tungsten lamps into the flow cell. The light then disperses on the surface of a diffraction grating and falls on the photodiode array. The range varies from instrument to instrument. The detector shown here is used to measure wavelengths from 190 to 950 nm using the twin-lamp design.

In our example, the array consists of 1024 diodes, each of which measures a different narrow-band spectrum. Measuring the variation in light intensity over the entire wavelength range yields an absorption spectrum. The bandwidth of light detected by a diode depends on the width of the entrance slit. In our example, this width can be programmed to selected values from 1 to 16 nm. If very high sensitivity is required, the slit is opened to 16 nm for maximum light throughput. If maximum spectral resolution is needed, the slit is narrowed to 1 nm. At this setting, the fine structure of benzene can be detected, even at 0.7 mAU full-scale (mAUFS; see figure 57). Because the relative positions of the sample and the diffraction grating are reversed compared with a conventional instrument, this configuration is often referred to as reversed optics. The most significant differences between a conventional UV absorbance detector and a DAD are listed at left.

DADs connected to appropriate data evaluation units help optimize wavelengths for different compounds over the course of the run. Maxima can be seen easily using three-dimensional plots of data, or as absorbance intensity plotted over time at different wavelengths, that is, as an isoabsorbance plot (see figure 58). Figure 55 illustrates the optimization result for antibiotics. The ability to acquire and store spectra permits the creation of electronic spectral libraries, which can be used to identify sample compounds during method development.
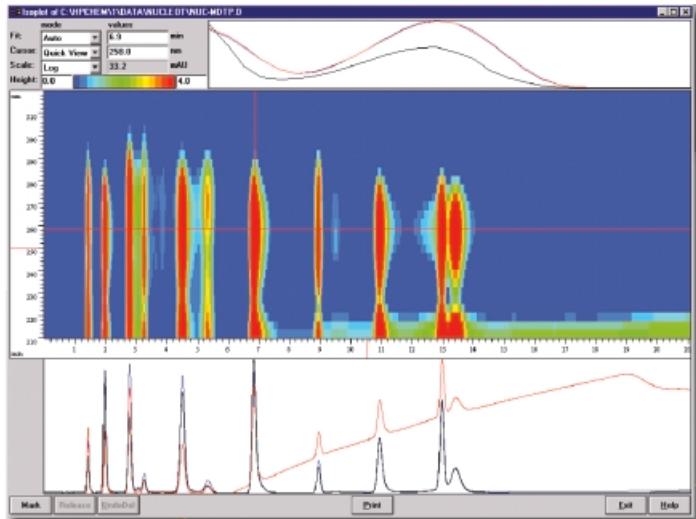
Figure 58
Isoabsorbance plot

Multisignal detection yields optimum sensitivity over a wide spectral range. However, the spectral axis in figure 58 shows that no single wavelength can detect all antibiotics at highest sensitivity.
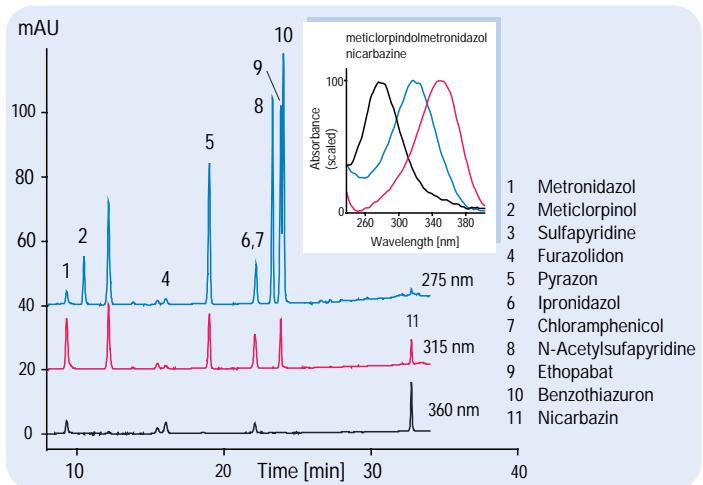
Figure 59
Multisignal detection of antibiotic drugs

1	Metronidazol
2	Meticlorpinol
3	Sulfapyridine
4	Furazolidon
5	Pyrazon
6	Ipronidazol
7	Chloramphenicol
8	N-Acetylsufapyridine
9	Ethopabat
10	Benzothiazuron
11	Nicarbazin

Figure 60
Peak purity analysis
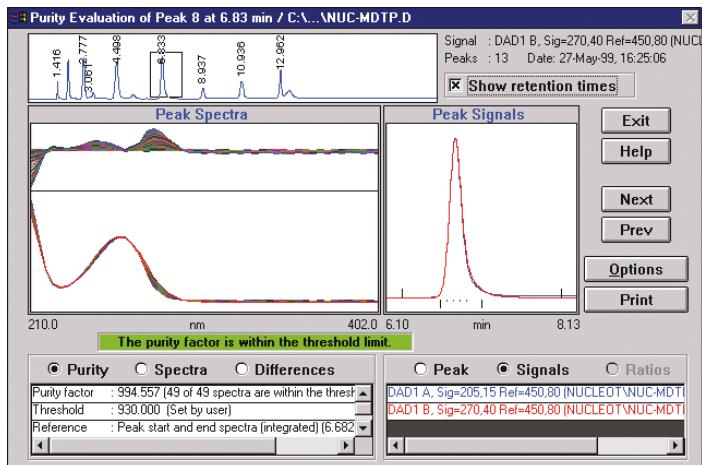
In light of the complexity of most food samples, the ability to check peak purity can reduce quantification errors. In the most popular form of peak purity analysis, several spectra acquired during peak elution are compared. Normalized and overlaid, these spectra can be evaluated with the naked eye, or the computer can produce a comparison. Figure 60 shows a peak purity analysis of antibiotics. If a spectral library has been established during method development, it can be used to confirm peak identity. Analyte spectra can be compared with those stored in the library, either inter-actively or automatically, after each run.

Figure 61 shows both the quantitative and qualitative results of the analysis. Part one of this primer contains several applications of UV absorbance DAD detection.

Match > 950

1 ?-*Metronidazole
2 ?-*Meticlorpindol
3 Sulfapyridine
4 Furazolidone
5 Pyrazon
6 ?-*Ipronidazole

7 Chloramphenicol
8 N-Acetylsulfapyridine
9 Ethopabate
10 Benzothiazuron
11 *Nicarbazin
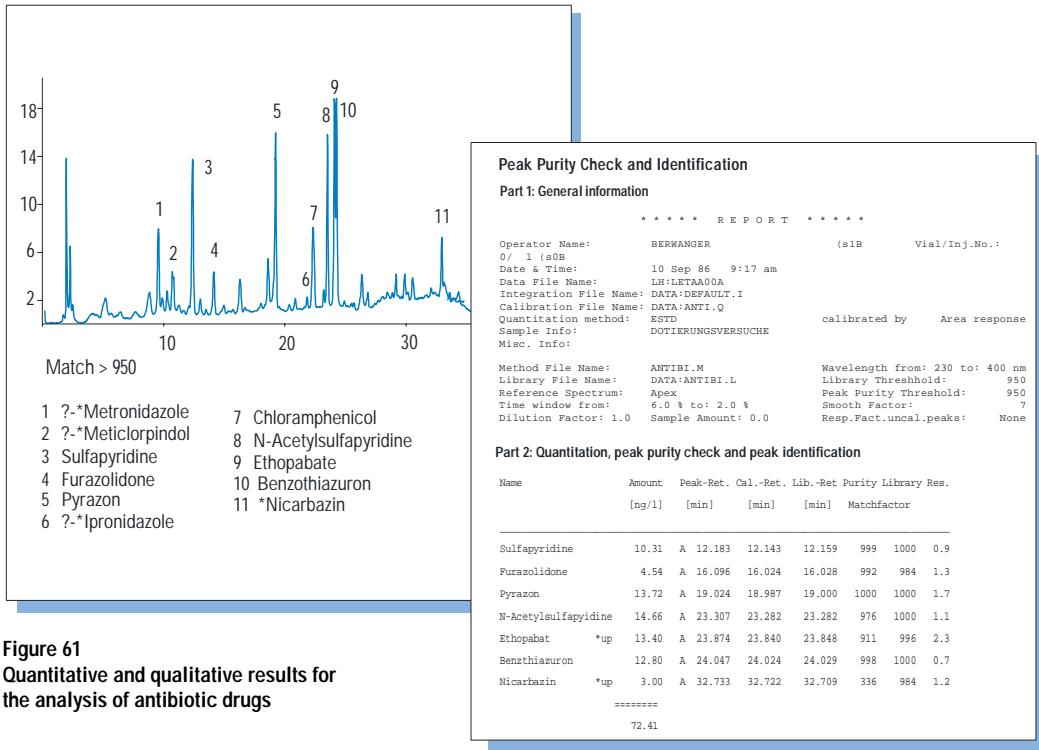
Figure 61
Quantitative and qualitative results for the analysis of antibiotic drugs

Peak Purity Check and Identification

Part 1: General information

```
                    * * * * *  R E P O R T  * * * * *
Operator Name:        BERWANGER            (s1B      Vial/Inj.No.:
0/  1 (s0B
Date & Time:          10 Sep 86   9:17 am
Data File Name:       LH:LETAA00A
Integration File Name: DATA:DEFAULT.I
Calibration File Name: DATA:ANTI.Q
Quantitation method:  ESTD                 calibrated by    Area response
Sample Info:          DOTIERUNGSVERSUCHE
Misc. Info:

Method File Name:     ANTIBI.M             Wavelength from: 230 to: 400 nm
Library File Name:    DATA:ANTIBI.L        Library Threshhold:        950
Reference Spectrum:   Apex                 Peak Purity Threshold:     950
Time window from:     6.0 % to: 2.0 %      Smooth Factor:               7
Dilution Factor: 1.0  Sample Amount: 0.0   Resp.Fact.uncal.peaks:    None
```

Part 2: Quantitation, peak purity check and peak identification

Name	Amount [ng/l]	Peak-Ret. [min]	Cal.-Ret. [min]	Lib.-Ret [min]	Purity Matchfactor	Library	Res.
Sulfapyridine	10.31	A 12.183	12.143	12.159	999	1000	0.9
Furazolidone	4.54	A 16.096	16.024	16.028	992	984	1.3
Pyrazon	13.72	A 19.024	18.987	19.000	1000	1000	1.7
N-Acetylsulfapyidine	14.66	A 23.307	23.282	23.282	976	1000	1.1
Ethopabat *up	13.40	A 23.874	23.840	23.848	911	996	2.3
Benzthiazuron	12.80	A 24.047	24.024	24.029	998	1000	0.7
Nicarbazin *up	3.00	A 32.733	32.722	32.709	336	984	1.2

```
                ========
                  72.41
```

✔	✘
Enables maximum peak purity and identity, measurement of multiple wavelengths, acquisition of absorbance spectra, and spectral library searches.	DADs are best suited for universal rather than sensitive analysis (for which electrochemical or fluorescence detection is more appropriate).

Fluorescence detectors
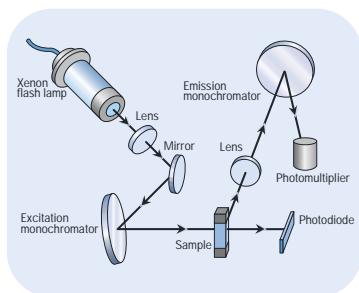
Fluorescence is a specific type of luminescence that is created when certain molecules emit energy previously absorbed during a period of illumination. Luminescence detectors have higher selectivity than, for example, UV detectors because not all molecules that absorb light also emit it. Fluorescence detectors are more sensitive than absorbance detectors owing to lower background noise. Most fluorescence detectors are configured such that fluorescent light is recorded at an angle (often at a right angle) to the incident light beam. This geometry reduces the likelihood that stray incident light will interfere as a background signal and ensures maximum S/N for sensitive detection levels.

The new optical design of the Agilent 1100 Series fluorescence detector is illustrated in figure 62. A Xenon flash lamp is used to offer the highest light intensities for excitation in the UV range. The flash lamp ignites only for microseconds to provide light energy. Each flash causes fluorescence in the flow cell and generates an individual data point for the chromatogram. Since the lamp is not powered on during most of the detector operating time, it offers a lifetime of several thousand hours. No warmup time is needed to get a stable baseline. A holographic grating is used as a monochromator to disperse the polychromatic light of the Xenon lamp. The desired wavelength is then focused on the flow cell for optimum excitation. To minimize stray light from the excitation side of the detector, the optics are configured such that the emitted light is recorded at a 90 degree angle to the incident light beam. Another holographic grating is used as the emission monochromator. Both monochromators have optimized light throughput in the visible range.

A photomultiplier tube is the optimum choice to measure the low light intensity of the emitted fluorescence light. Since flash lamps have inherent fluctuations with respect to flash-to-flash intensity, a reference system based on a

Figure 62
Schematics of a fluorescence detector

photodiode measures the intensity of the excitation and triggers a compensation of the detector signal.

Cut-off filter

Since the vast majority of emission maxima are above 280 nm, a cut-off filter (not shown) prevents stray light below this wavelength to enter the light path to the emission monochromator. The fixed cut-off filter and bandwidth (20 nm) avoid the hardware checks and documentation work that is involved with an instrument that has exchangeable filters and slits.

Signal/spectral mode

The excitation and emission monochromators can switch between signal and spectral mode. In signal mode they are moved to specific positions that encode for the desired wavelengths, as with a traditional detector. This mode offers the lowest limits of detection since all data points are generated at a single excitation and emission wave length.

Online spectral measurements and multisignal acquisition

A scan of both the excitation and the emission spectra can be helpful in method development. However, only detectors with motor-driven gratings on both sides can perform such a scan. Some of these detectors also can transfer this data to a data evaluation computer and store spectra in data files. Once the optimum excitation and emission wavelength has been determined using scanned spectra, detectors with grating optics can be programmed to switch between these wavelengths during the run.

The spectral mode is used to obtain multi-signal or spectral information. The ignition of the flash lamp is synchronized with the rotation of the gratings, either the excitation or emission monochromator. The motor technology for the gratings is a long-life design as commonly used in high-speed PC disk drive hardware. Whenever the grating has reached the correct position during a revolution, the Xenon lamp is ignited to send a flash. The flash duration is below two microseconds while the revolution of the grating takes less than 14 milliseconds. Because of the rotating monochromators, the loss in sensitivity in the spectral

mode is much lower compared to conventional dual-wave-length detection with UV detectors.

Multisignal   PNA analysis, for example, can be performed with simulta-neous multi wavelength detection instead of wavelength-switching. With four different wavelengths for emission, all 15 PNAs can be monitored (figure 63).
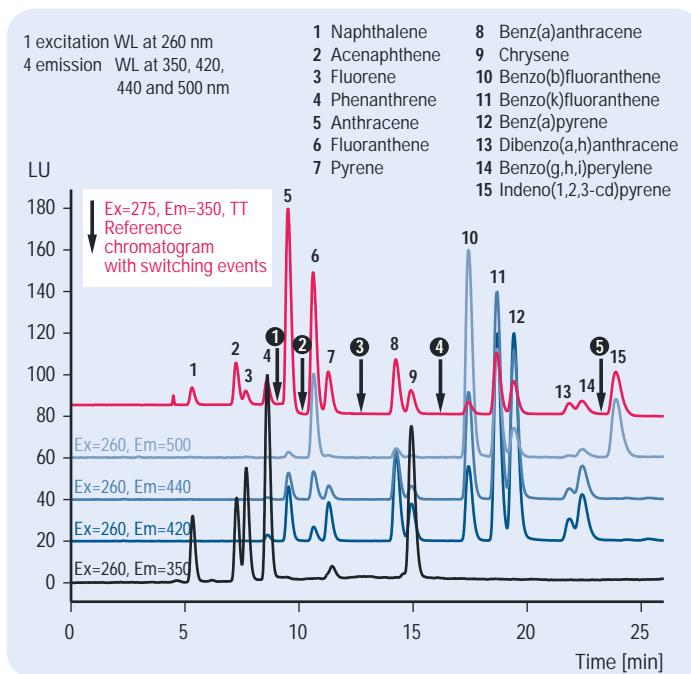
Figure 63
Simultaneous multi wavelength detection for PNA-analysis
The upper trace was received with traditional wavelength switching.
❶ **Ex/Em = 260/420 nm**
❷ **Ex/Em = 270/440 nm**
❸ **Ex/Em = 260/420 nm**
❹ **Ex/Em = 290/430 nm**
❺ **Ex/Em = 250/550 nm**

✔	✘
Highly specific. Flash lamps eliminate drawback of baseline drift from heat transfer. Fluorescent tagging improves detection limits.	Fluorescence spectra are not commonly used to confirm peak identity.

Electrochemical detectors

Electrochemical detection techniques are based on the electrical charge transfer that occurs when electrons are given up by a molecule during oxidation or absorbed by a molecule during reduction. This oxidation or reduction takes place on the surface of a so-called working electrode. Whether a compound is reduced or oxidized and the speed of the reaction depend on the potential difference between the working electrode and the solution containing the compounds. From the activation energies and redox potentials expressed by the Nernst equation, reaction speed can be determined. The resulting current is proportional to the number of reactions occurring at the electrode, which in turn is an indicator of the concentration of the compound of interest at the surface.

In the detection process, three electrodes are used: the working electrode, in which the reaction takes place; the counter electrode, which applies the potential difference between mobile phase and the working electrode; and the reference electrode, which compensates for any change in eluant conductivity (see figure 64). The reference electrode readings feed back to the counter electrode in order to keep the potential difference constant during peak elution as current flows through the working electrode.

Figure 64
Three-electrode electrochemical detector
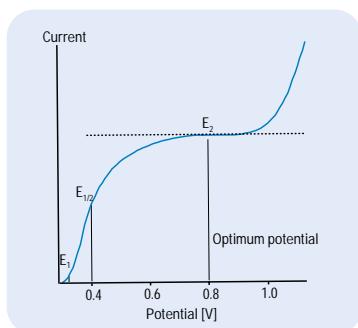
Figure 65
Current-voltage relationship

Detector response results from amplification of the electron flow and its subsequent conversion to a signal. Extremely low currents representing analyte quantities in the picogram range and below can be measured with today's advanced electronics. Although electrochemical detection can detect only those substances that can be electrolyzed, this limitation is actually an advantage when applied to complicated food matrices because it improves selectivity. To determine the optimum working electrode potential, the relationship between detector response (current) and potential applied (voltage) must be plotted for each compound as a current-voltage (CV) curve, as shown in figure 65. At a potential less than E1, oxidation cannot occur because the supply of energy is insufficient. Increasing the potential to E1/2 will electrolyze 50 % of all molecules at the surface of the electrode. Maximum response requires a potential just above E2. This potential is known as the limiting current because any further increase in voltage will limit detection by raising noise.

Electrode materials

Several materials are used in working electrodes, the most common of which is glassy carbon. These materials also include gold (for sugars and alcohols), platinum (for chlorite, sulfite, hydrazine, and hydrogen peroxide), silver (for halogens), copper (for amino acids), mercury (in reductive mode for thiosulfate), and combined mercury-gold (in reductive mode for nitrogenous organic compounds).

Flow cell aspects

Numerous cell designs have been described in the literature. The majority can be classified as one of three principal types: thin-layer design, wall-jet design, and porous flow-through design (see figure 66). The porous flow-through cell design differs significantly from the other two in that coulometric detection ensures 100 % reaction yield on the surface of the electrode. The other designs allow an efficiency of only 1–10 % by amperometric detection. However, amperometric detection is usually the more sensitive technique and is preferred over coulometric

detection. electrochemical detectors can employ 1-µl flow cells and are well-suited to narrow-bore HPLC.
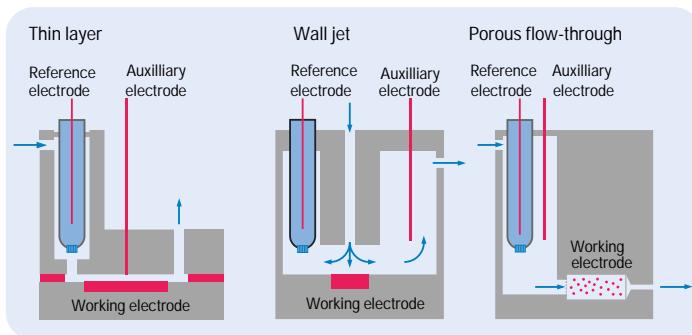
Figure 66
Thin-layer design, wall-jet design, and porous flow-through design

Automation features

Until recently, the electrochemical technique was considered difficult to apply and not stable enough for routine analysis. However, recent improvements have made the use of these detectors routine, for example in the analysis of catecholamines in clinical research and routine testing laboratories. When applied between runs or even during peak elution (for example in sugar analysis using gold electrodes), self-cleaning routines based on pulsed amperometry improve stability (see figure 67).

Although an optimum potential for a mixture of compounds can be determined by evaluating the voltamograms for each compound, these optimizing steps can be automated using certain electrochemical detectors in so-called auto-increment mode. The HPLC equipment runs a series of injections over a range of increasing potentials (defined by start and end potentials and increment parameter), as shown in figure 68. A drift sensor helps ensure that a specified threshold is maintained before the next analysis begins (see figure 69).

Figure 67
Cleaning of working electrode

Figure 68
Autoincrement mode
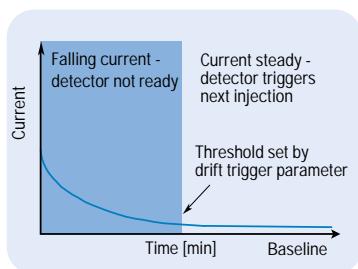
Figure 69
Drift trigger

Should the electrode surface of the flow cell become severely contaminated, as is likely for food matrixes, the cell must be disassembled and the electrode removed and cleaned in a strong acid or other suitable cleaning agent. Modern detectors are designed for ease of access and disassembly. Part one of this primer contains several applications of electrochemical detection.

Mass spectrometers
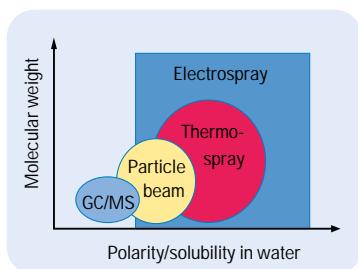
Figure 70
Suitability of MS interfaces

The identification of complex samples presents a problem for LC analysis. Coeluting compounds generally can be identified using UV absorbance detection with diode array technology, but this method may not be specific enough where spectra differences are low. Detection techniques such as fluorescence may offer higher specificity than UV detection, but if many different compounds are to be analyzed, these techniques also may not yield desired results. With mass spectroscopy (MS), several different analyte classes in a wide variety of sample types can be identified with greater certainty. Although GC/MS is a well-established technique for food analysis, LC/MS is only now emerging as a useful tool in this area. A GC-based analysis is appropriate only for those food compounds that are volatile and thermally stable (see figure 70). However, many compounds are nonvolatile, extremely polar, or thermally labile. Such compounds often can be separated successfully with LC, and the development of improved interfaces has made LC/MS more popular.

HPLC	MS
High pressure liquid phase separation	High vacuum required
Produces large quantities of volatilized solvent (100–1000 ml/min gas)*	Typical MS vacuum systems designed for low ml/min gas load
No mass range limitation	Depends on masss/charge and mass range of analyzer
Can use inorganic buffers	Prefers volatile buffers

* About 1000-fold increase going from liquid to gas phase with typical LC solvent

Mass spectrometers nevertheless are more easily interfaced with GC equipment than with LC equipment. The table at left lists the different operational conditions of LC and MS. Early efforts to interface LC with MS used direct liquid injection and moving-belt interfaces, but these methods proved ineffective and unreliable. In the 1980s, thermospray and particle beam interfaces improved both the range of applicability and the reliability of LC/MS. However, low sensitivity, the narrow mass and polarity range of analytes, and frequent maintenance requirements limited the effectiveness of these interfaces. More recently, two atmospheric pressure ionization (API) interfaces—electrospray and atmospheric pressure chemical ionization (APCI)—have replaced almost completely thermospray and particle beam techniques. These interfaces have a broad range of analyte molecular weights and polarities, high sensitivity, improved usability, and reduced maintenance needs. Selection of the appropriate LC/MS interface for an application depends on factors such as the polarity, molecular weight, and thermal lability of the analyte.

API interfaces

In electrospray, effluent is directed through a nebulizing needle into a high-voltage field where charged droplets are formed (see figure 71). The charged droplets are then dried
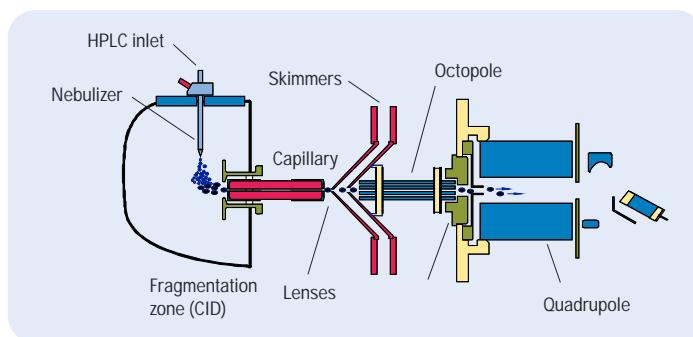
Figure 71
API-electrospray LC/MS interface
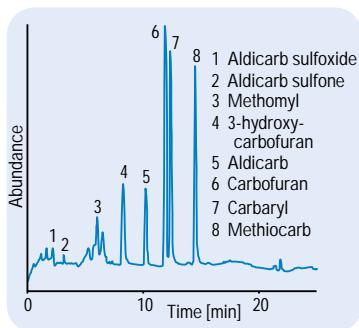
Figure 72
Carbamate analysis

and, as they shrink, analyte ions are desorbed. The ions are transported to the mass analyzer through a series of vacuum stages and ion-focusing elements.

Electrospray ionization can produce multiply charged ions of macromolecular analytes such as proteins and peptides. Because mass analyzers separate ions based on mass-to-charge ratio (m/z), lower-cost mass spectrometers with mass ranges of several thousand m/z can be used to analyze compounds in excess of 150,000 daltons. The primary use of electrospray has been the analysis of compounds of higher molecular weight. However, this technique also has been applied successfully to small polar molecules. Fig. 72 shows a separation of carbamate pesticides using electrospray.
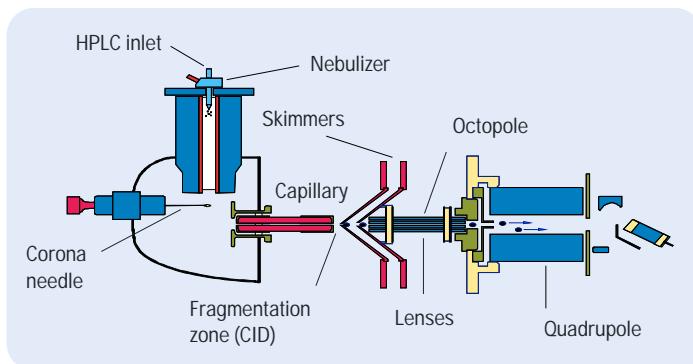
Figure 73
APCI LC/MS interface

APCI also can be used to analyze moderate polarity analytes. As in electrospray, APCI ionization occurs at atmospheric pressure via a chemical ionization process (see figure73).
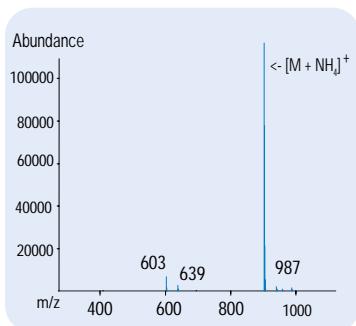
Figure 74
Mass spectrum of the fatty acid triolein (C18:1, [cis]-9) molecular weight = 884.781 molecular formula = $C_{57}H_{104}O_6$

APCI requires some compound volatility and is less suitable for highly thermally labile compounds. Figure 74 shows a typical triglyceride mass spectrum. Both the degree of unsaturation and the length of the fatty acid side chains can be determined from the $[M + NH_4]$ + ion, which corresponds to mass M + 18. In-source CID experiments also can be helpful in determining the fatty acid composition of chromatographic peaks. Full-scan methods allow easy identification at the low nanogram level. If more precise quantitation is required, selected ion mode (SIM) can be used to obtain detection limits at the low picogram level.

✔	✘
Polar and semipolar compounds up to 150,000 daltons can be analyzed. Highly sensitive. Strong molecular ions. Fragments, depending on in-source CID parameters.	Data analysis for complex heterogeneous mixtures of multiply charged analytes is not straightforward. Matrix can interfere with the ionization process.

Refractive index detectors

Refractive index (RI) detection is based on the difference in RI between the solution in the sample cell and the pure mobile-phase solution in the reference cell. Because the composition of the eluents must remain fixed throughout the analysis, this detector is not suitable for gradient analysis. Four main types of RI detectors are available: deflection according to Snell's law, reflection according to Fresnel's law, interference, and Christiansen effect. The first, which uses the dual-cell design, is by far the most popular. However, the nearly designed Agilent 1100 Series refractive index detector allows detection limits to the low ng range. Because RI detectors lack sensitivity and exhibit a tendency to drift owing to temperature changes, they are used primarily in the analysis of carbohydrates and nonaromatic acids.

✔	✘
Universal detector.	Low sensitivity, no gradient operation.

In brief…

The following table reviews the detection techniques discussed in this chapter—your decision ideally should reflect a balance between desired results and financial resources.

Detector	Sensitivity	Selectivity	Advantages	Applications
UV variable wavelength	+	-	Low cost, universal acids	Organic acids, fatty after derivatization, inorganic anions
UV-DAD	+	+	Peak purity confirmation	Antioxidants, preservatives, flavors, colorants, antiparasitic drugs, mycotoxins, pesticides, vitamins, amines after derivatization
Fluorescence	++	+	High sensitivity	Artificial sweeteners, mycotoxins, vitamins, carbamates, glyphosate
Electro-chemical	++	+	High sensitivity	Vitamins, inorganic anions
Mass spectro-meter scan	-	++	Identity, structure	Carbamates, lipids
Mass spectro-meter SIM	++	++	High selectivity	Pesticides, proteins
RI	-	-	Universal	Carbohydrates, nonaromatic acids
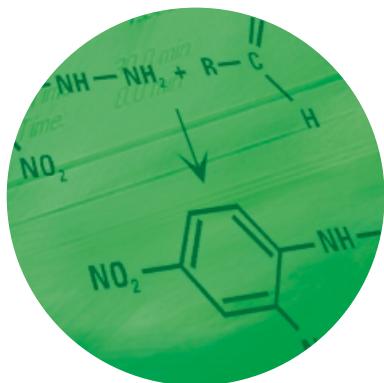
Chapter 9

**Derivatization
chemistries**

9
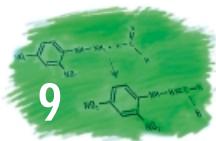
When analyte concentrations are particularly low,
sample handling equipment for chemical derivatization
can enhance the sensitivity and selectivity of results.
As discussed in chapter 6, such equipment is
available both pre- and postcolumn. In this chapter,
we detail the chemistries that can be applied to food
compounds and list the detection techniques for
which they are best suited.

Addition of UV-visible chromophores

Labeling compounds with reagents that enable UV absorp-
tion is one of the most popular derivatization techniques.
The reagent should be selected such that the absorption
maximum of the reaction product exhibits not only
improved sensitivity but also good selectivity. This combi-
nation reduces matrix effects resulting from the reagent,
from by-products, or from the original matrix. The following
table lists common compounds and reactions. In part one of
this primer we give examples of compound derivatization,
including that of fatty acids and amino acids.

Target compound		Reagent	λ
Alcohols	-OH	phenylisocyanate	250 nm
Oxidizable sulfur compounds	SO_2^{3-}	2,2'-dithiobis (5-nitro-pyridine)	320 nm
Fatty acids	-COOH	p-bromophenacyl bromide 2-naphthacyl bromide	258 nm 250 nm
Aldehydes and ketones	-CO-COOH, =C=O, and -CHO	2,4-dinitrophenyl hydrazine	365 nm
Primary amines	$-NH_2$	o-phthalaldehyde (OPA)	340 nm
Primary and secondary amines	NHR	9-fluorenylmethyl chloroformate (FMOC)	256 nm

Addition of a fluorescent tag

Fluorescence is a highly sensitive and selective detection technique. Adding fluorescent properties to the molecule of interest is of particular benefit in food analysis, in which components must be detected at very low concentrations. The following table lists common fluorescent tags. In part one of this primer we give examples for carbamates[41] and glyphosate.[42]

Target compound		Tagging reagent	λ
Alcohols	-OH	phenylisocyanate	λex 230 nm, λem 315 nm
Primary amines	-NH^2	o-phthalaldehyde (OPA)	λex 230 nm, I em 455 nm
Primary and secondary amines	NHR	9-fluorenylmethyl, chloroformate (FMOC)	λex 230 nm, I em 315 nm

Precolumn or postcolumn?
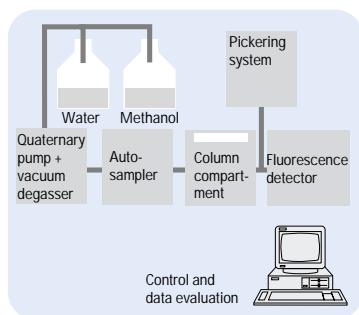
Figure 75
Pickering postcolumn derivatization equipment for the analysis of carbamates

Precolumn techniques can be run either offline or online, but postcolumn techniques should be run online for maximum accuracy. In postcolumn derivatization, reagents can be added only through supplementary equipment (see figure 75) such as pumps. Mixing and heating devices also may be required. Increasing the dead volume behind the column in this way will result in peak broadening. Although this broadening may have no effect on standard-bore columns with flow rates above 1 ml/min, postcolumn derivatization is not suitable for narrow-bore HPLC.
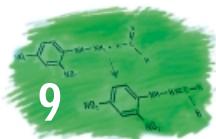
Automatic derivatization

Both pre- and postcolumn derivatization techniques can be automated with modern HPLC equipment. The single-step mechanical functions of an autoinjector or autosampler can be programmed prior to analysis and stored in an injector program (see left). These functions include aspiration of the sample and of the derivatization agent, and mixing. Precolumn derivatization is fully compatible with narrow-bore HPLC and can result in fivefold improvements in S/N, with much lower solvent consumption than that from standard-bore methods. The analysis of fatty acids in part one of this primer illustrates this principle.

1	Draw	1.0 µl	from vial 12
2	Draw	0 µl	from vial 0
3	Draw	1.0 µl	from vial 8
4	Draw	0 µl	from vial 0
5	Draw	1.0 µl	from sample
6	Draw	0 µl	from vial 0
7	Mix	8 cycles	
8	Draw	1.0 µl	from vial 12
9	Inject		

✔	✘
Derivatization improves detectability of trace species. It can be automated and integrated online within the analysis. Many chemistries have been developed for routine use both pre- and postcolumn.	Additional investment in equipment.

In brief…

Derivatization offers enhanced analytical response, which is of benefit in food analysis. Chemical modifications can be automated either before or after separation of the compounds under study. In precolumn derivatization, autoinjectors with sample pretreatment capabilities (see chapter 6) are used, whereas in postcolumn derivatization, additional reagent pumps are plumbed to the chromatograph upstream of the detector. The latter approach adds dead volume and therefore is not suitable for the narrow-bore column technique described in chapter 4.
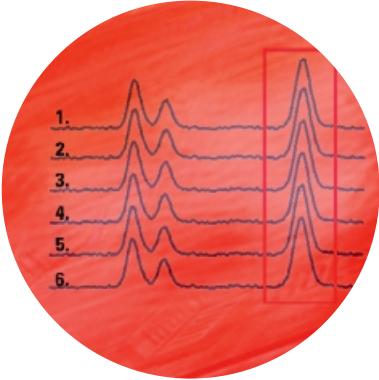
Chapter 10

**Data collection
and evaluation
techniques**
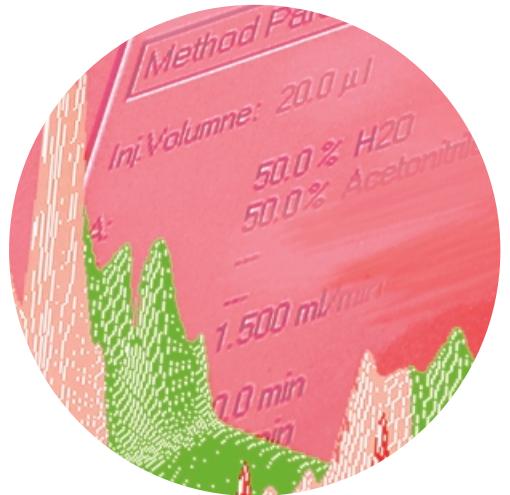
Regardless which detection system you choose for your laboratory, the analytical data generated by the instrument must be evaluated. Various computing equipment is available for this task. The costs depend on the reporting requirements and on the degree of automation required.

Depending on individual requirements, increasingly complex techniques are available to evaluate chromatographic data: at the simplest level are strip chart recorders, followed by integrators, personal computer–based software packages and, finally, the more advanced networked data systems, commonly referred to as NDS. Although official methods published by the U.S. Environmental Protection Agency (EPA) and by Germany's Deutsche Industrienorm (DIN) provide detailed information about calculation procedures and results, they give no recommendations for equipment.

Strip chart recorders

Strip chart recorders traditionally have been used in connection with instruments that record values over a period of time. The recorder traces the measurement response on scaled paper to yield a rudimentary result. In the age of electronic data transfer, such physical records have been largely surpassed by data handling equipment preprogrammed to make decisions, for example to reject peaks that lie outside a certain time window.

✔	✘
Inexpensive.	No record of retention times, no quantitative results on-line, no automatic baseline reset between runs, no electronic storage.

Integrators

Integrators offer several advantages over strip chart recorders and consequently are becoming the minimum standard for data evaluation. Integrators provide a full-scale chromatographic plot and multiple report formats. Area percent, normalization, and external and internal standard calculations are basic features of almost all modern integrators. Annotated reports list amounts, retention times, calculation type (peak areas or heights), and integration parameters as well as the date and time of measurement. Advanced features may provide for automated drawing of the baselines during postrun replotting and for the plotting of calibration curves showing detector response. For unattended analyses in which several runs are performed in series, integrators normally are equipped with a remote control connected to the autosampler in the system. Most models can also store raw data for replotting or reintegration at a later date. Some instruments have computer programming capabilities and can perform more advanced customized statistical calculations using the BASIC programming language, for example. Multichannel integrators are available for some analytical methods requiring two or more detection signals.

✔	✘
Inexpensive. Facilitates reporting of retention times, quantitative results, and automatic baseline resets.	No instrument control or report customization.

Personal computers

In recent years personal computers (PCs) have become increasingly popular as data analysis tools in analytical laboratories. PCs offer more flexibility and better data storage capabilities than traditional storage methods. Moreover, on-line functions such as word processing, spreadsheet analyses, and database operations can be performed simultaneously (see figure 76). Through computer networks, laboratory instruments can be interconnected to enable the central archival of data and the sharing of printer resources. Client/server-based software extends these capabilities by distributing the processing across multiple processing units and by minimizing the time spent validating software. With PCs, all aspects of the HPLC system can be accessed using a single keyboard and mouse. Parameters for all modules, including pump, detector, and autosampler, can be entered in the software program, saved to disk, and printed for documentation. Some HPLC software programs include diagnostic test procedures, instrument calibration procedures, and extensive instrument logbooks, all of which can facilitate the validation processes of various regulatory agencies. Such complementary functions, although not

Figure 76
Cross sample reports regression analyses, trend charts and other calculations consolidate sample data, enhancing the overall productivity and efficiency of the laboratory

directly related to the control of the equipment, are more easily built into a software program than into the equipment itself. In fact, many GLP/GMP features are added to every new version of the software programs sold with HPLC equipment (see figure 77). For example, in some chromatography software, the raw data files can store more than just signal data. A binary check-sum protected file stores instrument parameters (system pressure, temperature, flow, and solvent percent) as well as all aspects of the analytical method, including integration events, calibration settings, and a date-stamped logbook of events as they occurred during the run. Additionally, with spectral libraries, compounds can be identified not only on the basis of their elution profile but also according to their spectral characteristics. Such procedures can be fully automated to reduce analysis time and user interaction.
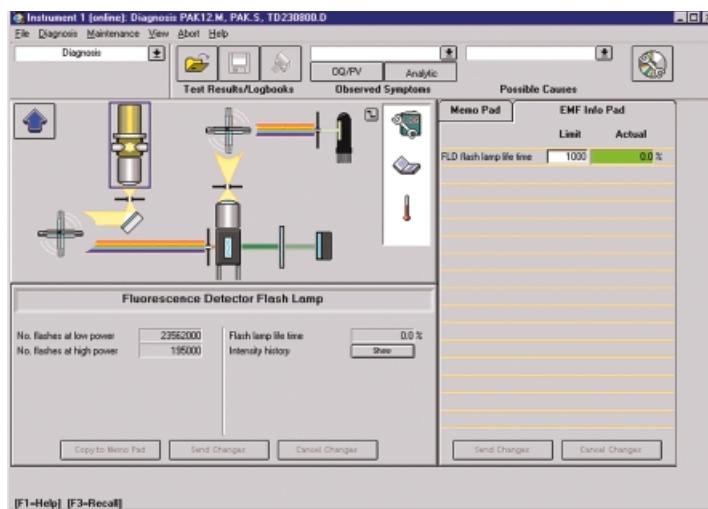
Figure 77
Maintenance and diagnosis screen

A single PC running the appropriate chromatography software can process data from several detectors simultaneously. This feature is particularly useful in analyses in which sensitivity and selectivity must be optimized to different matrices and concentrations. For example, in the analysis of polynuclear aromatic hydrocarbons, UV absorbance and fluorescence detection are applied in series. The PC displays graphically the chromatographic signals and spectra, enabling detailed interpretation of the data. Software purity algorithms can be used to help determine peak homo-geneity, even for coeluting peaks.

Flexible software programs can report data in both standard and customized formats. For example, some chromatography software can be programmed to yield results on peak purity and identification by spectra or, for more complex analyses, to generate system suitability reports. Any computer-generated report can be printed or stored electronically for inclusion in other documents. PCs are well-suited for the modification of calibration tables and for the reanalysis of integration events and data. The software must record such recalculation procedures so that the analysis can be traced to a particular set of parameters in accordance with GLP/GMP principles.

A computer can automate entire sequences of unattended analyses in which chromatographic conditions differ from run to run. Steps to shut down the HPLC equipment also can be programmed if the software includes features for turning off the pump, thermostatted column compartment, and detector lamp after completion of the sequence. If the HPLC equipment malfunctions, the software reacts to protect the instrumentation, prevent loss of solvents, and avoid unnecessary lamp illumination time. A good software application should be able to turn off the pump, thermostatted column compartment, and detector lamp in the event of a leak or a faulty injection. System suitability tests also can be incorporated in a sequence. When performed on a regular

basis, such tests can validate assumptions about performance of the analytical system and help verify results.

✔	✗
Enables control of multiple instruments. Additional software can be used for many other tasks. Provides for better data storage and archival.	Requires more bench space for peripherals such as printers or plotters.

Local area networks

A laboratory running food analyses frequently requires multiple instruments from multiple instrument vendors for sample analysis. Although the integrators and PC systems described above can evaluate data at analytical instrument stations throughout the laboratory, this data must be collected centrally—over a network, for example—in order to generate a single report for multiple analytical techniques. Local area networks (LANs) offer several advantages in addition to shared data processing (see figure 78). Centralized printing saves bench space and reduces equipment expenses, and centralized file security through a single computer—the server—accelerates data backup. Standard network software and hardware cannot handle data files from diverse analytical instrument vendors. The analytical software therefore should have file conversion utilities based on the Analytical Instrument Association ANDI file format (*.cdf).

Shared printing peripherals

Figure 78
A laboratory LAN —
connecting instruments and
collating analytical results

✔	✗
Integrates multiple techniques and instruments from multiple vendors. Saves bench space and computer processing resources. Access to network utilities such as e-mail.	Data processing features may not match those of dedicated data analysis software applications.

Networked data systems

The Agilent ChemStation remote access and data storage modules combine isolated islands of data into a powerful client/server networked information system. Each Agilent ChemStation becomes a network client. It is possible to oversee and control all laboratory operations securely and easily from any computer on the network. The progress of each analysis is monitored to ensure the quality of the results the first time the sample is analyzed. Appropriate action can be taken with the access remote capability from wherever you happen to be if the performance looks suspect. Laboratory data is automatically stored on one centralized and secure server system.

In brief…

Which data handling technique is most effective and economical for your laboratory depends on several factors:
• the size of the laboratory
• the role of the laboratory in the organization
• industrial testing, public safety testing, and so on
• the demands on sample throughput
• the range of analytes under study

For laboratories with few instruments and low sample throughput, integrator systems normally suffice, although a PC may be more appropriate for automated operation of multiple HPLC instruments. A client/server networked data system helps consolidate documentation and validation processes for multiple techniques and instruments from multiple vendors.

Chapter 11

Factors that determine performance in HPLC

11
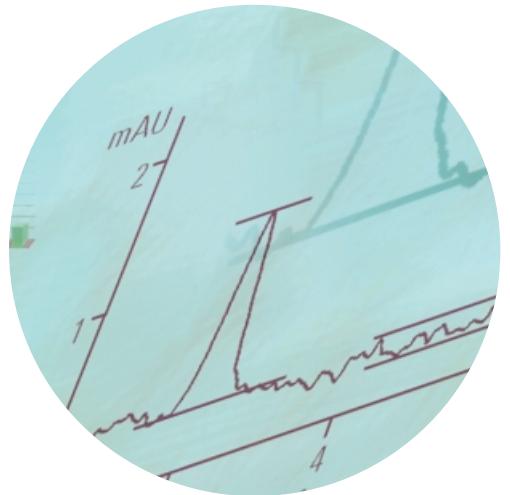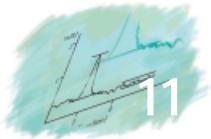
The analysis of food samples places high demands on HPLC equipment, notably in the areas of performance, stability, and reliability. Modern evaluation software enables you to determine the suitability of a particular piece of HPLC equipment for analysis. The factors that influence the outcome of a measurement thus can be identified before results are published to confirm assumptions made during analysis or to draw attention to erroneous data.

In this chapter we focus on those instrument-related parameters that strongly influence the limit of detection (LOD) and the limit of quantification (LOQ). We also discuss the accuracy, precision, and qualitative information that an HPLC system can provide. Some vendors address the performance of specific instrumentation in technical notes.[43] Such notes include detailed performance test procedures and results for individual modules as well as for complete HPLC systems.

Limit of detection and limit of quantification

The principle determinant of the LOD in HPLC is the response of the detector to the compound of interest. The response factor thus depends primarily on the choice of detection technique. However, regardless of the quality of the detector, the LOD or LOQ remains a function of peak height. This height can sink if the peak is allowed to disperse within the surrounding liquid in the flow path. All parts of the flow path in front of the detector therefore must be designed to limit broadening and flattening of the response.

A minimum of narrow capillaries between injector and column and from column to detector helps keep dead volume low. With low injection volumes, separation efficiency of the column can be utilized to the maximum, thereby improving peak height. In other words, the lower the column volume, the lower the peak volume eluted. Other factors that influence peak dispersion include pump performance, degassing efficiency, capacity factor (k'), and column particle size. Any improvements can be registered by calculating the S/N of the analyte. Indeed, the noise of the detector should be tested regularly in this way to ensure that performance is maintained. Dead volume of the complete injection system can be determined by first injecting a tracer mobile-phase additive into the flow path with the column disconnected and then recording the time this additive takes to reach the detector at a particular flow rate. The flow cell volume of the detector should be as low as possible, whereas its pathlength should be as long as possible, according to Beer's law.

Maximizing analyte response is not sufficient to ensure good results, however, since the level of background noise from the detector can counter any gains made. In particular, the performance of the pump in combination with certain solvents can increase detector noise level, as described in chapter 7. Degassing is necessary in order to avoid gas
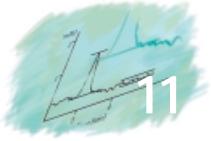
bubbles, which can cause noise or spikes, or oxygen quenching in fluorescence.

High k' values result from higher elution volume or from longer retention time. These values are accompanied by broader peak width and smaller peak height, that is, peaks with longer retention times have poorer S/N. The use of different columns, different mobile phases, and different flow rates can improve S/N. Packing material also directly influences peak dispersion; for example, smaller-sized particles reduce peak dispersion.

Accuracy and precision

Accuracy is the degree of agreement between test results and true values. It is influenced by the analytical method, the extraction procedure used, and the choice of column or detector. Prior to the adoption of any HPLC method for routine use, the degree of agreement with an established reference method should be determined, or a control run should be performed with a known quantity of spiked sample matrix. In practice, however, the degree of agreement will never reach 100 %. This mismatch can be corrected by calibration with standards of known concentration and, based on these results, by calculating the accurate results from an unknown sample. Inclusion of an external or internal standard calibration procedure ensures accuracy in food analysis. The precision of a method is the degree of agreement among individual test results when an analysis is applied repeatedly to multiple samplings. Precision is measured by injecting a series of standards and then calculating the relative standard deviation of retention times and areas or peak heights. Precision may be measured at three levels: repeatability, intermediate precision, and reproducibility. Repeatability is associated with an analysis performed in one laboratory by one operator using a single piece of equipment over a relatively short time period. Intermediate precision is

the long-term variability of the measurement process for a method performed within one laboratory but on different days. Reproducibility applies to an analysis performed in more than one laboratory. Any HPLC method used in food analysis should be tested for both repeatability and reproducibility.

The precision of a method is strongly influenced by the performance of the HPLC instrumentation. Repeatability of flow rates, gradient formation, and injection volumes can affect precision, as can response stability of the detector, aging of the column, and temperature stability of the column oven. The equipment should be inspected on a regular basis using the test methods recommended by the supplier to ensure reliability, high performance, and good analytical results.

Qualitative information

HPLC analytes can be identified on the basis of their retention times and either their UV-visible or mass spectra. Compounds, on the other hand, are identified primarily according to the degree of agreement between retention times recorded using calibration standards and those obtained from the sample. Unfortunately, co-eluting peaks can falsify results obtained with samples containing unknowns, especially for food matrices such as meat, vegetables, or beverages. In such cases, samples often can be identified using UV-visible spectral information. A diode array detection system enables online acquisition, and a number of software packages offer automatic evaluation, for example for the analysis of polynuclear aromatic hydrocarbons (PNAs) and pesticides.[41]

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Part Three

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