Rise Above the Risk: Effective GC Solutions to Optimize Helium Usage

Helium Shortage Affects Laboratory Productivity
The helium supply chain crisis has negative implications on research and laboratory operations world-wide. Despite the willingness of the GC and GC-MS laboratories to pay anywhere from $350 (on average) to a more costly $1,500 per cylinder (~3.5 m$^3$) for ultra-high purity (UHP) helium, rationing and delayed deliveries still cause difficulty in production planning and uncertainty in instrument productive uptime. Although the GC & GC-MS segment consumes less than one percent of the global helium supply usage per year, the shortages and delivery interruptions have wide-spread consequences for many industries utilizing varied analytical techniques.

Barriers to the Adoption of Renewable Gas Options
The severity of the helium crisis is evident when acknowledging that the helium, itself, is a non-renewable resource that is not in extreme abundance. Helium prices have tripled or quadrupled in some areas, and delivery uncertainty has not convinced the majority of GC-MS laboratories to switch to hydrogen – a readily available and renewable alternative carrier gas. For an effective carrier gas switch, methods need to be re-developed or translated and re-validated, the QA/QC criteria require adjustment, and problems caused by the reactive hydrogen gas in the MS ion source need to be addressed. In addition, for some regulated industries, helium carrier gas is still stipulated in the GC or GC/MS methods to which they must adhere.

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- Maintain your Methods
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- Save your Budget

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Advances in Food Analysis: Introduction from Guest Editor
Hans-Gerd Janssen

Liquid Chromatography of Carbohydrates in Human Food and Animal Feeding Stuffs
Kommer Brunt
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Advances in Micro-Ultrahigh-Performance Liquid Chromatography–Mass Spectrometry in Residue and Contaminant Analysis
Arjen Gerssen, Marco H. Blokland, and Hans G.J. Mol
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Flow-Modulated Comprehensive 2D Gas Chromatography–Triple Quadrupole MS Elucidation of the Fatty Acids and Unsaponifiable Constituents of Oil Derived from Lemon Seeds, A Food-Industry Waste Product
Peter Q. Tranchida, Flavio A. Franchina, Simona Salivo, Marina Russo, Paola Dugo, and Luigi Mondello
The application of flow-modulated comprehensive 2D gas chromatography–triple quadrupole MS is described for the detailed qualitative analysis of fatty acids and unsaponifiable constituents in lemon seed oil.

A Generic Method for Target (Group) Analysis in Edible Oils and Fats: Combined Normal-Phase Liquid Chromatography and Capillary Gas Chromatography
Herrald Steenbergen and Hans-Gerd Janssen
Edible oils and fats are complex mixtures of compounds. This article describes the role of normal-phase liquid chromatography as a generic sample pretreatment tool prior to detailed gas chromatography analysis.

Food Taints and Flavors — An Investigative Approach
Kathy Ridgway
A review of the analytical methods available to chromatographers for the determination of sources of off-flavors and food taints.

Comprehensive Two-Dimensional Liquid Chromatography Coupled to Triple Quadrupole Mass Spectrometry: Application to a Challenging Food Case Study
Paola Donato, Francesco Cacciola, Francesca Rigano, Daniele Giuffrida, Paola Dugo, and Luigi Mondello
A novel system for fully automated comprehensive two-dimensional liquid chromatography is presented.

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From the **GUEST EDITOR**

**Advances in Food Analysis**

Food provides the body with energy and good food is indispensable for a healthy life. With a worldwide population of over seven billion people, it is clear that state-of-the-art technology is needed to provide food of a sufficient high quality. Analytical chemistry plays a key role in this. Food quality and safety can only be assured through numerous analyses performed throughout the whole chain from agriculture to the kitchen. Modern analysis tools have made our foods safer, healthier, and better tasting than ever before. As food-analytical scientists, however, we cannot afford to relax. What are state-of-art methods today, will be outdated and insufficient tomorrow. Innovation in food analysis has to continue. And thanks to the never-ending enthusiasm of researchers in the field we can confidently say it does!

Chromatography and mass spectrometry (MS) are the key mature analytical techniques in modern food analysis. Mature here refers to reliable, with proven performance and good characteristics. Mature should in no way be interpreted as dull, with no new developments! The way we perform MS today for example is very different from 10 to 15 years ago. In the past the mass spectrometer could only be used by true experts, nowadays it is a very powerful detector in the hands of those properly trained. In chromatography we have also seen the introduction and rapid market acceptance of hydrophilic interaction chromatography (HILIC), comprehensive two-dimensional chromatography, and advanced methods for sample preparation. “Direct analysis in real time” (DART), -omics techniques, multi-residue methods, accurate mass MS, and many other methods have found rapid acceptance. It is true that the area of food analysis is not always the first area to develop novel methods, but we are masters in adopting and adapting methods from, for example, the pharmaceutical and biomedical fields. In the future we will continue the inclusion of new techniques into our analytical toolbox. The analysis of natural toxins, for example, requires new methods. In addition, the trend towards less processing, meaning that fewer possible contaminants are removed from products, will have consequences for our work. We are also confronted with regulations that are becoming ever more stringent. The pressure of time is an additional complicating factor. With the food area highly dynamic, we need to come up with solutions now, not in a couple of years.

The world of food analysis is an expanding one. Knowledge from the past remains relevant, while simultaneously new ideas and understanding need to be developed. In this special supplement for *LCGC North America*, key state-of-the art areas in food analysis are discussed and the applicability of these novel developments are demonstrated.

In the first article the current status of carbohydrate analysis is reviewed. The impact of recent developments on the way classical sugar analysis is performed is discussed and novel strategies for the identification of polysaccharides are described.

Scientists from the RIKILT Institute for Food Safety discuss the trend of miniaturization and multi-compound detection. Smaller particles allow faster separations and reduced flow rates improve the compatibility of liquid phase separation techniques with MS. High-resolution MS allows us to distinguish compounds that are not chromatographically resolved.

Despite the strong developments in MS, separation is still needed. For extremely complex samples, such as citrus essential oils, even the most powerful one-dimensional methods do not provide sufficient resolution. For volatile compounds, comprehensive GC×GC–MS is a valuable approach, as is convincingly demonstrated by scientists from the University of Messina.

Flavor, and especially off-flavor analysis, is an important research field in food analysis. It requires the joint deployment of an array of sensorial and analytical methods, as is described in a contribution by Kathy Ridgeway from Reading Scientific Services Ltd.

With all the novel methods being developed, the toolbox of the food analyst is ever expanding and the selection of the most appropriate method becomes increasingly difficult. Scientists from my team at Unilever have tackled this issue by moving towards more generic methods. Target analysis of several groups of compounds in edible oils and fats can now be done using one, tuneable method.

Finally, we return to complex vegetable sample analysis. For the non-volatile species, comprehensive LC×LC–MS is a good choice, as is discussed in another contribution from scientists at the University of Messina.

We hope the short articles and reviews presented here provide the reader with more background information and provide ideas for the challenging questions of today and the many new questions that will arise in the future.
When product consistency and brand integrity are on the line, your lab needs to be fortified with innovative analytical systems that put food and beverage quality first. Waters comprehensive solutions do just that—efficiently and cost effectively. With superior precision and reproducibility, you’ll be part of a streamlined process that stocks shelves around the globe with safe, enjoyable products that taste great every time. To discover what’s possible in your lab, visit waters.com/food.
Liquid Chromatography of Carbohydrates in Human Food and Animal Feeding Stuffs

Food for human consumption and animal feedstuffs contain a variety of mono-, di-, oligo-, and polysaccharides with different functions. In this article specific liquid chromatographic (LC) systems (column, mobile phase, and detector), which are used to determine different carbohydrates in food and feed matrices, are described. Cation-exchange columns with different cation counter ions (Na\(^{+}\), Ag\(^{+}\), Ca\(^{++}\), and Pb\(^{++}\)) in combination with a refractive index detector have been widely used for the analysis of mono-, di-, and oligosaccharides for many years. Currently high performance anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD) is often applied in these analyses. Hydrophilic interaction liquid chromatography (HILIC) with mostly aqueous organic mobile phases combined with mass spectrometric detection is a very powerful tool for both the qualitative and quantitative analyses of complex carbohydrates.

Carbohydrates are one of the most abundant distributed constituents in living nature. Together with fat and protein, carbohydrates are a major food constituent. Food and feed contain a broad variety of different carbohydrates as mono-, di-, oligo-, and polysaccharides. In Figure 1 an overview is given of the different carbohydrates that can be present in food and feed. The different categories of carbohydrates have a variety of functions in food products, such as (1) a source of energy, (2) a sweetener, (3) for viscosity and texture, (4) a fat replacer, (5) dietary fiber, and (6) as a prebiotic. The content of the different carbohydrates in our food is of course related to their functionality. For example, digestible carbohydrates such as sugars, starch, and malto-oligosaccharides, and which are important for the energy content of the food product, are usually major constituents with concentration levels ranging from a very small percent to over 50%. Alternatively, carbohydrates that are important for the viscosity and texture of a food product are normally present at low concentration levels, ranging from ≤ 0.1% (for carrageenan) to about 5% (for a starch-based thickener). Dietary fibers (by EU [1] and Codex definition [2] non-digestible carbohydrates with a DP≥3) are mainly present in vegetables, fruits, nuts, and cereal products. Meat, milk, and eggs do not contain dietary fiber. Usually the dietary fiber content in food products ranges from 0–10% depending on the product. According to the Food and Agriculture Organization of the United Nations (FAO) definition of 2007 (3), prebiotics are non-viable food components that confer a health benefit on the host associated with modulation of the microbiota in the intestinal track. Common prebiotics include inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), soya-oligosaccharides, xylo-oligosaccharides, pyrodextrins, isomalto-oligosaccharides, and lactulose.
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Both qualitative and quantitative characterization of the different carbohydrates present in food and feed are very important to ensure the correct labeling of the product concerning the digestible carbohydrates content (part of the energetic value), dietary fiber, and prebiotic content.

In addition, the carbohydrate characterization (both qualitative and quantitative) of raw materials for manufacturing the different (non-starch polysaccharide) food ingredients is essential for a good food manufacturing process.

Liquid Chromatography of Carbohydrates Detectors

Liquid chromatography (LC) is a very well established technique for the separation and determination of carbohydrates. The bottleneck in the LC analysis of carbohydrates is the detection system. Because carbohydrates do not show a significant radiation absorption in the normal UV and visible range, UV–vis detectors are not appropriate in carbohydrate analyses unless they are derivatized with UV-absorbing substituents. The most applied LC detectors, particularly in routine analysis, are the refractive index (RI) detector in combination with isocratic separations, the pulsed amperometric detector (PAD), and the evaporative light scattering detector (ELSD), which can both be applied with gradient elution LC systems. In addition, mass spectrometric (MS) techniques are becoming more commonly applied for carbohydrate characterizations in combination with LC.

Liquid Chromatographic Systems

Various LC systems have been described in the literature. The choice of the chromatographic system depends upon the required level of structural details (for example, total monosaccharides or separation between the monosaccharides [glucose, galactose, mannose, and fructose]), type of glycosidic bonding (α, β, or 1→4, 1→1, or 1→6), concentration level of the carbohydrate, and, of course, the sample matrix. In this mini review we will discuss three chromatographic systems: Cation-exchange chromatography; high performance anion-exchange chromatography; and hydrophobic interaction chromatography. These LC techniques are very important for the analysis of carbohydrates in food and feed.

Cation-Exchange Chromatography with RI Detection

For a long time LC separations of carbohydrates have been performed on strong cation exchange resins in the Ca\(^{2+}\) form at elevated temperatures, applying an aqueous solution of 50 ppm calcium ethylenediaminetetraacetic acid (Ca-EDTA) as the mobile phase and RI detection (4). For all carbohydrates the refractive index increment (dn/dc) has the same value, which means that the sensitivity of the RI detector is the same for all carbohydrates irrespective of DP value or monosaccharide composition. This facilitates the quantitation of unknown carbohydrate peaks in the chromatograms. A severe drawback of RI detectors is that they can only be applied in combination with isocratic separations.

At room temperature the mutarotation of many reducing sugars is low. As a result, the separation of a mixture of carbohydrates at room temperature on a cation-exchange column will result in a needlessly complicated chromatogram with double peaks or peaks with shoulders because of the (incomplete) separation of the α- and β-anomers of the respective sugars. Increasing the temperature increases the mutarotation by a factor of approximately 2.5 for every 10 °C rise in temperature. As a result of fast mutarotation at elevated temperatures, the α- and β-anomers of the respective carbohydrates elute together in one relatively sharp peak (5). Mutarotation is also strongly catalyzed by high pH values in alkaline solutions. This alkaline catalytic effect on the mutarotation is applied in high performance anion-exchange chromatography, which will be discussed later.

The separation mechanism is two-fold: Firstly, it is based on a complex formation between the hydroxyl groups of the carbohydrates and the immobilized Ca\(^{2+}\) ions on the resin (ligand exchange mechanism); and secondly, it is based on size-exclusion effects.

The complex formation between de hydroxyl groups in the carbohydrates and the immobilized counter ions in the cation-exchange resin depends on the conformation of the hydroxyl groups (equatorial or axial) and the carbohydrate itself (chair or boat conformation), and on the specific cation counter ions. H\(^{+}\), Na\(^{+}\), K\(^{+}\), Ag\(^{+}\), Ca\(^{2+}\), or Pb\(^{2+}\) are used as counter ions. Na\(^{+}\) and K\(^{+}\) form no complexes with the hydroxyl groups because of the (incomplete) separation of the α- and β-anomers of the respective sugars. Increasing the temperature increases the mutarotation by a factor of approximately 2.5 for every 10 °C rise in temperature. As a result of fast mutarotation at elevated temperatures, the α- and β-anomers of the respective carbohydrates elute together in one relatively sharp peak (5). Mutarotation is also strongly catalyzed by high pH values in alkaline solutions. This alkaline catalytic effect on the mutarotation is applied in high performance anion-exchange chromatography, which will be discussed later.

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Introducing a SPE / GC-MS method for the analysis of acrylamide in potato chips

Analysing acrylamide
Acrylamide is an endogenous compound, formed when heating starchy or sugary foods. The production of potato chips can result in its formation. Due to the polar nature of acrylamide, the compound is not easily retained using a conventional SPE product such as C18.

HyperSep Hypercarb SPE
The extraction of acrylamide from potato chips was carried out using Thermo Scientific™ HyperSep™ Hypercarb™ cartridges. Hypercarb SPE material is 100% porous graphitic carbon and offers retention of highly polar compounds that are not usually retained by traditional C18 columns. HyperSep Hypercarb SPE can produce clean samples by removing potential matrix interferences.

TraceGOLD TG-WaxMS GC Column
Acrylamide is a highly polar, water-soluble compound having a logP value of -0.65. Such highly polar compounds are not readily amenable to GC. The Thermo Scientific™ TraceGOLD™ TG-WaxMS GC column is a polyethylene glycol-phase GC column that allows the analysis of polar compounds. This low-bleed stationary phase provides greater retention of polar compounds, such as acrylamide, compared with lower polarity stationary phases.

The TraceGOLD range of GC columns offers:
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groups in the carbohydrates. Applying a column in the Na$^+$ or K$^+$ form instead of a column in the Ca$^{++}$ form results in a loss of selectivity for the different monosaccharides and polyols. Almost all monosaccharides and polyols co-elute with each other. Columns in the Na$^+$ or K$^+$ form are mostly applied for the separation of (malto) oligosaccharides. For the separation of different monosaccharides, cation-exchange columns in the Pb$^{++}$ form are frequently applied. As a result of the differences in structural conformation of the hydroxyl groups in the various monosaccharides, the respective monosaccharides form complexes with the immobilized counter ions in the cation exchange resin with different stability constants, resulting in different retention times of the sugars eluting from the chromatographic column.

The size-exclusion mechanism of the chromatographic separation is strongly affected by the degree of divinylbenzene cross-linking of the polystyrene matrix of the cation-exchange resin. Increased cross-linking decreases the pore size in the resin and therefore the size-exclusion range of the column. A linear relationship exists between the retention time and the logarithm of the molecular weight of the eluted oligosaccharides, using a cation exchange column. When applying a 4% cross-linked cation-exchange resin (in the Na$^+$ form), the size-exclusion effect ranges from DP1 up to about DP7 or DP8 for malto-oligosaccharides. Applying a degree of cross-linking of 8% decreases the size-exclusion range from DP1 to about DP3 (Figure 2). The benefit of a relatively high degree of cross-linking is that it results in a more rigid column which resists higher pressures and therefore can be operated at higher flow rates, making faster chromatographic run times possible.

Cation-exchange chromatography in combination with RI detection is still a good technique for analyzing simple mixtures of mono-, di-, and tri-saccharides in relatively clean sample matrices such as fruit juices and beverages. Various cation exchange columns with different specifications (for example, particle size, degree of cross-linking, and counter ions) can be commercially obtained from different manufacturers.

### High Performance Anion Exchange Chromatography With Pulsed Amperometric Detection (HPAEC–PAD)

The hydroxyl groups of carbohydrates are weak acids and can be ionized under strong alkaline conditions (pH>12), making it possible to achieve anion-exchange separation of carbohydrates using strong alkaline mobile phases. High performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) is a very powerful LC system in the field of carbohydrate analyses.

At the beginning of the 1980s, Dennis Johnson and co-workers at Iowa State University (5,6) developed the triple pulse amperometric detector for carbohydrates. This amperometric detection system was particularly designed for the detection of carbohydrates in strong aqueous alkaline solutions (0.1 M NaOH). The major advantage of this detector lies in its high sensitivity and high selectivity for carbohydrates. Because of these properties, a less intensive sample cleanup is needed prior to the chromatographic determination of the carbohydrates when compared with the use of RI detectors. The detector can also be applied very well in combination...
with gradient elution LC systems. However, a drawback of this detector is that, in contrast to the RI detector, the detector sensitivity differs for the different carbohydrates. The pH of the mobile phase in the detector flow cell is of significant influence on the detector sensitivity. This necessitates the preparation of calibration curves for all carbohydrates in every application, applying the specific chromatographic conditions as used in that application.

As mentioned previously, the strong alkaline mobile phase also catalyzes the mutarotation of the reducing carbohydrates very efficiently. Using a strong alkaline mobile phase at room temperature speeds up the mutarotation in such a way that once again the α- and β-anomers of the respective carbohydrates elute together in one sharp peak.

The mobile phase composition in HPAEC–PAD significantly affects both the selectivity of the separation and the sensitivity of the amperometric detection system. Mostly (ternary) gradient elution profiles are applied with water, with 0.1 M sodium hydroxide and 0.5 M sodium acetate applied as the solvent. In the last 25 years, HPAEC–PAD has established itself as one of the major tools for carbohydrate analyses.

Numerous different applications have been developed, and are described in the literature and summarized in review papers (8,9). With a fine-tuning of the applied ternary gradient, very complex separations can be achieved.

Eurofins Carbohydrate Competence Centre has developed and implemented numerous HPAEC–PAD applications. The application presented in Figure 3 deals with the identification and quantitation of approximately 20 different mono-, di-, and oligosaccharides present in an infant milk powder formula. In this formula three different types of oligosaccharides are present: a series of malto-oligosaccharides ranging from DP1 to DP8 (glucose up to maltotraose); a series of short chain fructo-oligosaccharides ranging from DP3 to DP7; and a series of short-chain inulins with end-standing glucose units, including sucrose, ranging from DP3 to DP8. The chromatographic separation of this complex matrix is completed in about 40 min.

Another application concerns the characterization of the side chain lengths in amyllopectins in native and modified starches (Figure 4). Amylopectins in starches of different botanical sources (for example, potato, wheat, and maize) show different side chain length distributions. A characteristic fingerprinting of the side chain length distribution is obtained by first applying an enzymatic debranching of the amyllopectin followed by HPAEC–PAD characterization of the obtained set of amyllopectin side chains. This is demonstrated in Figure 4 for a native potato starch sample. The dip in the peak heights at DP8 is more or less characteristic for native potato amyllopectins. The fingerprinting for the amyllopectin chain length distribution of wheat starch shows mostly a binominal distribution.

Recently the galactosylsucrose family of oligosaccharides has become of
great interest in food analyses. This interest is particularly concerned with the tri-saccharide raffinose, the tetra-saccharide stachyose, and the penta-saccharide verbascose. These oligosaccharides are present in vegetables as peas, beans, and lentils. Their content ranges from about 5% to 9% in the dry product. The galactosylsucroses are not digested in the human small intestine. They enter the colon where they are fermented by the gas-producing intestinal microflora, leading to flatulence.

The vegetables contain also the usual mono- and disaccharides as glucose, fructose, sucrose, and sometimes maltose. Therefore in the galactosylsucrose HPAEC–PAD application it was necessary to achieve a good chromatographic separation between glucose, fructose, sucrose, maltose, raffinose, stachyose, and verbascose (Figure 5). Of particular note is the retention time of the disaccharide maltose: It elutes between stachyose (tetrasaccharide) and verbascose (pentasaccharide).

HPAEC–PAD is becoming more and more a standard tool for the routine analyses of carbohydrates in food and feed matrices. This is emphasized by the fact that HPAEC–PAD is also used today in both ISO (10) and CEN (11) protocols for the analysis of carbohydrates in human and animal nutrition.

### Hydrophilic Interaction Liquid Chromatography

Hydrophilic interaction liquid chromatography (HILIC) can be considered as a variant of normal-phase chromatography: A hydrophilic stationary phase is applied in combination with a mostly organic mobile phase and elution is usually performed by increasing the water content. The technique was introduced in 1990 by Alpert (12) and is suited to the analysis of carbohydrates. HILIC is often combined with evaporative light scattering detection (ELSD) (13). An advantage of the ELSD over the RI detector is that it also can be used with a gradient elution. HILIC in combination with MS appears to be a very powerful technique for characterizing carbohydrates. Depending upon the exact configuration of the MS detection system, it provides extra structural information on the eluted peaks, such as molar mass or fragmentation patterns. The technique is used both for the analysis of relatively simple mixtures of mono- and disaccharides and for more fundamental investigations into the structure of cell wall polysaccharides and hydrocolloids.

Ikegami et al. (14) described the use of a polyacrylamide modified monolithic silica capillary column for HILIC coupled with electron spray ionization (ESI)–MS detection for the analysis of carbohydrates. In their paper they presented chromatograms of mono- and disaccharides and for more fundamental investigations into the structure of cell wall polysaccharides and hydrocolloids.

Fu et al. (15) developed a general and efficient HILIC method for the separation of various kinds of carbohydrates,
ranging from galacto-oligosaccharides, carrageenan oligosaccharides, sodium alginate, and chito-oligosaccharides, to higher molecular weight fructo-oligo-saccharides.

In the laboratory of Food Chemistry of Wageningen University, HILIC–ELSD–MS is applied for the characterization of plant cell wall polysaccharides and exo-polysaccharides (16–18). The non-starch polysaccharides are subjected to a dedicated enzymatic hydrolysis using defined enzymes. The mixture of different oligosaccharides in the hydrolysate are then characterized and quantified with HILIC–ELSD–MS techniques, resulting in an enzymatic fingerprinting chromatogram. This approach provides information beyond the level of the monosaccharide composition of the polysaccharides. It also provides information on the distribution of the constituting monosaccharide moieties along the polysaccharide backbone or side-chains.

**Sample Preparation**

As a final note, two remarks on sample preparation and sample cleanup should be noted. Firstly, the degree of sample pretreatment depends very much on which chromatographic detector is applied for the analysis. Applying high selective carbohydrate detectors such as PAD and MS, generally requires a less intensive sample cleanup procedure than when applying an universal RI detector.

Secondly, most sugars and oligosaccharides are water-soluble. Therefore it is common practice to apply an aqueous extraction of the sugars and oligosaccharides as a first step in the analytical procedure. However, depending on the sample origin, it is possible that the sample contains some (rest) enzyme activities (19) as amylases or amylglucosidases. In that case, measures should be taken to ensure that the carbohydrate composition in the sample is not changed as a result of enzymatic activities during the sample cleanup procedure.

**References**


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For more information on this topic, please visit [www.chromatographyonline.com](http://www.chromatographyonline.com)
Advances in Micro-Ultrahigh-Performance Liquid Chromatography–Mass Spectrometry in Residue and Contaminant Analysis

Advances in nano-ultrahigh-performance liquid chromatography (nUHPLC) and “plug-and-play” micro-UHPLC (µUHPLC) for the detection of veterinary drugs and steroids in porcine meat and urine respectively are described. Recent developments in “plug-and-play” µUHPLC devices offer several advantages compared to earlier micro-LC systems. As well as the ease of use, solvent consumption can be reduced by more than 95% and the amount of sample required can be reduced 10-fold. In addition, the performance and the robustness of the µUHPLC system described is comparable to conventional UHPLC, and can be successfully applied for the routine analysis of residues and contaminants in food.

Residues and contaminants that can affect consumer safety (such as veterinary drugs, growth promoters, pesticides, or natural toxins) are continually monitored to ensure product safety and compliance with legislation (1). Residues and contaminants may be present at low concentrations in the ng/kg to µg/kg range, and so most laboratories use liquid chromatography coupled to tandem mass spectrometer (LC–MS–MS). Significant improvements have been made over the last decade, with LC methods shifting from traditional particles (3–5 µm) to ultrahigh-performance liquid chromatography (UHPLC) using smaller particles (1.7–2-µm). This has led to narrower peaks, which has improved peak capacity and detectability. Improvements in MS instruments have increased sensitivity and acquisition speeds (required for quantitative measurement of the narrow peaks). Furthermore, MS instruments have become compatible with the higher flow rates typically used in UHPLC. Many laboratories have transferred existing HPLC methods to UHPLC in combination with highly sensitive and fast scanning MS–MS instruments.

The currently applied UHPLC techniques in the field of routine food analysis can be divided into different classes: low flow UHPLC (flow rate = 10–100 µL/min); conventional UHPLC (100–600 µL/min); and high flow UHPLC (>600 µL/min). Nano- or micro-liquid chromatography [nLC (<1 µL/min) or µLC (1–10 µL/min)] are missing from this list because they have not been routinely used in the field of routine food safety analysis for a number of reasons. Variable flow rates were one major issue with nLC and µLC attributed to nano- or micro-flows that were attained by splitting regular flow rates using capillaries; the viscosity of the mobile phase changes during analysis; and injection of dirty samples that could cause clogging. This hampered the implementation in routine laboratories because unstable flow rates caused unstable retention times that meant the methods could not be used for official control purposes where it is mandatory that retention times are consistent to allow proper identification (2,3).

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RIKILT – Institute of Food Safety – Wageningen UR, The Netherlands
This was especially true in laboratories where a technique is performed on a daily and routine basis.

Another drawback of early nLC or µLC techniques were issues associated with the connections between the autosampler, trapping columns, analytical columns, and electrospray emitters. Each connection would introduce void volumes that eventually resulted in serious peak broadening. Moreover, detecting a small leak with all these connections was a challenging task at low nano or micro flow rates. This was almost impossible for routine applications where various applications would be performed on a single instrument and columns would have to be frequently changed. For these reasons outlined, conventional nLC and µLC were not really applicable for routine applications (4).

Nowadays, there are several commercial nLC and µLC instruments available, without the flow rate issues discussed above, that are able to produce stable nano- or micro-flow without using flow splitting. The most recent nano pumps can produce stable low flow rates of 10–4000 nL/min and handle a pressure of 10,000 psi (approximately 700 bar). This high-pressure tolerance made it possible to use nano or microbore columns packed with sub-2-µm particles. nUHPLC and µUHPLC are now becoming the standard in nano and microLC separations.

A major issue remaining is the connection of pre-columns and analytical columns to both the nUHPLC and nano electrospray emitter, restricting the implementation of the technique in the field of routine food control. In response to this, n(U)HPLC and µ(U)HPLC devices have been developed that can be directly connected to the electrospray source or LC instrument without manual connections. These solutions lead to more robust equipment as no manual connections have to be made. Potential advantages of the nLC and µLC are the reduced use of high grade solvents and reference standards. Furthermore, at these lower flow rates in theory the detectability is improved and ionization suppression is reduced (5–7). To explore the applicability of nUHPLC and µUHPLC in food safety, the levels of veterinary drugs and steroids in porcine meat and urine were determined.

### Experimental

**Veterinary Drug Screening Reagents**

Water for sample preparation was deionized and passed through a Millipore water purification system (Millipore). Formic acid (98–100%) was purchased from Merck. Leucine-enkephalin was purchased from Sigma-Aldrich. Acetonitrile (HPLC supragradient), methanol (absolute) and water (UHPLC–MS-grade) were purchased from Biosolve. Veterinary drug analytical standards were purchased from Sigma-Aldrich, Smith Kline Beecham, Riedel de Haen, or Fluka. Oxfenbendazole and oxfenbendazole sulfoxon were purchased from Syntex, valnemulin from Novartis, and marbofloxacin from Vetoquinol. Albendazole (sulfoxide), oxendazole (sulfone), hydroxy- ipronidiazol, carazolol, piroxicam, propyphenazone, and piroxicam were obtained from Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL–EURL).

**Sample Preparation**

Blank porcine samples were selected from the Dutch national monitoring program. These samples (n=7) were fortified with the various veterinary drugs to develop the screening method. Extraction was done as previously described by Peters et al. (8), and µSPE was subsequently performed. Of the acetonitrile/water extract, 375 µL was diluted with water to a volume of 3.75 mL. A 30-µm 96-well SPE Oasis HLB µElution plate (Waters) was conditioned and equilibrated with 200 µL methanol and water, respectively, and 2850 µL of diluted extract was applied to the plate. The cartridge was washed with 500 µL water/methanol (95/5, v/v), and the veterinary drugs then eluted into a 96-well plate using 500 µL acetonitrile/methanol (50/50, v/v). The eluate was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted in 50 µL water/acetonitrile (90/10) containing 0.1% (v/v) formic acid. The well plate was sealed using a pealable heat seal to prevent evaporation.

**UHPLC Separations**

UHPLC was performed on an Acquity UPLC system (Waters) equipped with a 100 mm × 2.1 mm, 1.7-µm µUPLC BEH C18 column. Mobile phase A and B were water and acetonitrile, respectively, that both contained 0.1% (v/v) formic acid. Gradient flow rate was 400 µL/min starting at 0% B, which was increased linearly to 40% B in 4 min and then linearly increased in 6 min to 100% B. It was kept at 100% B for 2 min and subsequently returned to 0% B. An equilibration time of 4 min was allowed prior to the next injection. The column temperature was kept at 40 °C and 20 µL was injected onto the column.

**Reagents**

Veterinary drug screening was performed on a daily and routine basis. To explore the applicability of nUHPLC and µUHPLC in food safety, the levels of veterinary drugs and steroids in porcine meat and urine were determined.

**Figure 1:** nUHPLC–MS chromatograms obtained using different trapping columns for peak refocusing: (a) C18; (b) high strength silica C18. Analytes: 1) 5-OH thiamethazole, 2) sulfadoxine, 3) sulfamethoxazole, and 4) mefenamic acid.
µUHPLC Conditions

For µUHPLC a packed micro LC column (50 mm × 0.15 mm, 1.7-µm dₚ BEH C18 [Waters]) was used. The microbore column, microfluidics connections, and ESI emitter were integrated on a ceramic tile (iKey, Waters) that was inserted into a dedicated source module (ionKey/MS, Waters) coupled to the MS. Solvent and sample were delivered to the ionKey/MS module using a Waters nanoAcquity UPLC binary solvent manager and nanoAcquity sample manager, respectively. The gradient was started at 1% B which then linearly increased to 50% B in 10 min and then increased to 95% B in 0.1 min. This composition was held for 2 min and then the composition was changed back to 1% B in 1 min. An equilibration time of 5 min was allowed prior to the next injection. The column temperature was kept at 35 °C and 2 µL was injected onto the column.

Mass Spectrometric Detection

The UHPLC and nUHPLC were directly interfaced with a Xevo Q-ToF mass spectrometer (Waters) equipped with an electrospray ionization interface (ESI) and nano ESI, respectively. For µUHPLC the column and electrospray emitter were integrated in one module, which was inserted into a dedicated source mounted onto the Q-ToF MS as already described above. The analysis was performed in ESI positive (ESI⁺) mode. A capillary voltage of 3 kV and a cone voltage of 40 V were applied.

Steroid Drug Screening

Reagents

17β-boldenone (17β-Bol), 1,4-androstadiene-3,17-dione (ADD), methylboldenone (meBol), norethandrolone (norEth), 17α-methyltestosterone (17α-meT), 17α-nortestosterone (17α-norT), 17β-nortestosterone (17β-norT), 17α-testosterone (17α-T), 17β-testosterone (17β-T), and 4-chlorotestosterone (clos) were obtained from Steraloids. 17α-boldenone (17α-Bol), 17α-trenbolone (17α-Tren), 17β

Table I: Improved signal-to-noise ratio: Detectability in nUHPLC and conventional UHPLC

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<td>Albendazole amino sulfone</td>
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</tr>
<tr>
<td>Amino mebendazole</td>
<td>11</td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>14</td>
</tr>
<tr>
<td>Pirimicyn</td>
<td>8</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>4</td>
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<tr>
<td>Difloxacin</td>
<td>4</td>
</tr>
<tr>
<td>Sarafloxacin</td>
<td>3</td>
</tr>
<tr>
<td>Dapsone</td>
<td>1</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>3</td>
</tr>
<tr>
<td>Sulphachloropyridazine</td>
<td>10</td>
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<tr>
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² nUHPLC 2 µL injection and with UHPLC a 20 µL injection

Table II: Differences between UHPLC, µUHPLC, and nUHPLC applications for veterinary drugs

<table>
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<th>Compound</th>
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<tr>
<td>Column internal diameter (mm)</td>
<td>2.1</td>
<td>0.15</td>
<td>0.10</td>
</tr>
<tr>
<td>Flow rate (µL/min)</td>
<td>400</td>
<td>4</td>
<td>0.6</td>
</tr>
<tr>
<td>Injection volume (µL)</td>
<td>20</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total run time (min)</td>
<td>16</td>
<td>15</td>
<td>33</td>
</tr>
<tr>
<td>Trap pre concentration</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
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<td>Mobile phase used in a single run (µL)</td>
<td>6400</td>
<td>60</td>
<td>33</td>
</tr>
<tr>
<td>Ease of use</td>
<td>+</td>
<td>++</td>
<td>+/-</td>
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<td>Relative method detectability</td>
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The flow rate was kept constant at 4 µL/min. The column temperature was kept at 45 °C and 2 µL of each sample was injected.

nUHPLC Conditions

For nUHPLC, a nano-Acquity UPLC system (Waters) was equipped with a 20 mm × 0.2 mm, 5-µm dₚ HSS T3 trap column (Waters) and a 100 mm × 0.1 mm, 1.7-µm dₚ BEH C18 analytical column (Waters). The mobile phase composition was the same as described in the UHPLC section. The compounds of interest were trapped for 2 min with a flow of 7.5 µL/min. After trapping, a gradient was run at a flow rate of 0.6 µL/min starting at 1% B, which was increased linearly to 50% B in 11 min and then linearly increased in 5 min to 95% B. It was kept at 95% B for 5 min and subsequently returned to 1% B in 3 min. An equilibration time of 5 min was allowed prior to the next injection. The column temperature was kept at 35 °C and 2 µL was injected onto the column.

Steroid Drug Screening

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The flow rate was kept constant at 4 µL/min. The column temperature was kept at 45 °C and 2 µL of each sample was injected.
Porcine urine samples were screened for the presence of veterinary drugs. The mass spectrometer was operated in ESI+, with a capillary voltage of 3.2 kV. Argon was used as collision gas at a flow of 0.15 mL/min. The cone voltage and collision energies were optimized for the various steroids by direct infusion experiments. For each of the steroids two SRM transitions were monitored.

Results and Discussion
Comparison of UHPLC, nUHPLC, and µUHPLC in Veterinary Drug Screening
Porcine meat samples were screened for the presence of veterinary drugs using UHPLC, nUHPLC, and µUHPLC. In principle, when scaling down UHPLC methods to nUHPLC, the injection volume has to be reduced proportionally with column diameter and flow rate to avoid band broadening, achieve adequate peak shapes, and maximize

Mass spectrometric detection
The UHPLC–ESI and µUHPLC were connected to a Xevo TQ-S triple quadrupole MS (MS–MS) [Waters] in the same way as described for the veterinary drugs. The mass spectrometer was operated in ESI+, with a capillary voltage of 3.2 kV. Argon was used as collision gas at a flow of 0.15 mL/min. The cone voltage and collision energies were optimized for the various steroids by direct infusion experiments. For each of the steroids two SRM transitions were monitored.

Sample Preparation
Porcine urine samples were screened for the presence of steroids as a second example matrix. Samples were treated using β-glucuronidase and arylsulfatase to deconjugate the excretion products to their aglycon/desulphated form. The samples were then applied to a dual stage SPE cleanup using a C18 (Varian bond Elut) and subsequently an NH₂ cartridge (IST Isolute, Biotage). After evaporation, the cleaned extract was further purified using HPLC fractionation. The purified extracts were evaporated to dryness and reconstituted in 225 µL water/methanol (70/30, v/v) containing 0.3% formic acid.

UHPLC Conditions
Instrument set-up was as previously described. The gradient started at 10% B which was linearly increased to 20% B in 2 min, and 20% to 60% B in 6 min. The gradient was then increased in 0.1 min to 95% B which was kept for 2.9 min before returning to 10% B in 0.5 min. An equilibration time of 3.5 min was allowed before the next injection. The flow rate was kept constant at 392 µL/min, the column temperature at 40 °C, and an injection volume of 0.5 µL was used.

- trenbolone (17β-Tren), 4-chloro-4-androst-3,17-dione (CLAD), 17β-testosterone-d₃ (17β-T-d₃), 17β-boldenone-d₃ (17β-Bol-d₃), 17β-trenbolone-d₃ (17β-tren-d₃), methylboldenone-d₃ (meBol-d₃), 17β-nortesteron-d₃ (17β-norT-d₃), and 17α-methyltestosterone-d₃ (17α-meT-d₃) were obtained from the European Reference Laboratory. 16βOH-stanozolol (16βOH-Stan), stanozolol (Stan) and stanozolol-d₃ (Stan-d₃) were purchased from Cerilliant and tetrahydrogestrinone (THG) was purchased from the National Measurement Institute in Pymble, Australia.

Figure 2: (a) Sensitivity of α-testosterone and β-testosterone. (b) Difference in peak shapes in UHPLC and µUHPLC.
sensitivity. Factoring in the column and flow rate, this translates into an injection volume of 45 µL (compared to the 20 µL used in UHPLC). Even with the theoretical sensitivity gain, this results in an unacceptable increase in the limit of detection. To compensate for this, a relatively large volume of extract (2 µL) was injected adding 5 min to the analysis time. It also negatively affects peak shape because of insufficient refocusing on the head of the analytical column. To remove this issue, a precolumn was used to transfer the extract at a higher flow rate, the analytes were trapped and subsequently transferred as a narrower band to the analytical column.

The use of a precolumn is not ideal when developing multi-compound methods as a wide variety of polarities and functional groups are present. The first trapping columns available for the nUHPLC system contained C18 particles, but when used for screening for veterinary drugs it caused peak broadening for some of the compounds (Figure 1). For example, the peak width of sulfadoxine (peak 2) and sulfadimethoxine (peak 3) was relatively broad. To improve peak shape a different trap material containing high strength silica C18 particles was used, which significantly improved peak shape, especially for sulfadoxine (peak 2) and sulfadimethoxine (peak 3). Although this high strength silica C18 column is more retentive a small shift in retention could be observed which is potentially attributable to the improved end-capping of the silica in this trap column. This improvement reduced the secondary interactions of sulfone and sulfonic acid groups under acidic mobile phase with free silanol groups at the silica surface.

To elute the compounds as a sharp plug, the trap column has to be maintained at an optimal temperature, but with the nUHPLC system used this was not possible. With a modified setup it was possible to place both trap and analytical column at optimal temperatures. Unfortunately, under the modified setup, the capillaries were under a lot of stress, which affected the robustness of the system.

The developed nUHPLC method was compared with the UHPLC method in terms of sensitivity for a number of compounds (Table I). On average the signal-to-noise (S/N) improved with a factor 5 and the areas of the compounds with a factor 18. Factoring in differences in injection volume between UHPLC (20 µL) and nUHPLC (2 µL) the improvement in detectability (S/N) is 50-fold. A major drawback of the nUHPLC was that the run-time increased from 16 min with UHPLC to 33 min with nUHPLC. This, in combination with the inflexibility of rapidly changing columns for different applications, hampers the implementation in routine. Plug-and-play µUHPLC offers an alternative method.

µUHPLC was performed with a flow rate of 4 µL/min, and no trapping was performed. The loop of 2 µL was flushed at a flow rate of only 4 µL/min. Therefore, injection volume and solvent are of major importance for a good separation and peak shape. A small percentage organic present in the injection solvent may already cause poor peak shapes and loss of retention. For the early eluting veterinary drugs a small injection volume will cause in-loop dilution that results in broader peaks.

The developed µUHPLC method was validated according to EU criteria (2). Preliminary results were promising for example: Carbazol at levels of 5; 10, and 15 µg/kg in porcine meat had good linearity (correlation 0.991), accuracy (89–95%), repeatability (11–15%) and within-laboratory reproducibility (12–16%). The robustness and performance of the µUHPLC system is comparable with that of UHPLC.

**Comparison of UHPLC and nUHPLC in Steroid Screening**

For the steroid application UHPLC–MS–MS and µUHPLC–MS–MS were compared. Steroids of interest eluted at a higher organic solvent concentration and therefore were better focused at the head of the analytical column. When transferring the method from UHPLC to µUHPLC, column volumes and gradient were comparable by scaling the flow rate of the UHPLC method to the µUHPLC method. Based on the internal diameter of the column, the flow rate was adjusted, respectively to 392 µL/min in the UHPLC column and 2 µL/min in the µUHPLC column. Also the injection volume was reduced (from 5 µL with the conventional UHPLC and 0.5 µL with µUHPLC) but not proportionally to achieve at least the same limits of detection. Figure 2a shows that with a 10-fold smaller injection volume, the sensitivity of the steroids analyzed was improved. The repeatability of the method was also comparable to the UHPLC method.

The introduction of less matrix into the system reduced drift in sensitivity during analysis. Retention times observed for both the UHPLC and µUHPLC were extremely stable, with deviations smaller than 5 s within each series. When studying the chromatographic peaks in more detail, as shown in Figure 2b, the asymmetry factor (a/b) was only slightly affected. On average the steroids in UHPLC had an asymmetry factor of 1.37±0.34 versus 1.39±0.11 in µUHPLC. The effective plate numbers of both columns were in the same range, UHPLC Neff = 48711 and µUHPLC Neff = 46829. It could be concluded that the plug-and-play µUHPLC method is at least delivering the same performance quality as conventional UHPLC.

**Green Chemistry**

Downscaling analytical procedures to reduce solvent and plastic waste is key to sustainable chemistry. Here, all methods developed were miniaturized to reduce waste. SPE was downscaled from the original method using conventional SPE cartridges (60 mg/3 mL) to a 96-well microplate. Conventional SPE used a total of 12 mL of solvent, whereas µSPE used only 1.4 mL of solvent, a reduction of almost 90%. The UHPLC method used 6.4 mL of mobile phase, compared to µUHPLC that used only 60 µL, a 99.9% reduction. Furthermore, the sample size (injection volume) can also be reduced by up to a factor of 10 when using µUHPLC. The use of smaller injection volumes provide new opportunities in food safety analysis, such as combining this technique with bio-activity driven sample cleanup (10,11).
Conclusions
In conclusion, µUHPLC as a technique is at the same level as conventional UHPLC and the robustness of the technique means that it can be implemented in routine laboratories. Furthermore, the green analytical concept is of interest from both an economic and sustainability perspective. We expect that the type of “plug-and-play” µUHPLC techniques used here will be adapted in different applications, such as food safety analysis, and more “plug-and-play” µUHPLC equipment will become commercially available on the market.

References
(4) C.M. Harris, Anal. Chem. 75(64a–69a), (2003).

Arjen Gerssen is a junior scientist in the group of natural toxins and pesticides. His main expertise is in the field of phycotoxins. He also has a strong interest in applying new separation and MS technologies for residue and contaminant analysis.

Marco Blokland is a junior scientist involved in the European Union Reference Laboratory for growth promoting compounds. The research topics covered include the whole range of growth promoters, from small molecules and peptides to proteins. Currently he is working on the use of statistical models to detect natural hormone abuse in cattle.

Hans G.J. Mol is a senior scientist and heads the group of National Toxins and Pesticides at RIKILT. He has over 15 years experience in residue and contaminant analysis in the food chain using chromatography with a range of mass spectrometric techniques. Please direct correspondence to: Hans.Mol@wur.nl

For more information on this topic, please visit www.chromatographyonline.com
Flow-Modulated Comprehensive 2D Gas Chromatography–Triple Quadrupole MS Elucidation of the Fatty Acids and Unsaponifiable Constituents of Oil Derived from Lemon Seeds, A Food-Industry Waste Product

This article is focused on the detailed qualitative analysis of the fatty acids and the unsaponifiable constituents of a vegetable oil derived from a food-industry waste product, namely lemon seeds. The seed oil was subjected to two sample preparation processes, the first enabling the formation of fatty acid methyl esters (FAMEs), and the other the isolation of the constituents of the entire unsaponifiable fraction (sterols, hydrocarbons, vitamins, etc.). Both sets of compounds were subjected to flow-modulation (FM) comprehensive two-dimensional gas chromatography–mass spectrometry (GC×GC–MS), with identification performed through full-scan data. Relative percentage data, relative to FAMEs and sterols, were derived through gas chromatography–flame ionization (GC–FID). Finally, a sterol identified through a clear signal as cholesterol, was subjected to absolute quantification using multiple reaction monitoring (MRM).

The worldwide production of Citrus fruits is enormous, reaching 91 million tons in the period 1999–2000. A high percentage of this fruit (= 40%) is involved in industrial processing, with essential oils and juices the main end-products. Considering single Citrus species, the orange represents approximately 67% of the fruit produced, while the lemon makes up about 6% (1).

It is clear that the industrial processing of such a large amount of fruit generates a great amount of waste, in terms of peel, pulp, and seeds. Studies on the use of lemon seeds as a source of vegetable oil have been reported previously. For example, Malacrida et al. reported that the main fatty acids (FAs) in lemon seed oil (approximately 35% of the seed mass) were $C_{16:0}$ ($\approx 21.0\%$), $C_{18:1\omega9}$ ($\approx 20.8\%$), $C_{18:2\omega6}$ ($\approx 44.3\%$), and $C_{18:3\omega3}$ ($\approx 9.0\%$), while minor FAs were $C_{16:0}$ ($\approx 0.7\%$), $C_{17:0}$ (traces), $C_{20:0}$ ($\approx 0.3\%$), $C_{22:0}$ ($\approx 0.1\%$), and $C_{24:0}$ ($\approx 0.2\%$) (2). Furthermore, a tocopherol content of 125 mg/kg was present in the oil, with the $\alpha$-isomer present in by far the highest amounts (102.5 mg/kg). Reda et al. studied the FAs in Sicilian lemon seed oil (approximately 38% of the seed mass), reporting the following composition: $C_{8:0}$ (1.0%), $C_{14:0}$ (0.1%), $C_{16:0}$ (19.6%), $C_{18:0}$ (3.0%), $C_{18:1\omega9}$ (28.6%), $C_{18:2\omega6}$ (34.4%), $C_{18:3\omega3}$ (10.0%), and $C_{20:0}$ (0.2%) (3). Saidani et al. performed research on Tunisian lemon seed oil, reporting a surprisingly high oil yield, namely 79% (4). The FA composition was reported to be: $C_{8:0}$ (0.04%), $C_{10:0}$...
Table I: Lemon seed oil FAMEs, MS database similarity results and percentage values (%). Abbreviations: a = anteiso; i = iso

<table>
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<th>Similarity</th>
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<tr>
<td>C15:0</td>
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<td>a-C16:0</td>
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</tr>
<tr>
<td>i-C18:0</td>
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<td></td>
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<td>a-C19:0</td>
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<td>i-C20:0</td>
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<td>C17:10o5</td>
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</tr>
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<td>C20:16o7</td>
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<td>36.6</td>
</tr>
<tr>
<td>C18:33o3</td>
<td>85</td>
<td>12.2</td>
</tr>
</tbody>
</table>

(0.05%), C12:0 (0.04%), C14:0 (0.10%), C14:1 (0.10%), C16:0 (21.40%), C18:0 (2.3%), C18:1ω9 (36.6%), C18:2ω6 (31.4%), and C18:3ω3 (6.90%). Compared to the research performed by Malacrida et al. and Reda et al., an inverted relationship between oleic and linoleic acid was reported.

The objective of the current research is focused on the qualitative analysis of the fatty acids and of the unsaponifiable constituents of lemon seed oil. Consequently, two distinct analyses were performed using a flow-modulation (FM) comprehensive two-dimensional gas chromatography–mass spectrometry (GC×GC–MS) method. The MS system used was a “rapid” triple quadrupole one, fast enough for GC×GC requirements, in both the full-scan and targeted (for example, multiple reaction monitoring [MRM]) analyses. Gas chromatography–flame ionization detection (GC–FID) was exploited to quantify a series of FAs and sterols in percentages.

**Experimental Samples, Reagents, and Pure Standards**

The lemon seed oil was produced in the laboratory, through a laboratory cold-pressing device.

The boron trifluoride–methanol complex was purchased from Merck. The BSTFA (N,O-bis[trimethylsilyl] trifluoroacetamide) + 1% TMCS (trimethylchlorosilane) kit, n-hexane, diethyl ether, ethanol, anhydrous Na2SO4, pyridine, KOH, NaOCH3, and NaCl solution were supplied by Sigma-Aldrich. The C7–C30 alkane series, eicosanol, docosanol, tetracosanol, cholesterol and β-sitosterol, were kindly supplied by Sigma-Aldrich.

**Sample Preparation Fatty Acid Methyl Esters (FAMES)**

The FAMES were prepared in the following way: A 100 μL sample of oil was saponified with 1 mL of methanolic sodium methoxide (0.5% w/v) at 100 °C in a closed Pyrex tube for 15 min. The subsequent methyl esterification was performed with 1 mL boron trifluoro-methanol reagent at 100 °C for 15 min. The FAMES were extracted by adding 1 mL of n-hexane and 4 mL of a saturated NaCl solution to the mixture, and agitating manually for 2 min, before a 5-min centrifugation (3000 rpm). Finally, the 1 mL n-hexane layer was transferred to a GC injector vial.

**Unsaponifiable Fraction (TMSE – Trimethyl Silyl Ethers)**

An amount equivalent to 1 g of vegetable oil was added to 10 mL of a 2N KOH/EtOH solution and was heated at 80 °C, under reflux, and magnetic stirring (for about 20 min after solution clarification). Extraction was performed three times with 15 mL of diethyl ether. The combined extracts were washed with 10 mL of distilled water until neutralization was reached. The washed diethyl ether solution was dried with anhydrous sodium sulfate, and the solvent was distilled leaving a few microliters. The latter was transferred into a vial, previously weighed, and the solvent was evaporated under a gentle nitrogen flow at room temperature. The dried residue, namely the unsaponifiable fraction, was weighed.

The unsaponifiable fraction was dissolved in 1 mL of chloroform and was treated with 200 μL of BSTFA (1% TMCS) and 200 μL of pyridine, and heated at 70 °C for 20 min. The derivatized sample was then ready for GC injection.

**FM GC×GC–MS Conditions**

All FM comprehensive two-dimensional GC–MS applications were performed on a system consisting of two independent Shimadzu GC2010 gas chromatographs (GC1 and GC2), and a TQ-8030 triple quadrupole mass spectrometer. Data were acquired using the GCMSSolution software (Shimadzu). Bidimensional chromatograms in all applications were generated using the ChromSquare software v. 2.0 (Shimadzu Corporation). The two GC ovens were linked through a heated transfer line. The primary GC (GC1) was equipped with an AOC-20i auto-injector and a split-splitless injector (310 °C).

The primary column (situated in GC1), an 20 m × 0.18 mm, 0.18-μm d.f., SLB-5ms (Supelco) (silphenylene polymer,
practically equivalent in polarity to poly[5% diphenyl/95% methylsiloxane], was connected to position 1 of the wafer-type interface (SGE), after passing through the heated transfer line. A 10 m × 0.32 mm, 0.20-μm d_f SPB-50 capillary segment (poly[50% diphenyl/50% dimethyl] siloxane) column (Supelco) was connected to position six of the interface. An external loop in stainless steel was used. A description of the modulator can be found in a previous article (5).

**GC1 and GC2 temperature programmes** (FAMEs): 80–310 °C at 3 °C/min. GC1 temperature programme (unsaponifiable fraction): 80–310 °C at 3 °C/min (a +20 °C temperature offset was used in GC2). Initial He head pressure (constant linear velocity): 106.5 kPa. Initial auxiliary (advanced pressure control [APC]) He pressure (constant linear velocity): 60 kPa. Injection volume: 0.5 μL; split ratio: 1:200 (FAMEs); and 1:10 (unsaponifiable fraction). Modulation period: 8.5 s (accumulation period 7.95 s/injection period 0.55 s). Triple quadrupole MS conditions: ionization mode: electron ionization (70 eV). Interface and ion source temperatures: 280 °C and 250 °C. Collision gas and pressure: Ar (200 kPa). In the full-scan mode, a mass range of m/z 50–510 and 25 Hz spectral acquisition frequency were used. TMS-cholesterol MRM transitions: m/z 368>228 and 329>97 [10 eV collision energy]; acquisition frequency: 20 Hz (during the simultaneous scan/MRM mode). Mass spectral library matching was carried out by using the FAMEs library (Wiley), and NIST08 and Wiley MS databases.

**GC–FID Conditions**
The GC–FID analyses were performed on a GC2010 Plus instrument (Shimadzu). Data were acquired and processed by the GCsolution software ver. 2.41 (Shimadzu). The gas chromatograph was equipped with an AOC-20i auto-injector and a split-splitless injector (310 °C).

Seed oil FAMEs were separated on a 30 m × 0.25 mm, 0.25-μm d_f Supelcowax-10 column (100% polyethylene glycol) (Supelco), using an oven temperature programme of 50 °C to 280 °C (3 min), at 3 °C/min. The unsaponifiable fraction was separated on a 30 m × 0.25 mm, 0.25-μm d_f SLB-5ms column (Supelco), using an oven temperature program of 90 °C to 320 °C (2 min) at 3 °C/min. Initial He head pressure (constant linear velocity): 99.5 kPa (FAMEs) and 107.7 kPa (unsaponifiable fraction). Injection volume: 1.0 μL; split ratio: 1:100 (FAMEs) and 1:10 (unsaponifiable fraction). The FID was operated at 320 °C.

**Results and Discussion**
In recent research, the entire unsaponifiable fraction (no thin-layer chromatography pre-separation was performed) of
A series of vegetable oils (mainly extra-virgin olive oils) was subjected to cryogenic GC×GC analysis, after TMSE derivatization (6). A dual-detector (rapid-scanning quadrupole MS and FID) configuration was used, with the FID data exploited to determine relative quantitative information for the sterols. In this study, two FM GC×GC–MS methods were used to extrapolate detailed information on the lemon seed oil composition in terms of fatty acids and unsaponifiable constituents. Two GC–FID approaches were used to extrapolate percentage data.

### Fatty Acid Methyl Esters

The fatty acids were analyzed as FAMEs. Identification was performed through: i) MS database matching (similarity values lower than 75% were not considered); ii) the support of linear retention index (LRI) data; iii) specific 2D chromatogram analyte locations. Considering the latter, the presence of group-type patterns has been previously observed in GC×GC FAME analysis, and is related to carbon number and double-bond number or position (5). Even though no pure standards were used, peak assignment can be considered as “positive”. Altogether 20 FAMEs were identified (Table I), 13 of which were “saturates”.

Relative quantification FID data were derived from two consecutive applications (Table I), and is reported only for the five main FAs, the summed percentage values of which reached 98.5% (all other FAs were present in percentage values ≤ 0.3%). A 30 m × 0.25 mm polyethylene glycol column was used, with no problem encountered in the separation and identification (based on elution order and peak intensity) of the five major peaks.

In general, the C16 and C18 groups were the most predominant ones, in agreement with previous results (2–4). Palmitic acid was the most abundant (18.4%) “saturated” species, followed by stearic acid, present at the 3.4% level. Such data are in agreement with those reported previously (C16:0 = 19–21%; C18:0 = 2–4%) (2–4). Other linear saturated fatty acids found were C14:0, C15:0, C17:0, C18:0, C19:0, C20:0, and C24:0. Among these, arachidate (C20:0) methyl ester was slightly more abundant (0.3%). Moreover, four iso-/anteiso- species were identified.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>Identification</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C13:0</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C14:0</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>C15:0</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C16:0</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>C17:0</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>C18:0</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>C19:0</td>
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</tr>
<tr>
<td>8</td>
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<tr>
<td>9</td>
<td>C21:0</td>
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<tr>
<td>10</td>
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<tr>
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<tr>
<td>12</td>
<td>C24:0</td>
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</tr>
<tr>
<td>13</td>
<td>C25:0</td>
<td>a</td>
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</tr>
<tr>
<td>14</td>
<td>C26:0</td>
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</tbody>
</table>

Table II: Components found in the unsaponifiable fraction of lemon seed oil, type of identification process, and percentage (%) of various constituents of the sterol fraction.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>Identification</th>
<th>%</th>
</tr>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>C15:0-ol</td>
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<td>C17:0-ol</td>
<td>b</td>
<td></td>
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<tr>
<td>6</td>
<td>C18:0-ol</td>
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</tr>
<tr>
<td>7</td>
<td>C20:0-ol</td>
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<td>8</td>
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<td>cycloartenol</td>
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<tr>
<td>54</td>
<td>citrostadienol</td>
<td>c</td>
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</table>

α: standard injection; b: MS database; c: on-line database and literature data (7,8).
Linoleic and oleic acid were by far the most abundant of the unsaturated species, reaching levels of 36.6% and 27.9%, respectively. Such percentages were similar to those reported by Reda et al. (3). Linolenic acid (C\textsubscript{18:3}) was found at the 12.2% level, slightly higher than values previously reported (2–4). The total percentage of oleic, linoleic, and linolenic acid reached 76.7%. Finally, three minor monounsaturated fatty acids were identified, namely C\textsubscript{16:1}, C\textsubscript{17:1}, and C\textsubscript{20:1}, along with a polyunsaturated FA, specifically C\textsubscript{20:4}.

**Unsaponifiable Fraction**

The constituents of the unsaponifiable fraction (calculated to be approximately 1.5% of the oil, slightly higher than in olive oil [6]) were initially analyzed with the QqQ MS operated in the full scan mode. The unsaponifiable fraction of lemon seed oil is illustrated in two GC×GC–MS chromatogram expansions in Figures 1 and 2. Compound identification (Table II) was performed through: i) the comparison of database mass spectra with experimental ones (database matches with a spectral similarity lower than 70% were not considered); ii) the visual comparison of freely-available mass spectra (7) with experimental values; iii) the use of literature information (8,9); iv) chemical class 2D plane locations. When available, pure standard compounds (C\textsubscript{7}–C\textsubscript{30} alkane series, eicosanol, docosanol, tetracosanol, cholest erol, and \( \beta \)-sitosterol) were also injected. A discussion on the constituents of the unsaponifiable fraction will now follow.

Hydrocarbons: squalene (peak 35 — triterpene hydrocarbon with six double bonds [C\textsubscript{30}H\textsubscript{50}]) and linear alkanes (peaks 1–21 — C\textsubscript{13}–C\textsubscript{34} range) are present. Three tocopherols were found (\( \beta, \gamma, \alpha \) — peaks 36–38), with the “alpha” compound the most abundant on the 2D plane (it co-elutes with the primary column bleed), confirming the results of previous research (2).

Derivatized aliphatic alcohols from C\textsubscript{13} to C\textsubscript{16} (peaks 22–25), and from C\textsubscript{18} to C\textsubscript{26} (peaks 26–33), were identified. The alcohols C\textsubscript{17} and C\textsubscript{19} were not found, while the most intense peak was C\textsubscript{22} (peak 29). The alcohol diagonal was positioned below the hydrocarbons on the 2D plane (Figure 1). Because of the medium-polarity nature of TMS ethers, their location was expected to be higher (along the y-axis) in the chromatogram. The most probable explanation for this is wrap-around, which is when an analyte is characterized by a second-dimension retention time exceeding the modulation period.

Overall, 16 sterols, located in a specific zone of the GC×GC–MS chromatogram (Figure 2), were given a name, and could be sorted into three groups: 4,4-dimethyl-, 4-methyl-, and 4,4-dimethyl-sterols.

Three 4,4-dimethyl-sterols were tentatively identified, namely \( \beta \)-amyrin (peak 49), cycloartenol (peak 51), and 24-methylene-cycloartenol (peak 53); in addition, a single 4-methyl-sterol was found, specifically citrostadienol (peak 54). Cycloartenol and 24-methylene-cycloartenol were identified through MS database matching, even though low similarity values were attained (<80%); information found in the literature, related to significant ions, was therefore very useful to support peak assignment (8,9). Ions corresponding to the loss of a methyl-, trimethylsilyl hydroxy-, and methyl- + trimethylsilyl hydroxy- groups were considered. \( \beta \)-amyrin and citrostadienol were tentatively identified using literature data (8), and through acquired knowledge of the elution sequence (6).

Twelve 4,4-desmethylsterols were identified (10 tentatively), with those of interest from a regulation viewpoint (olive oil is herein considered as reference) accompanied by percentage information (10) derived from two replicates (Table II). The same stationary phase used in the GC×GC first dimension was again used and so the elution order was maintained. Beta-sitosterol (positive identification) and campesterol (peaks 47 and 43) were the predominant compounds, reaching 74.7% and 13.6%, respectively. The percentage of campesterol should be noted as it was considerably higher than that of olive oil (<4%) (10). Cholesterol (positive identification) was found at the 0.9% level, higher than in olive oil (maximum 0.5%) (10).

With regards to the other nine 4,4-desmethyl-sterols, an acceptable MS database match was attained only for stigmasterol (peak 45) and ergosterol (peak 41); for other sterols, namely lathosterol, 24-methylene-cholesterol, clerosterol, \( \Delta^7 \)-avenasterol, \( \Delta^7 \)-stigmasterol, and \( \Delta^7 \)-avenasterol (peaks 40, 42, 46, 48, 50, and 52, respectively), the elution order (6) and the visual comparison of freely-available mass spectra, along with fragmentation pattern information (8,9), were the tools used for tentative peak assignment. Finally, a stanol, namely campestanol (peak 44), was identified on the basis of the elution order (6) and the visual comparison of freely-available mass spectra. Among the remaining nine 4,4-desmethyl-sterols,
Δ7-avenasterol and Δ7-stigmasterol were the most abundant, with percentage levels equal to 3.1% and 1.6%, respectively (Table II). It should be noted that the percentage level of Δ7-stigmasterol is regulated in olive oils, with values which must be lower than 0.5% (10). A further important regulation is the total sum of β-sitosterol, clerosterol, sitostanol (not found in the present research), and Δ7-avenasterol, which must exceed 93% in olive oil; in the present research the total percentage reached 78.7%.

Finally, the MRM capability of the QqQ MS system was exploited for the absolute quantification of cholesterol, present in unusually high percentages for a vegetable oil. A 3-point calibration curve was constructed (10–50–100 mg/L), using standard cholesterol (dissolved in chloroform) and was subjected to derivatization. Two product ions were selected, a quantifier (m/z 228) and qualifier (m/z 97), characterized by an ion ratio of 24.5%. The untransformed simultaneous full-scan/MRM traces, relative to three modulations, are shown in Figure 3. Partial overlapping of cholesterol with column bleed is evident (upper trace), as well as the high selectivity of MRM analysis. Derivatized cholesterol was quantified at a concentration of 104 mg/L in the unsaponifiable fraction solution (ready for GC analysis), corresponding to an absolute quantity of 146 μg/g in lemon seed oil. Such a value is an approximation because cholesterol is present in the free form, in the unsaponifiable fraction.

Conclusions
The results described here give a detailed view on the lipid profile of lemon seed oil. The FA results confirm the data present in the literature, even though a higher number of low-amount FAs were found. With regards to the information on the unsaponifiable fraction, to the best of the authors’ knowledge, no such studies have been previously reported. The flow modulator described here is being used in a routine manner, and can be considered as a valid, flexible, low-costing alternative to cryogenic modulation. In addition, the QqQ MS used, characterized by rapid simultaneous scan/MRM capabilities, has confirmed its usefulness for both untargeted and targeted GC×GC applications. Future FM GC×GC–QqQ MS research will focus on the elucidation of the aroma of lemon seed oil.

Acknowledgments
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A Generic Method for Target (Group) Analysis in Edible Oils and Fats: Combined Normal-Phase Liquid Chromatography and Capillary Gas Chromatography

Edible oils and fats are complex mixtures of compounds. To understand the properties of an oil or fat, information on groups of molecules, as well as individual molecules, is needed. This article describes the role of normal-phase liquid chromatography (LC) as a generic sample pretreatment tool prior to detailed analysis by gas chromatography (GC). Moving from one group to another can be performed by simply adjusting the elution conditions and collection windows. Different applications of the new unified method will be discussed, including detailed analysis of partial acylglycerides, steradienes, and glycidylesters, as well as the use of the novel method for total-polarity mapping.

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Edible oils and fats are an important part of the human diet. From an analytical perspective, these food ingredients, are extremely complex mixtures, irrespective of whether they originate from animals or vegetables. Analytical measurements are therefore challenging. A wide range of analytical methods are available that allow a detailed mapping of the overall bulk composition, as well as an analysis of specific trace compounds. In all of these methods sample preparation is a crucial step. Historically, little standardization of the sample preparation and analysis methods was performed. As a result, edible oil and fat laboratories are now confronted with a huge diversity of almost identical, yet slightly different, methods. More generic approaches could help to contribute to the efficiency of these laboratories.

As in all analytical measurements, the key aspects in method development for edible oil and fat analysis are selectivity and sensitivity. The compounds of interest should be measured at the desired levels without interferences from other species. Sensitivity is a crucial factor in trace analysis, for example, in contaminant or vitamin analysis. Selectivity, that is, the ability to distinguish the compounds of interest from those that are not relevant, is a second important factor in trace analysis, but it is equally relevant in the assessment of the main compound classes in the oil. Selectivity in oil and fat analysis is basically related to isolating the compound or compound group of interest from the total sample by exploiting differences in the physico-chemical properties (reactivity, size, or polarity) between the compounds of interest and the rest. Isolation is very often performed in the sample pretreatment step. Saponification followed by extraction is an important sample preparation step for removal of the bulk triacylglycerides (TAG) and isolation of the (non-hydrolysable) non-polar species. Saponification will not be considered here because it is not a universal approach. All compounds that can be hydrolyzed are lost in the procedure. This article will focus on methods for target-group isolation that isolate the molecules in
their native, intact form. After the initial isolation of the compounds of interest, chromatographic techniques can be used to introduce additional selectivity and quantify the groups or individual compounds for which information is needed.

In the past a wide variety of methods have been developed for the pre-isolation of the target analytes from an oil or fat sample. Liquid–liquid partitioning, solid-phase extraction (SPE), thin-layer chromatography (TLC), size-exclusion chromatography (SEC), and complexation are the most common tools.

After the initial isolation both gas chromatography (GC) and liquid chromatography (LC), either in combination with or without mass spectrometry (MS), can be used for separation and quantification. It is clear that all the sample preparation methods, with the exception of SEC, exploit solubility and interactions as the mechanisms for separating the target compounds from the remaining sample material. It was from this that the idea to replace the wide variety of isolation methods by a single analytical isolation tool, normal-phase LC, arose. Much of the early work to replace complex sample work-up methods for oil and fat analysis by LC isolation of the fraction of interest was performed by Grob and coworkers (1). Normal-phase LC isolation of the compounds of interest provided an excellent selectivity. The drawback of LC isolation (the dilution of the fraction) was elegantly resolved by applying large volume sample transfer to the second dimension GC or LC method.

This article will describe previous work in the development of a single, unified analytical protocol that can replace most of the sample preparation procedures applied in the various edible oil analysis methods. Building on the pioneering work done by Grob, the use of combined normal-phase LC–GC for the analysis of compounds and compound classes that have been of interest for decades will be demonstrated. In addition, its use for the analysis of species that have only received interest recently will be illustrated. The latter group includes sterols, steroloxides, and glycidylesters.

**Table I: Normal-phase LC gradient settings for typical edible oil and fat analyses. The flow rate is 1 mL/min unless indicated otherwise. Some of the applications are shown in this article (indicated by the figure number). Slight adjustments of the elution windows might be necessary because of between-column or between-eluent differences. Abbreviations: Hx: hexane, MTBE: methyl-tert-butylether, IPA: 2-propanol, CHCl3: chloroform.**

<table>
<thead>
<tr>
<th>Application</th>
<th>Solvent A</th>
<th>Solvent B</th>
<th>Gradient programme and collection time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total polarity separation (Figure 1)</td>
<td>Hx</td>
<td>MTBE</td>
<td>0% B (0–5 min) → 20% MTBE (45–50 min) → 0% B (51–60 min). Collection window: Not applicable. The entire normal-phase LC chromatogram is sampled.</td>
</tr>
<tr>
<td>Dialkylketones</td>
<td>0.01% MTBE in Hx</td>
<td>MTBE</td>
<td>0% B (0–6 min) → 60% B (10–20 min) → 0% B (21–50 min). Collection window: 4.0–7.0 min</td>
</tr>
<tr>
<td>Steradienes, sterols, and sterolesters</td>
<td>Hx</td>
<td>7% MTBE in Hx</td>
<td>On-line transfer originally, 2.1 mm column. Flow 0.3 mL/min, A:B = 1:1 v/v. Collection window: 1.0–3.5 min</td>
</tr>
<tr>
<td>Sterols: des-, mono-, and dimethylsterols</td>
<td>10% MTBE in Hx</td>
<td>Isocratic at 1.5 mL/min, Collection window: Desmethylsterols: 12.5–18.0 min. Mono: 8.0–11.0 min, Di: 6.0–8.0 min</td>
<td></td>
</tr>
<tr>
<td>Glycidylesters (Figure 4)</td>
<td>0.25% MTBE + 0.01% CHCl3 in Hx</td>
<td>MTBE</td>
<td>100% A (0–25 min) → 70% B (26–35 min) → 100% A (36–65 min). Collection window: 21–28 min</td>
</tr>
<tr>
<td>Steroloxides</td>
<td>Hx</td>
<td>IPA</td>
<td>1.0% B (0–22.5 min) → 50% B (23.5–29.5 min) → 1.0% B (30.5–50 min). Collection window: 20.0–35.0 min</td>
</tr>
<tr>
<td>Mono- and diacylglycerols (Figure 2)</td>
<td>Hx</td>
<td>IPA</td>
<td>1% B (0–5 min) → 60% B (45–50 min). Collection window: 20.0–35.0 min</td>
</tr>
<tr>
<td>Steradienes (Figure 3)</td>
<td>Hx</td>
<td>MTBE</td>
<td>0.5% B (0–10 min) → 50 %B (20–30 min) → 0.5% B (31–60 min). Collection window: 3.0–6.0 min</td>
</tr>
</tbody>
</table>

**Instrumentation**

The proposed novel methods rely on the use of tunable normal-phase LC as a unified route for target compound and target group isolation in edible oils. If one of the main compound groups, for example the TAG or diacylglycerides (DAG), is of interest, the oil sample is injected into the normal-phase LC system without any further sample pretreatment. For the analysis of trace-level compounds, such as in the analysis of glycidyl esters, it is generally worthwhile removing the bulk of the fatty matrix in a quick liquid extraction step prior to the normal-phase LC fractionation.

**Sample Preparation**

To remove the TAG, 100 mg of the oil sample was dispersed in 4 mL of acetonitrile. The oil sample at the bottom of the tube was gently warmed to obtain a homogeneous clear liquid oil phase. Vigorous mixing (vortex) for about 20 s was applied to disperse the oil into the organic phase. After centrifugation for 5 min at 3500 g, the acetonitrile was transferred into a clean test tube and the solvent was evaporated under a
A gentle stream of nitrogen (35 ± 5 °C). Immediately after drying, the walls of the test tube were rinsed with approximately 1 mL of chloroform. The solvent was again removed under nitrogen and finally the residue was redissolved in 1 mL of hexane/2-propanol (85/15 v/v).

**Normal-phase LC Separation**

The normal-phase LC separations were performed on a modular, binary gradient system, consisting of two solvent delivery pumps (type Gilson 305 + 306), a manometric module (Gilson 805s), and a dynamic mixer (Gilson 811b), and equipped with a UV detector (UV-2000, Thermo Separation Products), operated at 205 or 210 nm. The normal-phase LC separations were performed on either one or two (serially connected) 250 × 4.6 mm, Lichrosorb 5 Diol columns (Agilent Technologies) thermostatically controlled at 40 °C by the column oven of a Marathon-xt autosampler (Separations). Different normal-phase LC gradients were used matching the polarity of the analyte group(s) of interest. An overview of the gradients used for specific applications is presented in Table I.

Since many of the target compounds showed no or very limited UV absorbance, collection windows were typically determined through the analysis of the pure model compounds. Fractions at 0.5 min intervals were then collected around the expected normal-phase LC retention time. These fractions

| Table II: Typical GC settings for analysis of the isolated normal-phase LC fractions |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Application                 | Injection method and volume | Column                      | Temperature programme        | Detection       |
| Total polarity separation   | On-column, 50 µL (high level fractions were diluted) | CP-Simdist, 10 m × 0.53 mm, 0.17-µm \(d_f\) | 60 °C (2 min) \(\rightarrow\) 20 °C/min \(\rightarrow\) 385 °C (5 min) | FID            |
| Dialkylketones              | On-column, 2.0 µL            | VF-1ms, 25 m × 0.25 mm, 0.25-µm \(d_f\) | 60 °C (1 min) \(\rightarrow\) 20 °C/min \(\rightarrow\) 180 °C (0 min) \(\rightarrow\) 10 °C/min \(\rightarrow\) 280 °C (0 min) \(\rightarrow\) 5 °C/min \(\rightarrow\) 330 °C (5 min) | FID            |
| Steradienes, sterols, and sterolesters | On-column, 0.5 µL | CP-Sil 13CB, 25 m × 0.25 mm, 0.25-µm \(d_f\) \(\rightarrow\) CP-Sil 8CB, 30 m × 0.25 mm, 0.25-µm \(d_f\) | 60 °C (1 min) \(\rightarrow\) 15 °C/min \(\rightarrow\) 250 °C (0 min) \(\rightarrow\) 2.0 °C/min \(\rightarrow\) 300 °C (18 min) | FID            |
| Sterols: des-, mono-, and dimethylsters | On-column, 0.5 µL | CP-Sil 13CB, 50 m × 0.32 mm, 0.20-µm \(d_f\) | 80 °C (1 min) \(\rightarrow\) 20 °C/min \(\rightarrow\) 250 °C (0 min) \(\rightarrow\) 2.0 °C/min \(\rightarrow\) 300 °C (5 min) | FID            |
| Glycidylesters (Figure 4)   | Pulsed splitless, 1.0 µL    | Inertcap Purewax, 15 m × 0.25 mm, 0.50-µm \(d_f\) | 110 °C \(\rightarrow\) 25 °C/min \(\rightarrow\) 160 °C (0 min) \(\rightarrow\) 10 °C/min \(\rightarrow\) 260 °C (2 min) | MS             |
| Steroloxides                | PTV cold splitless, 1.0 µL  | CP-Sil 13 CB, 25 m × 0.25 mm, 0.25-µm \(d_f\) | 60 °C (0.5 min) \(\rightarrow\) 20 °C/min \(\rightarrow\) 260 °C (0 min) \(\rightarrow\) 2.0 °C/min \(\rightarrow\) 300 °C (2.5 min) | MS             |
| Mono- and diacylglycerols   | On-column, 1.0 µL           | CP-Sil 5CB, 10 m × 0.32 mm, 0.12-µm \(d_f\) | 80 °C (2 min) \(\rightarrow\) 10 °C/min \(\rightarrow\) 360 °C (5 min) | FID            |
| Steradienes (Figure 3)      | On-column, 0.5 µL           | CP-Sil 13CB, 25 m × 0.25 mm, 0.25-µm \(d_f\) \(\rightarrow\) CP-Sil 8CB, 30 m × 0.25 mm, 0.25-µm \(d_f\) | 60 °C (1 min) \(\rightarrow\) 15 °C/min \(\rightarrow\) 250 °C (0 min) \(\rightarrow\) 2.0 °C/min \(\rightarrow\) 300 °C (18 min) | MS             |

**Normal-phase LC–UV chromatogram**

Figure 1: Comprehensive normal-phase LC×GC separation of an edible oil sample. All identifications of compound classes are tentative.
were then analyzed by GC. In this way the approximate elution position could be determined. The exact elution window could then be established using either a series of standard compounds covering the whole chain-length range, or a real sample. Depending on the identity of the target analytes, whether derivatization prior to GC is needed, and the desired detection limits, the sample can be injected as such, or after removal of the solvent. This can be done under a stream of nitrogen at room temperature or slightly above. To avoid the loss of volatile sample constituents, a so-called keeper, for example, isooctane, can be added. The typical volume used was 200 µL. Derivatization was typically performed by adding 200 µL of a 25% BSTFA (bis-trimethylsilyl-trifluoroacetamide) solution in pyridine followed by heating at 70 °C for 30 min.

**GC Separation**
Separation of the compounds within a fraction was performed by capillary GC, either with flame ionization detection (FID) or MS detection. Different GC columns, injection methods, and injection volumes were applied depending on the desired chromatographic selectivity, the physicochemical properties of the compounds of interest, and the desired detection limits. An overview of the settings used is given in Table II.

GC–FID experiments were performed on a Trace GC2000 (Thermo) equipped with an AS2000 autosampler, a cold on-column injector and an early solvent vapor exit. All GC–MS experiments were performed on an Agilent 7890A GC coupled to a 5975C inert XL mass selective detector (Agilent Technologies) and controlled by Chemstation software version E.02.00 (Agilent). The GC–MS system was equipped with two injectors: a split/splitless and a PTV injector (OPTIC3, ATAS GL). Several analytical columns were employed.

**Results and Discussion**

**Total Polarity Separations**
As outlined earlier, the main aim of the current work was the development of a tunable-selectivity method that could provide a generic approach for the analysis of specific groups of compounds in edible oils or fats. Within this strategy normal-phase LC is used to isolate the compound group of interest on the basis of its polarity. The idea for this strategy was derived from a series of early experiments performed using a novel technique called comprehensive LC×GC (2). In comprehensive LC×GC subsequent fractions from an LC separation are taken for detailed analysis by GC. This method was initially developed for detailed separation of triacylglycerides and later applied to whole oils as well (3). Figure 1 shows one of the early comprehensive two-dimensional normal-phase LC×GC chromatograms obtained. In this dot-plot figure the normal phase LC separation is plotted in the x-direction and GC chromatograms recorded for every normal-phase LC fraction are plotted in the y-direction. The size of a dot represents the peak area at a certain GC retention time. In the separation plane a large number of compound groups can be seen. Based on their relative polarity...
(normal-phase LC retention time) and size (GC retention) they could be identified tentatively. From Figure 1 it is clear that one single normal-phase LC gradient allows the separation of a number of important compound classes, which means that a generic method for tunable target analysis is feasible. It is important to emphasize that it is not the aim of the current study to obtain information on all compound classes in a single comprehensive LC×GC run. Information on all compound classes would only be required for very advanced applications such as authenticity research or patent infringement investigations, in most other cases only one compound group is of interest. For the latter purpose heart-cut LC–GC suffices.

Chromatograms such as those shown in Figure 1 can be recorded in a fully automated manner as we have shown previously (4). Automated on-line heart-cut LC–GC using on-column or loop-type large volume injection was demonstrated by Grob and Frohlich in 1992 (5). We applied large volume injection using a programmed temperature injector (PTV) for solvent elimination between the LC and GC–MS. Full automation is evidently necessary for comprehensive LC×GC, but if just one or a few fractions of the LC separation are of interest, (automated) fraction collection with manual transfer to the GC for further analysis is generally easier.

**Partial Acylglycerides**

The main compounds present in edible oils are the TAG, tri-esters of fatty acids and glycerol. In addition to the TAG, low levels of the so-called partial acyl glycerides can also be present. These compounds consist of glycerol with two fatty acid chains (di-acylglycerides or DAG) or one fatty acid (mono-acylglycerides or MAG). Partial glycerides can result from incomplete acylation in the biosynthesis of the oil or from oil processing. Their analysis is important because even very low levels of these partial glycerides can strongly affect product stability of margarines and dressing products. For a full understanding of the influence of DAG and MAG on product stability, information on the chain length distribution, the last eluting DAG in GC. Figure 2 shows the GC–FID analysis of a combined MAG/DAG fraction from a palm oil sample. Figure 2 shows the GC chromatogram with the steradienes eluting between 18.0 min and 23 min. Quantification was performed against cholesterol as the internal standard.

**Steradienes in Olive Oils**

Olive oil is an important ingredient in Mediterranean cuisine. Different qualities of olive oils are available, depending on the origin and processing of the oil. The highest quality oils are the virgin or cold-pressed oils. The only processing steps allowed in the preparation of virgin oils are physical techniques such as pressing, filtration, decantation, and centrifugation (6). Because of its high price, virgin olive oil is susceptible to fraud and adulteration. There are various analytical methods to differentiate virgin from refined or thermally treated olive oils. An important indicator is the level of 3,5-stigmastadiene, a dehydration product of sterols formed upon thermal treatment of the olives. Virgin olive oils, obtained by cold pressing, do not contain measurable amounts of 3,5-stigmastadiene (less than 0.01 mg/kg [7]) while the level of 3,5-stigmastadiene in refined olive oils ranges between 0.3 mg/kg and 0.9 mg/kg (8). Reliable analysis of steradienes is therefore very relevant. Steradienes are four-ring compounds consisting only of carbon and hydrogen atoms. In terms of polarity, steradienes are less polar than the bulk of the oil, the TAG. In normal-phase LC isolation this means the steradienes elute almost unretained, together with other non-polar species such as alkanes and squalene. Since the latter compound is...
present at a much higher level than the stearadienes in many oil samples an efficient separation and selective detection is needed. GC–MS is clearly the technique of choice. Figure 3 shows the GC–MS chromatogram of the isolated stearadienes fraction of an olive oil. Full scan GC–MS is sufficiently sensitive while simultaneously the extracted ion chromatograms offer the required selectivity.

**Glycidylester Analysis**

Glycidylesters (GE), fatty acid esters of glycidol, are process contaminants formed during the deodorization step of vegetable oil refining (9). They present a safety hazard as a result of the toxic effects of glycidol, which can be released upon digestion. Accurate measurement of GE is required for performing the risk assessment and for evaluating the effectiveness of various mitigation technologies. A suite of methods for GE analysis has recently been developed (9). These methods can be classified into indirect methods that measure glycidol after conversion of all glycidylesters into a single halogenated derivative, and direct methods that measure the levels of individual intact GE. A drawback of the indirect methods is that information on the fatty acid chain esterified to the glycidol backbone is lost as all esters are converted into a single analyte during sample preparation. We have recently developed a method based on off-line LC–GC for the analysis of intact GE (10). Figure 4 shows the normal-phase LC isolation of intact GE esters from a spiked palm oil sample. The GE elute well before the residual TAG, DAG, and MAG. Because the GE are present at extremely low levels, a liquid–liquid partitioning step using heptane–acetone-nitrite was performed prior to injection into the normal-phase LC system. In this partitioning step the TAG preferentially distribute into the heptane phase, while the GE are enriched into the acetone-nitrite phase. To enable a full separation of the non-polar GE from the only slightly more polar residual TAG, the normal-phase LC eluent needs to be strictly non-polar and free of even the slightest traces of water or other polar species. Under these conditions the double bonds in the molecules start to strongly influence retention. In Figure 4 this becomes apparent from the rather large resolution between the glycidylesters with a saturated chain versus those containing one, two, or three double bonds. As a result of this rather strong separation, the fraction that has to be collected is wider than usual, necessitating an evaporative preconcentration prior to injection in GC–MS. Since the compounds have a low volatility, this preconcentration is straight forward and, if performed under nitrogen at a moderate temperature, holds little risk of losing analytes. The quantification limits of the novel method are better than 0.05 mg/kg for individual glycidylesters. In practical operation the method is very stable: Hundreds of samples could be analyzed without problems or excessive maintenance. The GC–MS performance was remarkably stable. This is a positive consequence of the efficient clean-up that is obtained in our generic normal-phase LC isolation strategy.

**Conclusions**

The combination of normal-phase LC as a selective method for the isolation of specific compound classes from edible oil samples for further detailed analysis of individual homologues by GC has been demonstrated. This combination offers a generic method with a selectivity that can be tuned from the keyboard. Fully automated off-line operation is easy to perform, and online coupling even allows unattended and therefore fast, cheap, and reliable operation. In addition to being highly selective, normal-phase LC pre-isolation combined with GC–MS quantification also provides good sensitivity. By using an intermediate evaporation step between the normal-phase LC isolation and the GC analysis, or by implementing a large volume injection step, detection limits in the ppb range are achievable. Future improvements could be achieved by combining the universal isolation method with more advanced analysis tools such as GC×MS–MS, comprehensive GC×GC–MS, or ultrahigh-pressure LC (UHPLC)–MS.

**References**


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Food Taints and Flavors — An Investigative Approach

If food flavor is not as expected, it can damage consumer confidence and give the perception of poor quality. Setting analytical standards of what constitutes an acceptable flavor can be challenging, and while methods based on total volatiles or specific marker compounds can be a useful quality control check, a more investigative approach is often required. Determination of the taints and off-flavors responsible can be particularly challenging as the compounds are often unknown and may be present at extremely low levels (sub ppb). This article discusses the analytical methods available for taint and flavor analysis and highlights the approaches taken to identify compounds responsible and determine root cause.

Taints and off-flavors in food represent poor quality to the consumer and result in lack of confidence and brand damage. Analysis is performed to determine the source of the issue, to ensure consumer safety, and to prevent future occurrence. Food taint determination is challenging, because the matrix is complex and the compounds responsible may only be present in food at extremely low levels. If key aroma or taste compounds are known, analysis can be targeted when characterizing flavor, but determining taints and off-flavors requires a more investigative approach. Evidence must be gathered about a particular issue and the analytical data be connected with sensory characteristics. A term used frequently in analytical chemistry is “fit for purpose.” For targeted analysis, where compounds are known and required limits of detection are clear, this is relatively easy to define. However, for unknown contaminants, at undefined levels, optimization of methods can be more of a challenge.

Approaches

Determination of food taints is not easy. Care must be taken to avoid possible contamination from external laboratory sources (including personal care products used by the analysts). To avoid contamination use a dedicated analysis area; handle and store all samples (controls, suspect samples, and reference standards) separately; and take care during transportation to the laboratory. It is also important not to alter the sample during extraction — particularly when analyzing off-flavors — so it may be necessary to avoid heating the sample.

It may be possible to use a targeted approach for taint determination if, for example, the source of contamination is known. However, a generic screening approach is most often used. In this case analysts can compare samples to control or reference samples to identify differences, rather than identify all volatile components in what can be a very complex sample.

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For flavor analysis there are several possible approaches. A complete volatile profile can tell you much of the components in a sample, but different sample preparation methods will produce different profiles. It is also important to consider whether a more targeted approach is appropriate. A sample may have a very complex volatile profile, but only a few compounds may be key to the perceived characteristic flavor. It may therefore only be necessary to monitor the levels of these key compounds. Setting specifications for flavors, particularly natural ones, is rarely straightforward. A good understanding of acceptable levels of natural variation is required, and must always be linked to sensory data.

**Consumer Perception and Sensory Evaluation**

When taints or off-flavors are brought to light via consumer complaints, descriptions of the odor or taste are frequently unreliable or unhelpful. The first stage when investigating complaints should therefore be a sensory assessment of the sample — be that by an in-house specialist trained sensory panel or a more informal round table discussion — that will give objective assessments and sensory descriptors that can be matched to reference guides (1,2,3) or specialized websites (www.odour.org.uk and www.flavornet.org).

Sensory analysis is key to quantitative methods of analysis as an accurate description can provide key information to the analyst. However, very similar compounds can have very different sensory thresholds and even isomers can be perceived very differently in terms of levels needed to give a taint. Other components within the food matrix can also affect acceptability and potency of flavor compounds. This is further complicated as more than one compound may be responsible for the taint.

Sensory theory perception is therefore used in combination with chemical analysis, rather than as a standalone method, to confirm the identity and levels of the compounds present.

**Sample Preparation and Extraction**

As in any food analysis, taking a representative sample is critical to obtaining meaningful results. However, the compounds responsible may not be homogenously distributed throughout the sample, particularly if the result of external contamination or migration from packaging. It is also important to understand normal variations in the product. It is critical to include as many of the ingredients and product varieties that are acceptable, to determine what is “normal” for a product.

The sample preparation method chosen can be critical to the correct interpretation of results. Flavor analysis profiles will be different depending on the extraction technique used (Figure 1). In the case of taints, the wrong approach could lead to the suspect compound not being detected at all, and if you have limited sample size repeat analysis may not be an option. The different sample extraction methods are discussed here.

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Figure 1: Comparison of flavor volatile extraction techniques for flavor profiling: (a) liquid injection; (b) headspace-solid-phase microextraction (HS-SPME); (c) stir bar sorptive extraction (SBSE).
Liquid-based Extraction

Direct solvent extraction is sometimes used for the analysis of food taints and flavors, but is most commonly performed when detecting specific target analytes in specific food matrices. As selectivity is largely controlled through solvent choice, it is often necessary to isolate compounds of interest from the matrix using further cleanup. This can be achieved using techniques such as solid-phase extraction (SPE) that can also provide an enrichment step. Any additional steps in a method can result in losses of volatile compounds and also introduce more opportunity for sample contamination, which is of particular concern in investigative work such as determination of taints.

Steam distillation extraction (SDE), using apparatus such as Likens Nickerson, is a technique frequently used in taint analysis. It has the advantage that it can be used for a wide variety of food matrices and it produces a clean extract of volatile components. Large sample sizes can be taken and with the inclusion of a concentration step excellent sensitivity is achievable (sub µg/kg [ppb] levels).

The major disadvantage of SDE is the need for specialist glassware, the possibility of cross-contamination, and losses in concentration. It is important to analyze both a “control” sample and suspect sample to enable identification of genuine differences as formation of breakdown products or artefacts can be a problem. Various modifications of the original apparatus have been made and these include vacuum distillation systems to reduce artefact formation.

A related technique used in flavor extraction is called solvent assisted flavor evaporation (SAFE)(5). This is regarded as the “gold standard” for flavor extraction, producing an extract that is the best representation of the product (6,7). SAFE enables the isolation of volatile compounds from foods using distillation under vacuum conditions. The distillate is collected in a flask cooled with liquid nitrogen. As SAFE is performed at reduced pressure and temperatures, the sample is not changed during the extraction process and therefore no “cooking”, artefact formation, or loss of thermally labile components. Fractions of both volatile and non-volatile components are obtained and both can be used for subsequent analysis.

Headspace Extraction

Headspace extraction is particularly suitable for extraction of volatile compounds. Sampling only the headspace above a sample ensures separation of the compounds of interest from the non-volatile food components that could cause interference or compromise the chromatographic system. This is an equilibrium technique and does not provide exhaustive extraction, but the concentration in the headspace is proportional to that in the sample. It can be used for a wide variety of matrices.

Direct static headspace involves heating a sample (ideally to the point...
of equilibrium) and taking an aliquot of the headspace for direct injection into the (GC–MS) instrument. It can be used for both a screening approach or optimized for more targeted quantitative analysis. For accurate quantitation, the use of appropriate internal standards (preferably isotopically labeled), or the method of standard additions is recommended. It can be used for all matrix types, including direct analysis of packaging, but can lack the sensitivity required for some compounds. Matrix modification, such as the addition of salt, can be used to encourage analytes into the headspace. Dynamic headspace or headspace techniques using further selection or enrichment can increase the selectivity and sensitivity of this approach.

**Solid-phase Microextraction (SPME)**

Microextraction techniques such as solid-phase microextraction (SPME), that use minimal solvent and enable extraction and enrichment in one step, are extensively used for flavor and taint analysis. Headspace–SPME in particular is being increasingly used for the determination of food taints and off-flavors (8,9). It has the advantages of direct headspace, as well as increased sensitivity and lower limits of detection (for some analytes).

Fibers and extraction conditions, in particular extraction temperature, must be selected to suit the compounds of interest. Generic protocols and mixed fiber coatings can be used for screening or comparative analysis. Because of the partitions between both the sample and fiber and the fiber and headspace, a higher temperature may not result in an increase in signal, as would be expected for direct headspace.

Conditions have to be optimized for each application for quantitative analysis, as response is dependent on the matrix and analytes of interest and conditions need to be optimized. For samples with high levels of matrix components, such as alcoholic drinks, fibers can be “poisoned” because of competition effects. Dilution, or careful selection of fibers and conditions, is therefore required.

For comparative analysis, or screening, the matrix should be identical and care should be taken with solid samples where the addition of water can significantly change the partition and resulting profile.

For accurate quantitation, the method of standard additions is often required, or the use of a suitable internal standard. Quantitative methods using labeled internal standards have been reported but require optimization. For unknowns, or in a variety of matrices, semi-quantitative analysis is possible, although the technique is more often used for comparative analysis.

**Stir Bar Sorptive Extraction (SBSE)**

Stir bar sorptive extraction (SBSE) was developed to have similar advantages to SPME, but provide higher extraction capacity (10). It generally uses no solvent and provides extraction and enrichment in one step. The most common approach is direct immersion sampling of liquid samples or extracts, although sampling of the headspace (known as headspace sorptive extraction [HSSE]) is also possible. Extraction is performed off-line allowing the simultaneous extraction of samples. Following extraction, analytes can be thermally desorbed directly into a GC–MS, or be extracted with solvent (this is less common because of the resulting dilution). For “dirty” matrices, the stir bar can be washed and dried following sampling as necessary, prior to desorption and GC–MS analysis.

SBSE has been used for determination of specific compounds associated with taints, such as chlorophenols and chloroanisole (11–14) but has also been demonstrated for a range of tainting compounds (15). Until recently the only coating commercially available for SBSE stir bars was polydimethylsiloxane (PDMS), limiting the technique to non-polar analytes (without derivatization). The development of a newer EG–Silicone coating ([PDMS)/ethylene glycol (EG) – copolymer) has been reported to be suitable for more polar analytes (16). Figure 2 shows a comparison of the PDMS and EG–Silicone coating for a range of compounds that have been reported to give taints in food and indicates an improved response for some of the more polar analytes (such as phenols).

**Instrumental Analysis**

A variety of techniques can be used for flavor analysis (4), and as causative compounds can have a range of chemical and physical properties, it is not always safe to assume that all compounds can be extracted using one analytical approach. The majority of compounds associated with taint and flavors are volatile. Gas chromatography coupled to mass spectrometry (GC–MS) is therefore a key method in food analysis.

For targeted compound analysis, flame ionization detectors (FID) or flame photometric detectors (FPD) can be used — the latter is particularly suited to analysis of sulfur compounds. For complex matrices and investigative work, where confirmation of identification is required, a mass spectrometric detector (MSD) is the preferred option. In many cases, a single quadrupole MSD is sufficient, but the use of a time of flight (TOF) detector with accurate mass or triple quadrupole (MS–MS) for increased sensitivity of target compounds offers advantages. This selectivity is particularly useful where generic non-selective extraction techniques have been used.

Whichever detector is used, good data analysis is key to obtaining accurate results, whether that be identification of unknowns or accurate quantitation of target analytes. This can be particularly challenging when determining taints in food, where the analyte is unknown and can be present at extremely low concentrations compared to matrix components. The use of software that enables comparison of samples for differences can help, although parameters must be optimized to enable detection of what may be a very small shoulder on a peak, or a small change in spectra.
Gas Chromatography–Olfactometry (GC–O)

Another technique quite commonly used in flavor analysis (also applicable to some taint applications) is GC–olfactometry analysis (GC–O). The technique was reviewed in 2008 by Barbara d’Acampaora Zellner et al. (17).

In GC–O, the output from the GC column is split between two detectors—one of these being the human nose via an olfactory port. This approach enables odor active components in complex mixtures to be assessed by correlation with chromatographic peaks as they elute from the instrument.

To fully represent any product or ingredient, the correct sample preparation procedure is critical. For flavor characterization projects, SAFE extraction is the method of choice. It is most commonly used to characterize the most odor active compounds within a product or ingredient to enable specifications, or more focused analysis, to be developed. However, when used for more investigative studies, other sample preparation methods, such as those based on headspace or SPME extraction, can be used.

GC–O can also be used in taint investigations. In cases where a distinct odor is perceived by human assessors, but where comparative GC–MS has not provided an answer, GC–O can help to identify the region of interest in a chromatogram allowing more thorough data analysis or improved enrichment solutions to be used.

E-nose Technologies

The approaches outlined so far are not rapid and require a level of expertise to interpret results. Often sensory analysis is used in the supply chain to assess flavor of raw materials ingredient and check the quality of finished products. Human olfactory sensors are the most sensitive detector for off-odors. Attempts have been made to replicate the way these receptors recognize smells and process the information to categorize "good" and "bad", via e-nose technologies.

The use of chemical sensors that attempt to correlate response to human perception have been discussed in a review by Citterio and Suziki (18).

E-nose systems can be broadly split into those using sensors and those using more traditional GC detectors, such as FIDs or mass spectrometers (including ion mobility systems). Most systems require a sample to be taken off-line and the headspace swept over a detector or sensor array. Sampling can be done in vials, beakers, Tedlar bags or, in some cases, on-line. Injection can be manual or automated, headspace or liquid, and detection can take place via gas sensors, flash GC, or liquid sensors. Data processing can include qualitative models, quality control cards (color cards for example are used in electronic eye devices) or quantification models. Various visualization techniques are also available.

All systems require initial training with a large number of samples and a clear definition (linked to sensory) of what is considered an acceptable profile for the particular raw material or product. Gas analysis or sensor systems that “sniff” samples of air can be used; however, most systems lack the sensitivity required to cover the full range of potential taints so they are generally used to monitor and control known issues.

Flavor Release — Real-time Volatile Analysis

A growing area of research in flavor analysis is the release of flavor over time. Flavor release profiles are strongly linked with consumer preference and, while the food industry has many mechanisms for prolonging or delaying flavor release, it is not always easy to measure analytically.

Techniques that provide real-time analysis of volatile organic compounds have been used for such studies. These include ion molecular reaction mass spectrometry (IMRMS), selected ion flow tube mass spectrometry (SIFT–MS), and proton transfer reaction mass spectrometry (PTRMS). All of these approaches use a soft ionization process to reduce fragmentation, resulting in simple spectra that enable rapid monitoring of specific ions.

IMR–MS uses ionization via ion-molecular reactions (19), whereas SIFT–MS is based on chemical ionization using selected reagent ions (H₂O⁺, NO⁺, and O³⁻). PTRMS utilizes proton transfer reactions from H₂O⁺ to the sample molecule and is therefore governed by the proton affinity (PA) of the analyte. Alternative ionization mechanisms have been reported using other reagents, such as krypton (20), and selective reagent ionization mass spectrometry (SRI–MS) technology has increased the number of compounds that can be analyzed. Instruments using both quadrupole and time of flight mass spectrometers are available. Volatiles can be introduced to the system in a number of ways: directly (air sampling), from headspace vials, or from ‘nose space’ during consumption of a foodstuff. A review of food applications was published in 2011 (21).

Conclusion

The analytical methods used in taint and off-flavor analysis will depend on many factors, including instrument availability and analyst experience. If targeted analysis can be performed then several techniques may be suitable, but for unknown taints, the choice is more limited. The correct choice of sample preparation can impact the results obtained and the conclusions drawn.

The link to sensory perception and analysis is critical in accurate data interpretation in both flavor characterization and determination of compounds responsible for taints and off-flavors. The human olfactory system remains the most sensitive detector and current analytical systems are still not a suitable replacement in many cases.

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Comprehensive Two-Dimensional Liquid Chromatography Coupled to Triple Quadrupole Mass Spectrometry: Application to a Challenging Food Case Study

The complexity of many food samples places a great demand in terms of both separation capabilities and specificity of detection. In this article, a novel system for fully automated comprehensive two-dimensional liquid chromatography (LC×LC) is discussed. The on-line coupling of the two separation dimensions was achieved using two six-port, two-position switching valves. High orthogonality was achieved by using a micro-bore cyano column for the first dimension separation, interfaced to a secondary C18 column packed with fused-core particles. The hyphenation to a triple quadrupole mass spectrometer generates a powerful analytical system, capable of extremely high resolving power, as well as targeted and untargeted analysis. The so-called multiple reaction monitoring (MRM) mode in fact enhanced selectivity, reducing sample consumption and the need for tedious clean-up procedures, specifically for beta-carotene quantification in a red pepper extract.
ions of interest from unspecified matrix components by monitoring only those m/z values that originate from a characteristic fragmentation pattern. The so-called multiple reaction monitoring (MRM) mode enhances selectivity and lowers detection limits, therefore reducing sample consumption; in addition, the MRM approach can also decrease analysis times by reducing the need for clean-up procedures, which are often mandatory, prior to the analysis of complex samples, such as many foodstuffs (6).

Among these complex samples, carotenoids represent a challenging analysis task for a number of reasons. These include high variability in the chemical structures, isomerization, poor stability, and the lack of commercially available standards for reliable identification and quantification in real samples. Commonly found in plants, algae, fungi, and bacteria, carotenoids consist of a C₄₀-tetramerpenoid structure with a symmetrical skeleton (7), and are usually divided into two groups: Hydrocarbon carotenoids, generally known as carotenes (such as β-carotene, lycopene), and oxygenated carotenoids, known as xanthophylls (for example, lutein, β-cryptoxanthin) (8). These compounds can be found in nature in their free form, or in a more stable fatty acid esterified form. The study of esterified carotenoids in natural sources is rather limited, especially because of the high degree of complexity; a saponification step is instead often used prior to LC analysis. However, such a strategy does present some drawbacks as during the saponification procedure, strong conditions are used and, as a consequence, carotenoid loss, as well as isomerization, can occur (9).

As far as separation is concerned, reversed-phase LC with both C₁₈ and C₃₀ stationary phases has been extensively used to achieve the separation of molecules differing in hydrophobicity within a given structural class (10). On the other hand, normal-phase LC on silica-based columns is largely used for carotenoid class separation, according to different polarity (with retention increasing from hydrocarbons to xanthophylls). A major limitation of this approach consists in diminished resolving power when separating carotenoid classes lying at two the extremes of polarity scale (hydrocarbons and xanthophylls [11]) from the rest of the matrix.

Furthermore, the true potential of coupling different C₁₈ columns (12) and two-dimensional LC approaches (13,14) has been investigated to increase the separation power and thus resolution and efficiency for the analysis of carotenoids in extremely complex natural samples.

Identification of carotenoids in foodstuffs is generally accomplished by the complementary information provided by LC retention times, photodiode array (PDA), and MS data. Although commonly used for carotenoid identification, PDA detection nevertheless fails in the case of analytes which exhibit similar — or even identical — spectra. On the other hand, MS was excellent for the analysis of these substances, allowing structure elucidation on the basis of both molecular mass and fragmentation pattern. Several ionization methods have been reported for MS analysis of carotenoids, including electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption–ionization (MALDI), electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI) and, more recently, atmospheric-pressure photoionization (APPI) and atmospheric pressure solids analysis probe (ASAP) (15). Sometimes, LC–MS–MS or MSⁿ can be advantageously applied to carotenoid analysis through the use of specific transitions and daughter ions for the identification of analytes via precursor ion selection, eventually allowing carotenoids of equal molecular masses but different fragmentation patterns to be discriminated between (16).

This article describes a novel LC×LC–PDA–MS–MS system capable of extremely high-resolving power, as well as targeted and untargeted analysis, simultaneously. The system was successfully used for the characterization of native carotenoids in red chili pepper, in addition to quantification of beta-carotene at sub-ppm level.

**Experimental**

**Standards and Chemicals**

Beta carotene reference material, as well as LC–MS grade n-hexane, butyl acetate, acetone, acetonitrile, and isopropanol, were obtained from Sigma-Aldrich/Supelco. Methanol, ethyl acetate, petroleum ether, and tert-butyl methyl ether (all reagent-grade) were supplied by Sigma-Aldrich.

**Sample and Sample Preparation**

The red chili pepper sample (Capsicum annuum L.) was purchased in a local market. For the extraction of intact carotenoids (not saponified), 200 g of red chili pepper homogenate were treated with three consecutive 300 mL aliquots of a methanol/ethyl acetate/petroleum ether (1:1:1, v/v/v) mixture. The extracts were combined and filtered through a paper filter. They were then evaporated to dryness under vacuum (30 °C), and the dry residue dissolved in 3 mL of a methanol/tert-butyl
methyl ether (1:1, v/v) mixture and filtered through a 0.45 μm Acrodisc nylon membrane (Pall Life Sciences).

**LC×LC–MS–MS Analysis**

LC×LC analyses were performed on a comprehensive two-dimensional LC×LC system (Shimadzu), consisting of a CBM-20A controller; four LC-30AD dual-plunger parallel-flow pumps; a DGU-20A5R degasser; a CTO-20AC column oven; a SIL-30AC autosampler; and a SPD-M30A photo diode array detector (1.8-μL detector flow cell volume). The two dimensions were connected by two high speed/high volume: 2 μL.

For the first dimension separation, a 250 × 1.0 mm, 5-μm dₚ Ascentis ES-Cyano column was used (Sigma-Aldrich/Supelco). Mobile phases: (A) n-hexane, (B) n-hexane/butylacetate/acetone (80/15/5, v/v/v). Gradient: 0–5 min, 0% B, 5–65 min, to 100% B. Mobile phase flow rate: 20 μL/min. Column oven: 30 °C. Injection volume: 2 μL.

For the second dimension separation, a 50 × 4.6 mm, 2.7-μm dₚ Ascentis Express C18 column was used (Sigma-Aldrich/Supelco). Mobile phases: (A) acetonitrile, (B) isopropanol. Gradient: 0.01 min, 0% B, 0.01–0.17 min, to 50% B, 0.17–0.27 min, 50% B, 0.27–0.54 min, to 80% B, 0.54–0.93 min, 80% B, 0.94 min, to 30% B. Mobile phase flow rate: 4 mL/min. Column oven: 30 °C. Modulation time of the switching valves: 60 s. PDA detection range: 250–550 nm; sampling rate: 12.5 Hz; time constant: 0.080 s. MS detection: DUlS positive mode; full-scan mass spectral range: 410–1200 m/z; event time: 0.1 s; nebulizing gas (N₂) flow: 2.5 L/min; drying gas (N₂) flow: 20 L/min; heat block temperature: 400 °C; desolvation line (DL) temperature 250 °C; interface voltage 4.5 kV. For the identification of selected compounds, product ion scan experiments were performed. For the quantitative determination of beta-carotene, MS parameters were optimized for MRM of the following transitions: m/z 536.40 to m/z 444.30 (qualifier ion) and m/z 536.40 to m/z 105.00 (qualifier ion).

From the LC system, 800 μL/min of the 2D flow were directed to the probe. For the splitting device, a stainless steel microvolume connector was used (1/16”, 0.15-mm bore) from VICI (Valco Instruments Co. Inc.).

**Results and Discussion**

This research was performed to develop an on-line LC×LC–PDA–MS–MS system, based on triple quadrupole technology, capable of fully automated operation, and exploitable for the analysis of highly complex samples, both in the targeted and untargeted mode. This novel system was assessed for the analysis of the carotenoid content of a red chili pepper extract, and the quantitative determination of the main bioactive molecule, beta-carotene.

Such a sample was chosen because of its complexity, by far overwhelming the limited peak capacity afforded by any monodimensional LC technique. Multidimensional techniques are therefore deemed as necessary, not only from the separation standpoint, but also as far as MS detection is concerned. Step-by-step implementation of the system addressed the following issues: Choice of the stationary phases, column dimensions, flow rates, connections, and detection parameters.

In our LC×LC platform, normal-phase LC and reversed-phase LC were used in the first and the second dimension, respectively. Normal-phase LC × reversed-phase LC represents one of the most orthogonal approaches in two-dimensional LC, capable of delivering very high resolution power as a result of independent separation mechanisms operating on the individual stationary phases. However, this type of coupling is not easy to

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**Figure 2:** Normal-phase LC × reversed-phase LC contour plot of free carotenoids and carotenoid esters in the red chilli pepper extract.
achieve because of the incompatibility of the solvents that are used in the two dimensions, and possible problems of peak focusing at the head of the secondary column (13,14). This issue was circumvented by the use of a microbore column in the first dimension. This served two purposes: Firstly, to reduce the mobile phase immiscibility and the occurrence of solvent strength mismatch, which would have affected system compatibility in such a combination. Non-polar hexane was in fact the 1D solvent, while aqueous acetonitrile and isopropanol mixtures were employed for the 2D analysis. In addition, the low flow rate employed also reduced band broadening and helped to achieve effective peak focusing at the head of the 2D column, thanks to the minimized mobile-phase strength, connected to the transfer from 1D to 2D. On the other hand, the separation in 2D must be fast and on-column focusing must be achieved in the online comprehensive multidimensional system. The fully automated LC×LC system was configured around two electronically activated two-position, six-ports switching valves for within-loop automated fraction collection and re-injection, equipped with two storage loops of identical volume (20 μL). The whole effluent from the 1D micro-bore column was transferred on-line to the second dimension column.

The first dimension effluent was fractionated every 60 s, which was the modulation time (cycle time) of the switching valves, which in turn was equal to the analysis time in 2D. The choice of the modulation time and the gradient profile was made to meet the following stringent requirements: Each fraction injected onto the second dimension column must be completely eluted before the following transfer occurred; the 2D analysis time had to be kept as short as possible, so as not to impair the separation achieved in the 1D (in this respect, a large number of cuts is highly beneficial). Moreover, re-conditioning of the stationary phase from each repetitive gradient should be fast enough to fit the limited time allowed.

In order to fulfil all these requirements, the second dimension column was operated at a very high flow rate (4 mL/min), with a gradient programme starting with 100% concentration of the weaker solvent, acetonitrile. A fast ramp up to 80% of the stronger solvent (isopropanol) was run to ensure the elution of all the components within a fast analysis time of 60 s, including a reconditioning time of 0.06 min. Repetitive injections of the sample under such conditions afforded perfectly super-imposable elution profiles (data not shown), demonstrating sufficient column re-equilibration time after the gradient. The shortest possible length of stainless steel tubing of 0.1 mm i.d. was used for extra-column connections and valves; as predicted, longer or higher internal diameter tubing negatively affected the overall performance of the system. This was in part as a result of additional dead volumes and the resulting peak broadening, but also down to significant dilution which jeopardized the MS response, with the overall dilution factor multiplicative of the dilution factors in each dimension. When designing an on-line LC×LC–MS technique, the detector response and the limited dynamic range of the instrument must also be considered. A fast acquisition rate was necessary, both for the PDA and the MS detector, because the second-dimension separation was very rapid, and peak widths only a few seconds or less apart. To adequately sample the 2D effluent, the mass analyzer should be capable of acquiring at least 6–10 data points for each peak. While traditional quadrupoles suffered from low resolution (typically 1 Da) and presented a mass range limited to approximately m/z 4000, the instrumentation used here allowed for high speed scanning (up to 15,000 amu/s) and ultrafast polarity switching for high-speed analyses, in both positive and negative ion mode.
Results obtained from the normal-phase LC × reversed-phase LC analysis of free carotenoids and carotenoid esters in the red chili pepper extract are shown in the contour plot of Figure 2. Chromatography on the cyanostationary phase allowed a good separation of the carotenoids in groups of different polarity in the first dimension, as can be seen from the ellipses in Figure 2, with retention times increasing in the order: Hydrocarbons < monoal esters < diol diesters < diol monoketo diesters < diol diketo diesters < diol monoxepoxide monoesters < free monoalols < diol monoketo monoesters < diol diketo monoesters < polyoxgenated free xanthophylls. On the other hand, the C18 column allowed the separation of carotenoids within each class, according to their increasing hydrophobicity and decreasing polarity (for components of the same class, the elution order increased with the number of carbon atoms of the fatty acid chain).

By optimizing the elution parameters (as a compromise between the chromatographic separation, modulation time, maximum column operating temperature, and highest flow rate allowed by the system back pressure limit), a satisfactory separation of the sample carotenoids was obtained, with 10 different classes distributed along characteristic chemical patterns in the 2D retention plane.

Identification of the separated compounds was achieved by both PDA and MS data. The possibility to operate in both positive ion and negative ion mode is a useful feature for the untargeted analysis of unknown samples, and in the special case of carotenoids it offered the double advantage of improved sensitivity and identification power. MS spectra obtained under negative ionization mode are in fact dominated by the presence of very intense pseudomolecular ions \([M-\text{H}]^-\), which make identification and quantitation of low-abundant components easier; on the other hand, abundant fragmentation is generally observed under positive ionization, especially for carotenoid esters, whose fragment ions can help in structure elucidation through a dedicated software or database.

The carotenoid content of this extracted red chili pepper, as determined by the method developed, was qualitatively very similar to that determined in our previous work (14), with only a few exceptions. The first regarded the absence of the chemical class of the diol monoxepoxide monoesters; in contrast an additional carotenoid class was found in this cultivar — the diol diketo monoesters. Capsorubin monooester with myristic acid (C14:0), whose chemical structure is shown in Figure 3, is representative of this chemical class, which comprises very polar molecules and elutes almost at the end of the first dimension gradient, immediately before the xanthophylls (ellipse 9 in Figure 2). Its UV spectrum shows a single absorption maximum centred at 473 nm, while the protonated molecular ion \([M+H]^+\) at \(m/z\) 811.2 showed a weak signal in the MS spectrum as a result of intense fragmentation, depicting the expulsion of water and myristic acid moieties in the product ion scan experiment (18 and 228 Da). Occasionally, these losses were also observed in the full scan experiment as a result of in-source fragmentation. In all cases, the formation of dehydrated product ions seemed to be favored, confirming the presence of a non-esterified hydroxyl function. An intense signal was detected as well, at \(m/z\) 713.4 \([M-1879]\), which according to the literature, may derive from the loss of a water molecule and the in-chain elimination of a \(C_6\)H fragment ion that occurs via a common seven-membered ring transition state (17).

The main advantage of this developed system, with respect to the one previously used (14), lies in the possibility of carrying out quantitative analysis with very high selectivity and sensitivity, in the so-called MRM mode, for target molecules. Beta-carotene \((m/z\) 536.40) transitions at \(m/z\) 444.30 (quantifier ion) and \(m/z\) 105.00 (qualifier ion) were selected for the construction of a calibration curve, in the 1 ppb to 10 ppm concentration range. Very good linearity was observed, with regression line equation: \(y = 410259x + 49414\); linear correlation coefficient \((R) = 0.998976\). The amount of beta-carotene was determined afterwards in the real sample as equal to 1.22 ppm. Figure 4 shows an expansion of the 2D plot, with the beta-carotene blob and the MS spectrum with the two transitions.

**Conclusions**

In this article, an LC×LC system was coupled on-line to a triple quadrupole mass spectrometer, developing a novel analytical system capable of extremely high-resolution power, as well as targeted and untargeted analysis. For the separation, identification, and quantification of highly complex samples, unique features of this system consist of: High resolving power, fast analysis time, full automation, high sensitivity and selectivity, fragment information, and fast cycle time (including polarity switching).

Moreover, the 2D plots rendered by the front-end LC×LC separation may be of great help in the identification of unknowns, whenever standard components or reliable MS library spectra are not available. The separated molecules are in fact distributed along chemically-similar compound patterns, and this will provide useful information on their structures and properties, according to the separation mechanisms involved.

**References**


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Analysis of Polyether Antibiotics in Animal Feeds by HPLC with Post-Column Derivatization

Maria Ofitserova and Sareeta Nerkar, Pickering Laboratories, Inc.

Polyether antibiotics are commonly used for preventing coccidiosis and other infections in poultry and for improving feed efficiency for beef cattle and swine. The use of polyether antibiotics is strictly regulated, with only specific ionophores approved for use in feeds intended for different animals.

Analysis of polyether antibiotics by HPLC with post-column derivatization and UV-vis detection has been proven to successfully identify and quantify monensin, narasin, and salinomycin in medicated feeds, supplements, and premixes as well as to determine trace contamination levels in non-medicated feeds (1,2).

Post-column derivatization of polyether antibiotics is done using highly acidic vanillin or DMAB reagents. Pinnacle PCX derivatization system (Pickering Laboratories, Inc.) has an inert flow path and automated system wash capabilities that make it uniquely suitable for handling corrosive reagents. The two-pump system is recommended to extend reagent stability, but the single-pump system for this application is also available.

Adding a fluorescence detector to the instrumentation allows for using the same extraction procedure and HPLC conditions to also determine lasalocid which doesn’t require post-column derivatization.

Method

Sample Preparation
To 25 g of finely ground feed sample, add 100 mL of extraction solution (90% methanol–10% water). Shake for 1 h at high speed using mechanical shaker. Let the solids settle and filter an aliquot of the extract for injection. Dilute with extraction solution if needed to fit the calibration curve. Use 2.5 g portion when testing premixes.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample Preparation</th>
<th>Sample Preparation</th>
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</table>

Table I: Polyether antibiotics in certified medicated feeds

<table>
<thead>
<tr>
<th>Feed Type</th>
<th>Certified Amount</th>
<th>Found in Sample</th>
<th>Recoveries</th>
<th>RSD, N = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin</td>
<td>Beef feed</td>
<td>267 g/ton</td>
<td>275 g/ton</td>
<td>103%</td>
</tr>
<tr>
<td>Lasalocid</td>
<td>Milk replacer</td>
<td>72 g/ton</td>
<td>69 g/ton</td>
<td>96%</td>
</tr>
</tbody>
</table>

Table II: Spike recoveries for monensin

<table>
<thead>
<tr>
<th>Non-Medicated Bird Feed</th>
<th>Non-Medicated Rabbit Feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin A</td>
<td>Monensin B</td>
</tr>
<tr>
<td>Spike Level</td>
<td>172 g/ton</td>
</tr>
<tr>
<td>Recoveries</td>
<td>100%</td>
</tr>
<tr>
<td>RSD, N = 3</td>
<td>1.9%</td>
</tr>
<tr>
<td>Spike Level</td>
<td>3.44 g/ton</td>
</tr>
<tr>
<td>Recoveries</td>
<td>96%</td>
</tr>
<tr>
<td>RSD, N = 3</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

Figure 1: Standard mixture of monensin, salinomycin, and narasin.

Figure 2: Certified medicated beef feed sample containing 267 g/ton of monensin.

Analytical Conditions

Analytical Column: Polyether Column, C18, 4.6 × 250 mm, Catalog No. 2381750

Temperature: 40 °C
Flow Rate: 0.7 mL/min
Mobile Phase: 90% methanol, 10% of 5% acetic acid solution in water, isocratic
Injection Volume: 20 µL

Post-Column Conditions
Post-column System: Pinnacle PCX
Non-medicated bird feed sample spiked with monensin A (3.44 µg/g) and monensin B (0.16 µg/g).

Certified medicated milk replacer containing 72 g/ton of lasalocid.

Figure 3: Non-medicated bird feed sample spiked with monensin A (3.44 µg/g) and monensin B (0.16 µg/g).

Figure 4: Certified medicated milk replacer containing 72 g/ton of lasalocid.

Reactor Volume: 1.4 mL
Reactor Temperature: 90 °C
Reagent 1: Concentrated sulfuric acid/methanol (4:96 v/v)
Reagent 2: 60 g of vanillin in 950 mL of methanol
Reagents Flow Rate: 0.3 mL/min
Detection: UV–vis 520 nm (for Lasalocid – FLD, Ex. 322 nm, Em. 370 nm)

Calibration
Monensin A: 0.1–50 ppm, R² = 0.999
Monensin B: 0.0035–0.7 ppm, R² = 0.999
Lasalocid acid: 0.25–50 ppm, R² = 0.999

Conclusion
Analysis of polyether antibiotics by HPLC with post-column derivatization is a robust and sensitive method that utilizes standard equipment and could easily be adopted by testing laboratories. It allows for testing of different ionophores at wide range of concentrations, including at trace levels. Using Pinnacle PCX post-column derivatization system, factory configured for the analysis, guarantees stable and reproducible results.

References
Rapid Arsenic Speciation on Hamilton PRP-X100

Derek Jensen and Mark Carrier, Hamilton Company

Arsenic Speciation Made Simple

Organic arsenic compounds added to feed stocks of chicken and poultry pose serious environmental and ecological threats. The recent occurrence of toxic arsenic levels in as many as 10% of apple juice products gained the attention of the United States Food and Drug Administration who quickly responded by implementing more stringent concentration limits and testing requirements.

Analysis of arsenic species is made challenging due to diverse sample matrices. To circumvent these problems, an HPLC-ICP-MS method has been developed. The new PRP-X100-arsenic analysis column line from Hamilton is designed to provide superior resolution of organic and inorganic arsenic species from a variety of sources and sample matrices. The rugged 55% cross-linked porous packing material of the PRP-X100 column resists shrinking and swelling in organic solvents and is fully stable throughout the entire pH range, making it an ideal solution for arsenic speciation in juice following simple dilution or grain samples, such as rice, which require preliminary acid digestion.

- High resolution separations
- Outstanding chemical stability and column lifetime
- Rigid polymer support for pressure stability up to 350 bar
- Can be used with 0–100% organics and at pH 1–13
- Isocratic or gradient elution
- Compatible with a variety of mobile phases
- Compatible with any ion chromatograph or HPLC system
- Available in metal-free PEEK hardware

Column: Hamilton PRP-X100, 5 μm, 4.1 × 50 mm
Part Number: 79810
Flow Rate: 3.0 mL/min
Mobile Phase: 2–40 mM (NH₄)₂CO₃ over 1.3 min
Sample Volume: 50 μL, 100 μg/L of each standard
Detection: ICP-MS

Analytes
1. As(III)
2. Dimethylarsenic acid
3. Monomethylarsenic acid
4. As(V)
5. Phenylarsonic acid

Figure 1: Rapid (<2 min) arsenic analysis on Hamilton PRP-X100 Rapid Resolution column. Data borrowed with permission from Peter Wegeworth, Mayo Clinic.
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