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Authenticity control of edible fats and oils by analysis of minor constituents via on-line liquid chromatography-gas chromatography

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Abbreviations

CI	Chemical ionization
Da	Dalton
EI	Electronical impact ionization
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High pressure liquid chromatography
IOOC	International olive oil comission
LC-GC	Liquid chromatography-Gas chromatography
MS	Mass spectrometry
PTV	Programmed temperature vaporizer
UV	Ultraviolett

1 INTRODUCTION

In food production quality assessment of raw materials and final products is a fundamental parameter for having and maintaining high quality standards. There are different aspects determining the overall quality of foods: the "quality" (in terms of sensory characteristics, stability and nutritional value), the "safety" (with respect to microbiology, contaminants and toxins) and the "authenticity".

An authentic product, whether raw material or a product on the supermarket shelf, is one which strictly complies with the declaration given by the producer in terms of ingredients, natural components, absence of extraneous substances, production technology, geographical and botanical origin, production year and genetic identity. Authenticity is an important issue for the food industry due to legal compliance, economic reasons ("right goods for the right price"), guarantee of a constant well-defined quality, use of safe ingredients (no hazardous substitutes) and religious reasons such as *e.g.* Halal or Kosher.

The problem how to guarantee/ensure food authenticity can be tackled by applying accurate specifications for raw materials and selecting suppliers that have a quality assurance system in place. The compliance with the specification is assured by regular audits of the supplier(s), certificates of analysis and periodical analytical checks. Hence it is necessary to identify and anticipate emerging authenticity issues, to confirm authenticity of potentially adulterated products, to develop methods adapted to solve new problems and to organize and/or perform analytical surveys of sensitive products.

Adulteration has always been practised and it is carried out for economical purposes (e.g. to increase the bulk volume, to overevaluate a product of inferior quality or to subtract/save expensive ingredients). On the other hand, contaminations may occur

accidentally, e.g. in factories, where several oils are produced or used at the same time. These cross-contaminations are usually below 1 – 2 % of the total amount.

In addition, safety problems can be involved in adulterations. In the early 1980s, a syndrome referred to as the "toxic oil syndrome" occurred in Spain and resulted in massively reported illness and over 300 deaths. The primary cause for the disease was the consumption of illegally marketed edible oil, adulterated with industrial-grade rapeseed oil containing 2% aniline as denaturant (Tabuenca, 1981; WHO, 1992). Work is still continuing to understand the mechanism of this intoxication (De La Paz *et al.*, 1996; Barnes, 1999; Bujons *et al.*, 2001). This example represents one of the most dramatic cases of a hazard to human health associated with an adulteration. In a recent example, the consumption of a mustard oil adulterated with argemone oil caused over 500 intoxications and several deaths in India (Barnes, 1998). For the safety of the consumer, food control laboratories as well as organizations in industrial quality control need to be aware of adulteration issues and have to be quite innovative to develop new analytical methods to detect frauds. On the other hand, fraudsters tend to be quite innovative and well informed about weak points in food inspection systems. Therefore, the development of effective and efficent methods is a permanent challenge for food authenticity control in general.

The objective of this study was to develop a method for authenticity control of oils and fats. Potential analytical parameters to be investigated comprise the minor lipid constituents of such oils, *e.g.* sterols or steryl esters. In order to overcome the drawbacks of conventional analytical approaches to the analysis of these components, such as multiple sample preparation steps, the hyphenated technique "on-line LC-GC" should be tested for its usefulness and conditions should be optimized to allow routine analysis. The technique should be evaluated for its use in authenticity control of (i) milk fat, (ii) coffee oil and (iii) cocoa butter.

2 BACKGROUND

2.1 Parameters suitable for authenticity assessment of fats and oils

To assess the authenticity of fats and oils it is fundamental to know the technologies applied, the fat modification techniques used and the chemical composition of the authentic oil(s) and of the potential adulterants. These aspects have been reviewed in detail (Kamm *et al.*, 2001b).

The fundamental problem for the authenticity assessment of fats and oils is to define one or more parameters within the lipid fraction which allow to check for the identity and purity of the specified fat or oil. Ideally, such markers are chemical compounds which are present in the adulterant fat and absent in the original one. However, very often marker substances are not totally absent in the authentic fat but present only in concentrations different from the adulterated product. Therefore, profiles of authentic fats must be compared with the fat to be tested. For the definition of authenticity, the natural variations of the markers, *e.g.* due to climate, soil and breeding must be taken into account.

Chemically, the main constituents of fats and oils are triacylglycerols (commonly referred to as triglycerides), lower levels of diacylglycerols (diglycerides), monoacylglycerols (monoglycerides) and free fatty acids, accompanied by other minor components like the steroidal alcohols. The latter class of the minor components are of particular interest for authenticity assessment as they often show a composition with a limited range of variability for each individual fat or oil.

2.1.1 Steroidal alcohols

Steroidal alcohols possess a hydroxyl group at C_3 and a branched aliphatic chain of 8-10 carbon atoms at C_{17} of the steroidal skeleton. Four principal classes of steroidal

alcohols can be found: 4-desmethylsterols (also called sterols), 4-monomethylsterols, 4,4´-dimethylsterols (also called triterpencic alcohols) and triterpenic diols. Generally, the sterol distribution is rather typical for each plant family and is not significantly changed by plant selection/hybridization. It has been considered unlikely that a traditional breeding approach would lead to dramatic increase in phytosterol content or modified phytosterol composition (Vlahakis and Hazbroek, 2000). Therefore, the evaluation of the total sterol content and the sterol profile is a suitable tool to assess the authenticity of fats and oils.

2.1.1.1 4-Desmethylsterols

The 4-desmethylsterols occurring in fats and oils are commonly called “sterols” or in the case of plant oils “phytosterols”. In crude vegetable oils they are present at levels ranging from 400 up to 15000 mg/kg (Kamm *et al.*, 2001b).

There are three major phytosterols (β-sitosterol, stigmasterol, campesterol), two of which β-sitosterol ($C_{29}H_{50}O$) **1** and stigmasterol ($C_{29}H_{48}O$) **2** are Δ^5-sterols (Figure 1). The former is the principal sterol of a large number of vegetable oils, the latter is an important sterol for authenticity assessment and is present in several oils, *e.g.* from soybean, castor, grape seed, palm, palm kernel, rice bran, safflower and sunflower.

The third major phytosterol, campesterol ($C_{28}H_{46}O$) **3**, can be isolated from a number of oils (at a 16-49% w/w range of the desmethylsterol fraction). It differs from β-sitosterol in the structure of the side chain, as it possesses a methyl group at position 24 instead of an ethyl group (Figure 1).

In general, the desmethylsterols are useful markers to assess authenticity. Considering that β-sitosterol is the most abundant sterol in the majority of oils, its value has only limited use for the authenticity assessment and differentiation of vegetable oils.

However, it has been shown to be useful for tracing vegetable oils in fats of animal origin (Zürcher *et al.*, 1976), as the latter only contain cholesterol as the primary sterol.

In vegetable oils there are several other minor Δ^5- and Δ^7- phytosterols, such as Δ^5- and Δ^7-avenasterol **4**, **5**, Δ^7-stigmastenol **6**, α-spinasterol **7** and 24-methylcholest-7-enol **8** (Figure 1). Some sterols can be used as indicators of fruit ripeness. In the case of olives, stigmasterol and Δ^5-avenasterol contents increase as the fruit matures up to a point which coincides with the maximum oil content of the olive fruit (Cimato, 1990).

Some "exotic" fats and oils, like argan, sheanut butter or pumpkin oil contain extraordinary amounts of Δ^7-sterols (37-69% w/w of the desmethylsterol fraction), followed by lower contents in sunflower seed, blackcurrant seed, palm and borage oils (18-37% w/w of the desmethylsterols).

The fact that Δ^7-sterols are virtually absent in palm kernel, walnut, rapeseed and mustard oil is useful for authenticity assessment of these oils. Admixtures of sunflower seed oil, rapeseed and soybean oil with pumpkin seed oil have been detected at levels as low as 2% due to the presence of Δ^7-sterols in pumpkin seed oil (Mandl *et al.*, 1999).

Cholesterol **9** (Figure 1) occurs only in very small amounts in plant oils, whereas in animal fat it is the predominant sterol (Ballesteros *et al.*, 1996). Admixtures of vegetable fat with animal fat can therefore be recognized. Adulteration of milk fat/milk or adulteration of butter oil with vegetable fats is indicated by a decrease in cholesterol and an increase in stigmasterol and β-sitosterol concentrations.

A considerable variability of the cholesterol content in peanut, rapeseed and palm oil has been reported (Turrel and Whitehead, 1990). This may be due to the biosynthetic

conversion of β-sitosterol (Heftmann, 1971) as reported for particular species of palm fruits and its liberation from precursors in the course of the deodorization step (Gertz, 1988).

Brassicasterol **10** (Figure 1) is mainly present in rapeseed and in mustard seed oils. It is absent or present only in trace amounts in other oils. Hence the presence of brassicasterol in oils other than rapeseed or mustard oil may indicate adulteration with these materials.

Another important sterol, ergosterol ($C_{28}H_{44}O$) **11** (Figure 1) has been recognized as a marker for yeast and fungi contamination in raw materials. Sterols obtained from yeast and fungi are commonly referred to as "mycosterols".

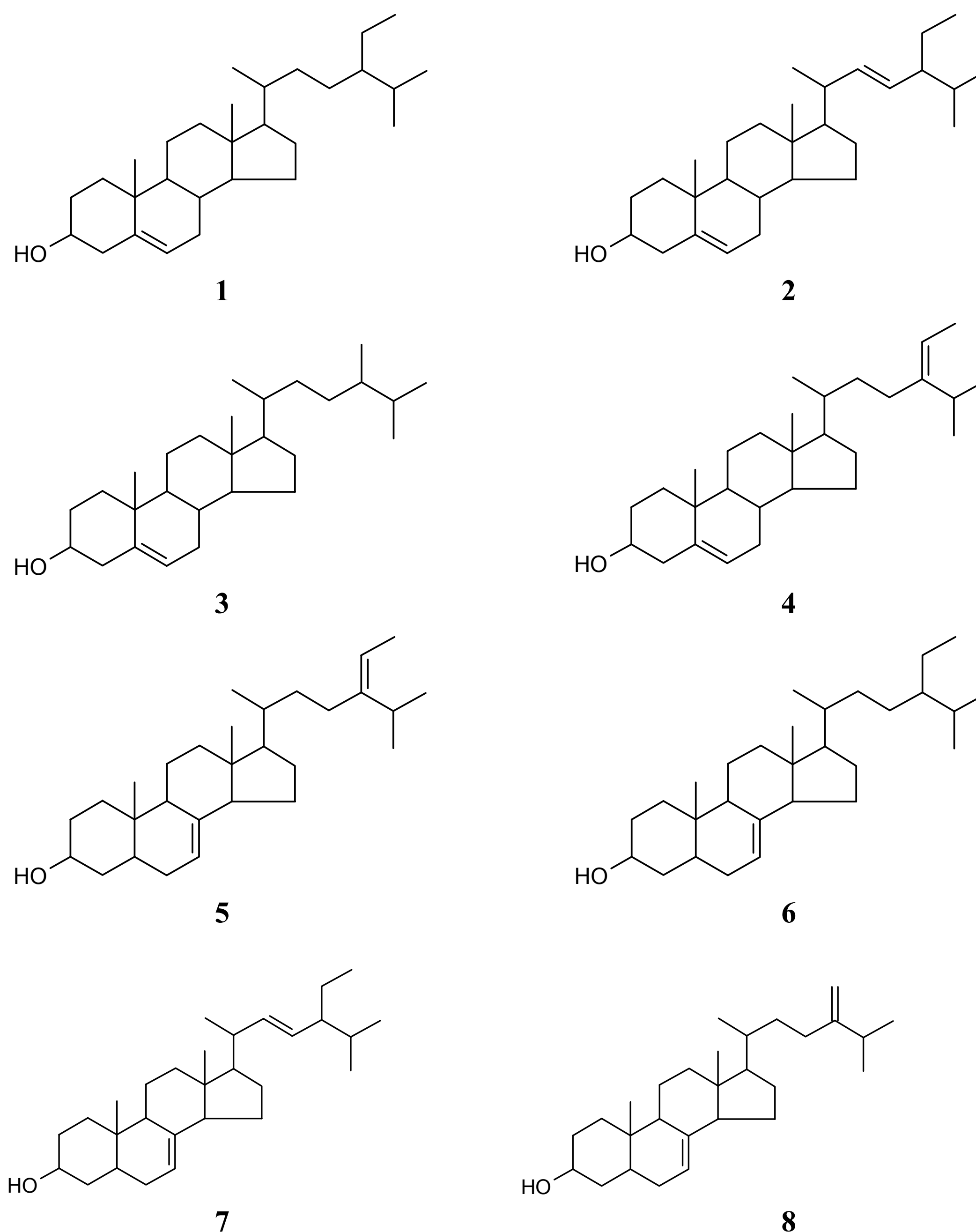


Figure 1.

Structures of the principal 4-desmethylsterols: **1** β-Sitosterol, **2** Stigmasterol, **3** Campesterol, **4** Δ^5-Avenasterol**,** **5** Δ^7-Avenasterol, **6** Δ^7-Stigmastenol, **7** α-Spinasterol**,** **8** 24-Methylcholest-7-enol**.**

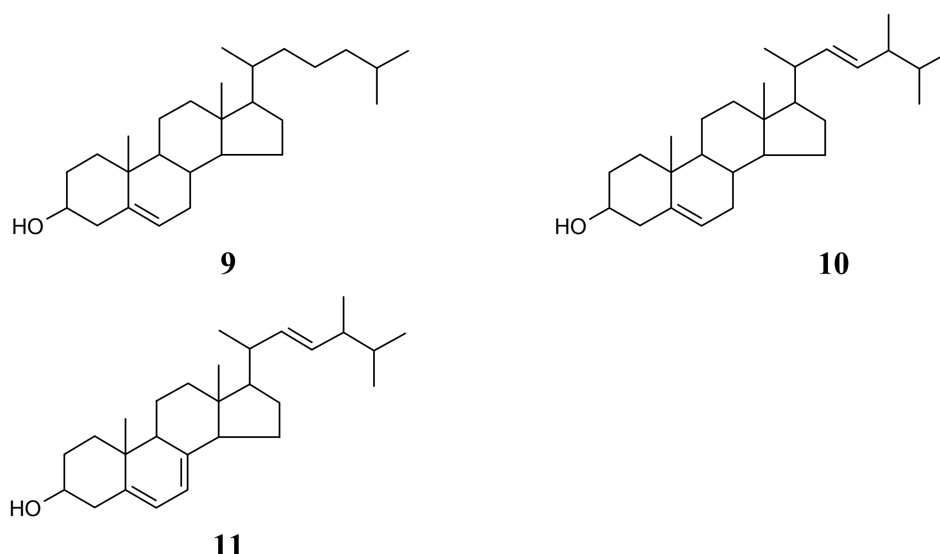


Figure 1 (continued).
Structures of the principal 4-desmethylsterols: **9** Cholesterol, **10** Brassicasterol, **11** Ergosterol.

Olive oil adulterated with soybean, grapeseed or sunflower oil can be detected on the basis of the concentrations of campesterol and stigmasterol. However, an addition of low amounts of these oils to olive oil cannot be detected with these markers because of their natural presence in olive oil. Sunflower oil is characterized by high concentrations of Δ^7-sterols (up to 37% w/w of the total desmethylsterols). The limit set in the EU for the marker substance Δ^7-stigmastenol in olive oil is at 0.5%, relative to the total amount of sterols (EC, 1997), *i.e.* 5-7.5 mg/kg, assuming a total sterol concentration of 1000-1500 mg/kg.

A strategy to avoid the detection of an adulteration of olive oil with sunflower oil is the elimination of the Δ^7-sterol fraction by refining under extreme conditions. However, in the course of this process the Δ^5-sterols are dehydrated to sterenes and the Δ^7-sterols undergo isomerizations to $\Delta^{8(14)}$ and Δ^{14} sterols. These reaction products

can be detected by gas chromatography and thus allow the determination of an addition of desterolized high oleic sunflower oil to olive oil (Mariani *et al.*, 1995).

2.1.1.2 4α-Monomethylsterols

4α-Monomethylsterols are intermediates in sterol biosynthesis and they are present in some oils at levels up to 1200 mg/kg (*i.e.* corn oil). The predominating 4-monomethylsterols in vegetable oils are citrostadienol **12**, cycloeucalenol **13**, obtusifoliol **14**, gramisterol **15**, lophenol **16** and 31-norcycloartenol **17** (Figure 2). They are all Δ^8- and Δ^7-sterols, except cycloeucalenol and 31-norcycloartenol, which have a 9, 19-cyclopropane ring in the steroid skeleton. Their distribution in vegetable oils has been reviewed (Kamm *et al.*, 2001b). Some 4-monomethylsterols, *e.g.* citrostadienol **12**, are potent antipolymerization agents and are very effective in retarding oxidation (Blekas and Boskou, 1999). Citrostadienol might be used to detect the presence of sunflower in safflower oil (Mariani and Bellan 1997). It has been reported that ripening of olive fruits leads to a decrease of citrostadienol (Mariani *et al.*, 1991).

The ratio of gramisterol **15** to cycloeucalenol **13** has been described as useful parameter for the characterization of vegetable oils (Turrel and Zilka, 1987). Cycloeucalenol is predominant in palm oil, whereas other oils, except for olive oil, contain lower amounts.

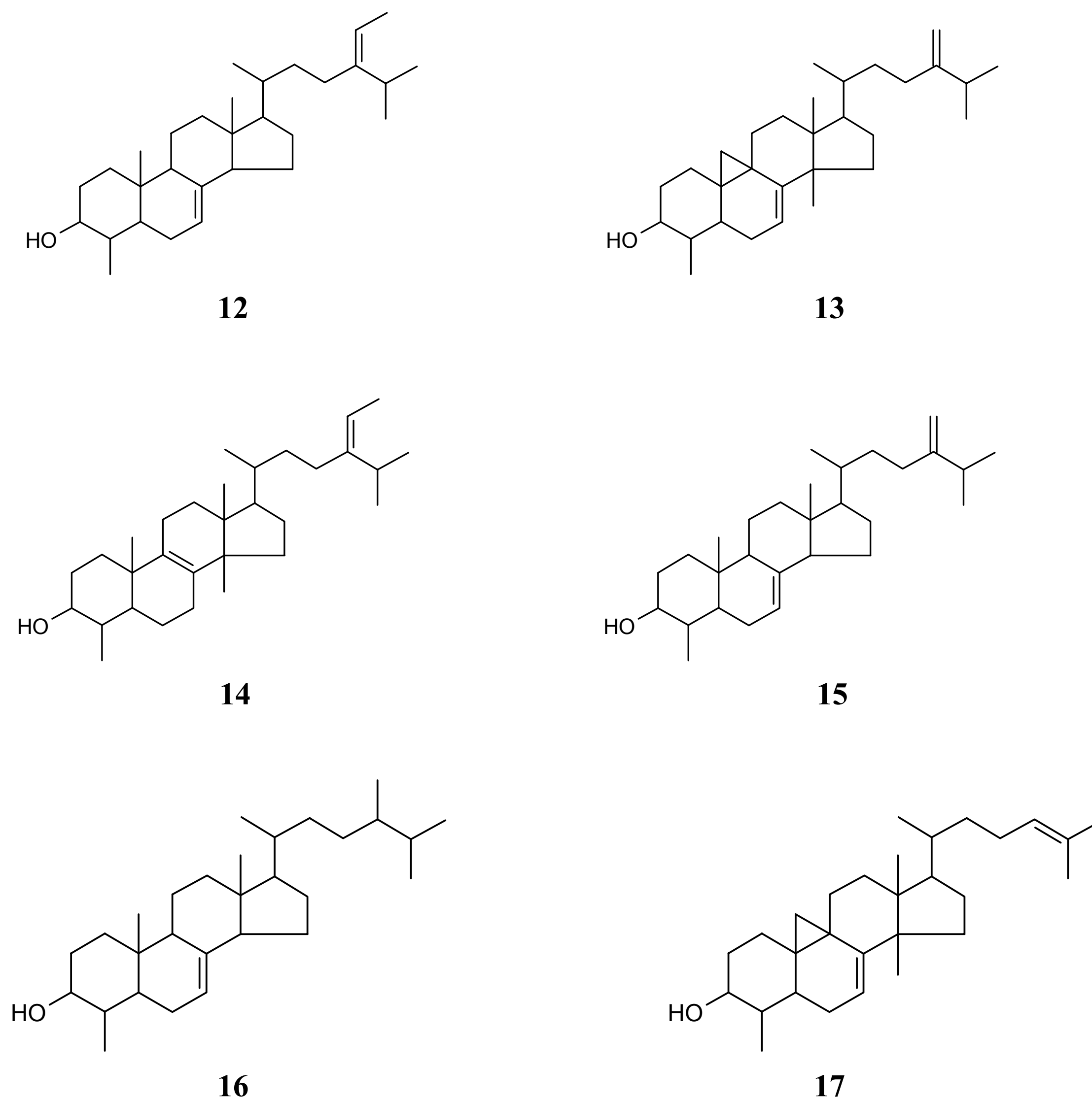


Figure 2.

Structures of the principal 4α-monomethylsterols: **12** Citrostadienol, **13** Cycloeucalenol, **14** Obtusifoliol, **15** Gramisterol, **16** Lophenol, **17** 31-Norcycloartenol.

2.1.1.3 4,4'-Dimethylsterols

The class of the 4,4'-dimethylsterols, commonly referred to as triterpenic alcohols, contains compounds with a cyclopropane structure, *e.g.* cycloartenol **18** or 24-methylene-cycloartanol **19** or with pentacyclic terpene structure, *e.g.* α- and β-amyrin **20**, **21** (Figure 3). The 4,4'-dimethylsterols are present at concentrations up to 1150 mg/kg oil (*i.e.* corn oil) (Kamm *et al.*, 2001b). The content of 24-methylene-cycloartanol **19** in olive oil is indicative for the ripeness of the olives; its concentration increased 3-fold during riping (Blekas and Boskou, 1999).

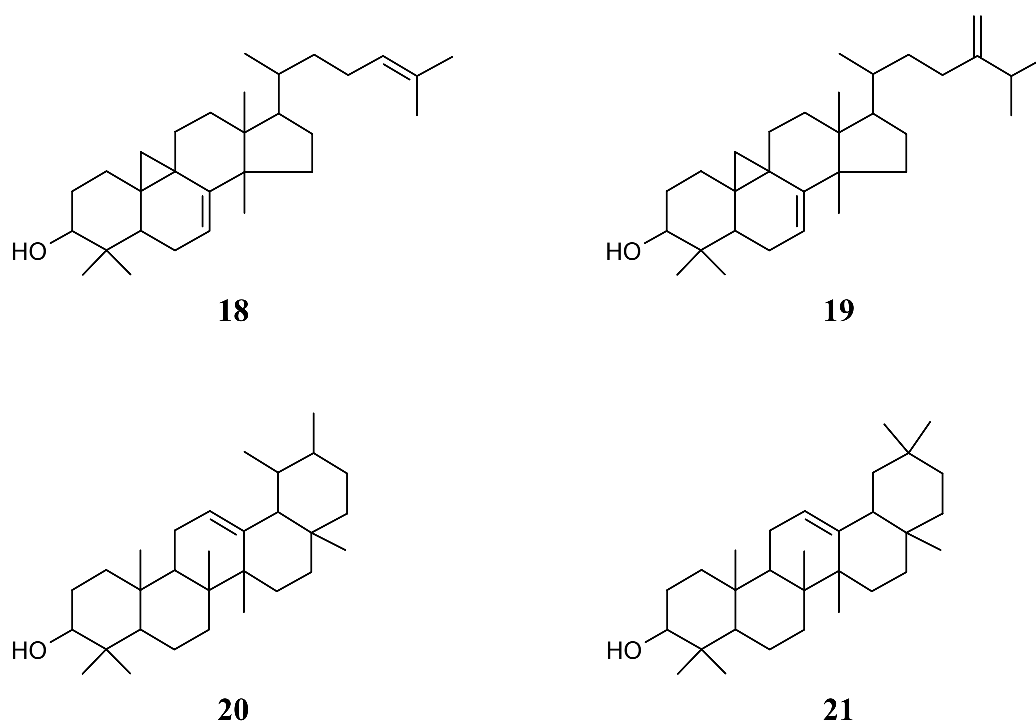


Figure 3.
Structures of the principal 4,4'-dimethylsterols: **18** Cycloartenol, **19** 24-Methylene-cycloartanol, **20** α-Amyrin, **21** β-Amyrin.

2.1.1.4 Triterpenic diols

In Figure 4 the structures of erythrodiol **22** and uvaol **23** are given as examples for triterpenic diols. These diols are most abundant in the epicarp of the olive fruit. Solvent extraction leads to high amounts in the oil; they are absent in oil isolated by cold pressing.

The determination of erythrodiol and uvaol provides a good means of differentiating pressed and solvent-extracted olive oil (Blanch *et al.*, 1998a; Reina *et al.*, 1999). However, the application of certain technological procedures may reduce the erythrodiol content. In vegetable oils like evening primrose or cottonseed oil, these diols are virtually absent, demonstrating the ability to distinguish among these plant oils (Reina *et al.*, 1997).

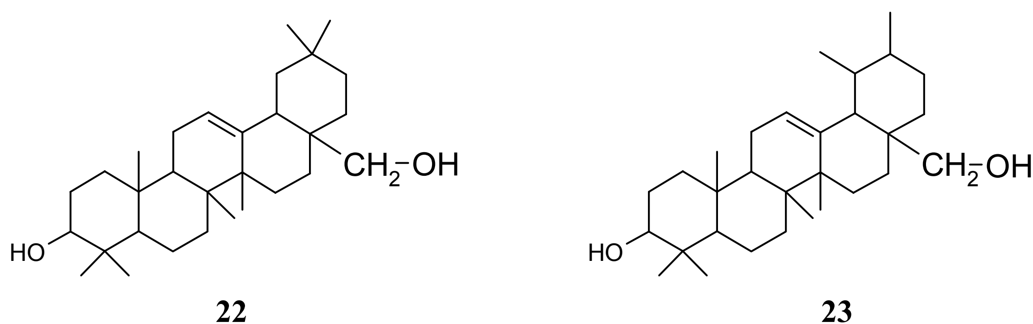


Figure 4.
Structures of the principal triterpenic diols: **22** Erythrodiol, **23** Uvaol.

2.1.2 Steryl esters

Sterols are present in oils as free or esterified alcohols. Steryl esters are esters of fatty acids with steroidal alcohols, *e.g.* sitosteryl oleate. The most routine approach for the analysis of steryl esters is based on saponification, isolation of the sterols by thin layer chromatography (TLC) and subsequent gas chromatographic (GC) or liquid chromatographic separation (LC). This method does not differentiate between free and esterified steroidal alcohols (Evershed *et al.*, 1987b). Hence, important analytical informations are lost. However, there are analytical methods, which allow to distinguish esterified from non-esterified sterols (Grob *et al.*, 1989b, 1990a; Artho *et al.*, 1993). The analysis of free and esterified components can provide some key information concerning authenticity (Gordon and Griffith, 1992b; Gordon and Miller, 1997). Under usual refining conditions only a minor decrease in the level of steryl esters has been observed (Ferrari *et al.*, 1996, 1997). However, by strong refining (*e.g.* harsh bleaching conditions) all sterols including their esters can be removed (Grob *et al.*, 1994b).

The analysis of free sterols and steryl esters has been proposed for the identification of oils in blends (Gordon and Griffith, 1992b; Gordon and Miller, 1997). There are characteristic ratios for free/esterified 4-desmethylsterols in some vegetable oils (Kamm *et al.*, 2001b). Brassicasterol **10** in rapeseed oil exists largely in the free state, whereas Δ^7-stigmastenol **6** in sunflower oil is mainly present in the esterified form. The presence of campesteryl-C_{18}-ester and Δ^7-stigmastenyl-C_{18}-ester in olive oil has been reported to be indicative for adulteration with rapeseed and sunflower oil, respectively (Ferrari *et al.*, 1996).

It is also remarkable that the fatty acids esterified with sterols are present in quite different proportions as compared to those esterified with glycerol forming the triglycerides of the oil.

2.1.3 Diterpenes

Diterpenes are present almost exclusively in coffee lipids. The group comprises cafestol, kahweol and other related diterpenoids. They are esterified with fatty acids and only small amounts are present in free form (Kölling-Speer *et al.*, 1999). Cafestol is contained in Arabica and Robusta coffee and 16-*O*-methylcafestol (16-OMC) (Figure 5) is found only in Robusta coffee and is therefore considered as a principal marker component (Pettitt, 1987; Speer, 1989d; Speer and Mischnick-Lübbecke, 1989c; Speer *et al.*, 1991).

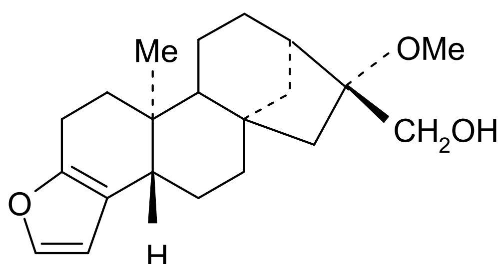


Figure 5.
Structure of 16-*O*-methylcafestol (16-OMC).

2.2 Classical methods for the analysis of sterols and steryl esters in lipids

The official methods for the analysis of total sterols in vegetable oils involve sample preparation steps such as saponification, extraction of the neutrals from the soap solution, pre-separation by preparative thin-layer chromatography, silylation and GC-FID analysis (DGF, 1991; EU, 1997). A drawback of this approach is the time consuming and manual work involved in these steps. Therefore, only few samples can

be analyzed at a time and risks for losses or contaminations during the individual sample preparation steps may occur. Furthermore, as the oil is saponified, no information on the composition of genuine steryl esters can be obtained. Nevertheless, a lot of data based on these methods has been published in literature and is used for the authenticity assessment of many oils.

Steryl ester analysis is usually carried out after their isolation by thin layer chromatography and analysis of either the steryl esters as such or as the individual sterols (after saponification and/or silylation) and fatty acids as methyl esters. No "official" method for the analysis of steryl esters has been established yet. Again, manual labor restricts sample throughput and affects the accuracy of results obtained.

2.3 Other analytical methods used in authenticity assessment of lipids

Comprehensive overviews on principal techniques useful for the assessment of authenticity of fats and oils have recently been reviewed (Ulberth and Buchgraber, 2000; Aparicio and Aparicio-Ruíz, 2000; Kamm *et al.*, 2001b). Scope and limits of the described methods have been discussed in detail.

Methods based on spectroscopic procedures might gain a broader application in the future as they have a potential advantage as an authenticity testing tool suited for direct and non-invasive quality measurement in food and food ingredients, *e.g.* suited for on-line process control in industrial productions sites (Downey, 1996). Sato (1994) investigated the discrimination of a series of plant oils (soybean, corn, cottonseed, olive, rice bran, rapeseed, peanut, sesame and coconut oil) by performing principal component analysis on spectra recorded from 1600-2200 nm. However, only some of the oils could be differentiated. Others used Raman scattering for edible oil classification and performed a classification showing high correlations with the fatty

acid profile of the oils used (Baeten *et al.*, 1998). Despite its potential, the practical usefulness of these spectroscopic techniques in authenticity assessment still remains to be tested.

Another method to detect adulteration of various edible oils (coconut, palm, groundnut and mustard) with castor oil was investigated using ^{13}C NMR spectroscopy (Husain *et al.*, 1993, Mavromoustakos *et al.*, 2000). Using ^{1}H NMR, also extra virgin olive oil was characterized (Sacchi *et al.*, 1998). Furthermore, mass spectrometric techniques have been employed for authenticity assessment of vegetable oils and fats, *e.g.* isotope ratio mass spectrometry analysis (IRMS) (Remaud *et al.*, 1997; Kelly *et al.*, 1997; Spangenberg *et al.*, 1998; Rossmann *et al.*, 2000; Spangenberg and Ogrinc, 2001) or pyrolysis mass spectrometry (Anklam *et al.*, 1997, Radovic *et al.*, 1998).

These methods proved to be useful for the analysis of lipids, however, they require sophisticated equipment such as IRMS or high resolution NMR, which is only available in a limited number of laboratories. Furthermore, in many cases laborious chemometric techniques are needed for handling the large amounts of data produced and calibration by conventional analytical methods is usually required.

2.4 On-line LC-GC

2.4.1 Introduction

After extraction of the sample material, preparation steps for a gas chromatographic analysis of minor lipids often involve chromatographic steps such as thin layer chromatography, column chromatography, solid phase extraction or off-line high performance liquid chromatography (HPLC). The latter approach provides far better resolution than conventional techniques and allows also an on-line detection of specific fractions. The direct control by the HPLC detector enables to optimize conditions and to set accurately the “window” for a cut of the component(s) of interest. When applying off-line (HP)LC-GC, reconcentration of the large volume of eluent is an additional step which has to be carried out prior to GC analysis unless a large volume injection method can be used for sample introduction into GC. A potential drawback of such an off-line evaporation is loss of volatile or adsorptive material and/or contamination.

On-line transfer and evaporation of the LC eluent in the GC reduce losses or contamination possibilities, with the drawback of having to fine-tune transfer and evaporation conditions. On-line LC-GC techniques allow the direct transfer of high-performance liquid chromatograhic (HPLC) fractions into capillary gas chromatographic (GC) columns. A schematic example of an on-line LC-GC set-up is shown in Figure 6. Coupling LC to GC involves the following steps: (i) isolation of the components of interest using HPLC (visualization by UV detection), (ii) transfer of the LC fraction into the interface valve, and (iii) thermal volatilization/evaporation of the transferred LC eluent.

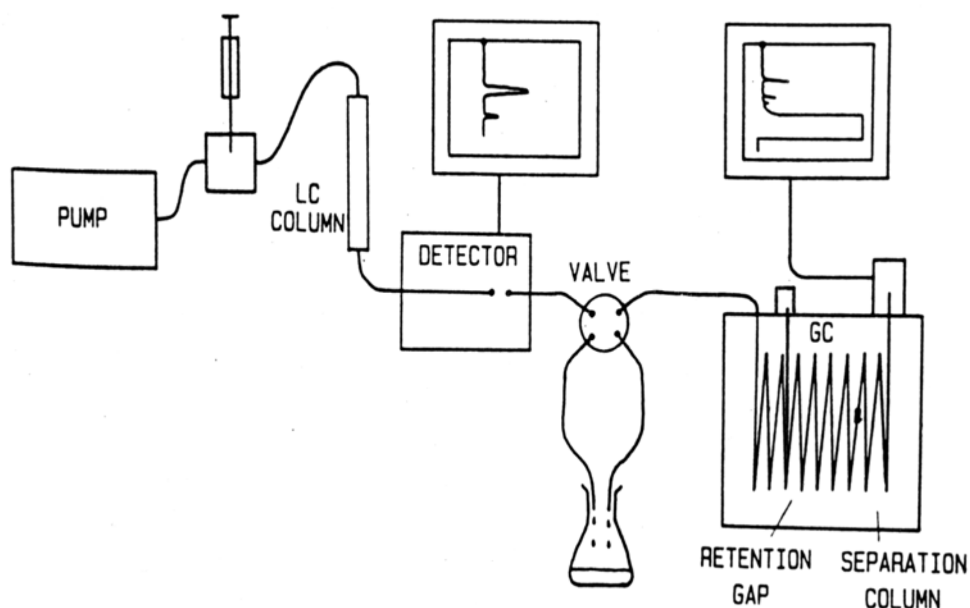


Figure 6.
Set-up of an on-line LC-GC system.

The automation through on-line coupling often massively reduces or even virtually eliminates sample preparation work, which saves time and enhances overall reliablility of the analysis. In many cases, analysis of complex samples becomes feasible in this way.

2.4.2 History of on-line coupled LC-GC

The first on-line coupled LC-GC system was described about 20 years ago (Majors, 1980). LC was coupled to a conventional vaporizing GC injector via an autosampler. Since only a few microliters could be transferred, a very small proportion of the LC fraction of interest was analyzed by GC. Therefore, poor sensitivity was observed and the reproducibility of quantitative determinations was unsatisfactory.

Raglione *et al*. (1987) improved this type of LC-GC transfer by an eluent splitter. The large volume of a broad LC fraction was reduced by splitting with a system of capillaries, however sensitivity remained low. In the early 1980s, investigation of the solvent effects “solvent trapping” (Grob, 1982a,b; Grob, 1983e, Grob and Schilling, 1983d; Grob and Neukom, 1986) and “phase soaking” (Grob and Schilling 1983a-c) which are the prerequisites for the reconcentration of volatile solutes allowed on-column injection of large volumes (Grob *et al.*, 1985). The term “on-column” describes the technique which involves the evaporation in the oven-thermostated column or pre-column. Simplicity and excellent performance suggested its use for an on-line LC-GC system. The first applications involved uncoated deactivated pre-columns of 50-60 m in length and transfer volumes of 300-400 µl (Grob *et al.*, 1984). The introduction of an early solvent vapor exit (Noy *et al.*, 1988) and partially concurrent eluent evaporation techniques (Biedermann *et al.*, 1989) allowed the use of shorter pre-columns and drastically reduced evaporation times. The introduction of the loop-type interface with concurrent eluent evaporation enabled the transfer of large transfer volumes, 20 ml being the limit still today (Grob *et al.*, 1989c). One major problem in on-line LC-GC has always been the use of polar eluents in LC and often normal phase eluents were used for that reason. However, as LC-GC primarily is a GC method, most components amenable to GC analysis are better dissolved in normal phase eluents anyhow. On the other hand, many approaches have been developed over the years to deal with polar solvents in LC-GC, as this is of a special issue for environmental analysis, especially water. Transfer techniques involving evaporation with a programmed temperature vaporizer (PTV) have to be named here (Grob, 1990b; Señoráns *et al.*, 1995; Blanch *et al.*, 1998b; Engewald *et al.*, 1999; Louter *et al.*, 1999). Reviews of the different on-line LC-GC techniques have been

published by several authors over the years (Grob, 1991, 1995a, 2000; Schmarr, 1992; Mondello *et al.*, 1999).

2.4.3 Transfer techniques applied in on-line coupled LC-GC

The different approaches for transfer can be classified by a differentiation of the conditions used for introducing the eluent into the gas chromatograph or the column system, respectively. One criterion is whether the transfer is accomplished at temperatures below or above the (pressure corrected) boiling point of the eluent. Another criterion differentiates whether the eluent evaporation takes place within the GC capillary system or within an external "evaporation device", for instance a PTV injector. Amongst the many varieties developed over the past years, normal phase LC-GC applications usually involve either the so called "on-column interface with partially concurrent eluent evaporation" or the "loop-type interface with fully concurrent eluent evaporation". These two interfaces will be described more detailed in the following chapters. Varieties thereof or applications involving PTV-techniques have been reviewed in literature (Señoráns *et al.*, 1995; Grob and Biedermann, 1996; Mondello *et al.*, 1999; Engewald *et al.*, 1999).

2.4.3.1 On-column interface with partially concurrent eluent evaporation

In principle, this interface uses a simple 3-way switching valve diverting the HPLC eluent either to waste or to the GC on-column injector. The GC column system consists of an uncoated deactivated pre-column ("retention gap"), a coated pre-column followed by a Y-piece splitting to an early solvent vapor exit or to the analytical column, respectively. The transfer of the LC fraction is driven by the HPLC pump and occurs through the on-column injector via a transfer capillary under conditions below the eluent boiling point (partially concurrent solvent evaporation), hence liquid floods into the uncoated pre-column. The uncoated pre-column serves as a storage volume for the liquid introduced and as an area of low retention power to ease evaporation of high boiling material later during the temperature program, thus allowing refocussing of the initially broadened solute bands (during flooding, "band broadening in space" (Grob, 1981) due to the retention gap effect (Grob, 1982). The latter can also be described as a phase ratio focusing effect (Jennings, 1985), because material evaporates at significant lower temperatures from the uncoated pre-column and is then refocussed upon entering the coated section of the retaining pre-column or the analytical column (Grob, 1982). Volatile solutes are also spread throughout the flooded zone but are refocussed by the solvent trapping effect (Grob, 1987). That means, solvent and volatile material are released at the introduction side of the liquid (the rear end of the flooded zone) because there, fresh carrier gas enters the column, saturates with volatile material and passes over the flooded zone. Solutes in the vapor phase are then focussed (trapped) in the liquid ahead and are released at the end of evaporation as a sharp zone with the last amount of liquid being evaporated. Therefore, LC-GC transfer by this technique prevents losses of volatile materials. However, specific method optimization as regards the adjustment of the evaporation

rate and the length of the flooded zone (with respect to the length of the uncoated pre-column) has to be performed according to the volume of the HPLC fraction of interest (Schmarr *et al.*, 1989).

2.4.3.2 Loop-type interface with fully concurrent eluent evaporation

In the so-called "loop type interface" (Munari and Grob, 1988), the HPLC eluent is directed through a valve system containing a sample loop which has to be of the size of the fraction of interest. During transfer, this loop is separated from the liquid flow and its content is pushed via flow-regulated carrier gas into the GC pre-column. With temperatures exceeding the pressure corrected boiling point, the evaporating eluent builds up a vapor pressure preventing further flow of liquid into the GC pre-column. Therefore, the system regulates itself and introduction rates equal evaporation rates as no more liquid enters the GC as can be released in the vapor form from within the column system. The latter usually consists of the same column configuration as described for the on-column interface, with the exception that the uncoated pre-column can be significantly shorter, as no storage of flooded liquid is required. The evaporation zone is also significantly shorter compared to the flooded zone using partially concurrent eluent evaporation conditions. However, high boiling material will form a broadened zone ("band broadening in space") and has to be refocussed using the retention gap effect. As there is no liquid in the column system, which is the pre-requisite for solvent trapping, volatile material will be completely lost. Only solutes of intermediate to high boiling points (elution temperatures about 70 – 100°C above the evaporation temperature) will remain in the system due to cold trapping - also called thermal focussing (Grob and Grob, 1987a,b; Grob, 1995b). The transfer of

LC fractions into a GC column under these conditions is therefore limited to the analysis of high boiling material.

As sterols and steryl esters are well within this application range, this transfer technique was chosen for the investigations carried out in this thesis. Another advantage compared to the on-column interface is the ease of operation and the possibility to transfer relatively large fraction volumes (Schmarr *et al.*, 1989). A scheme of this interface is outlined in Figure 7.

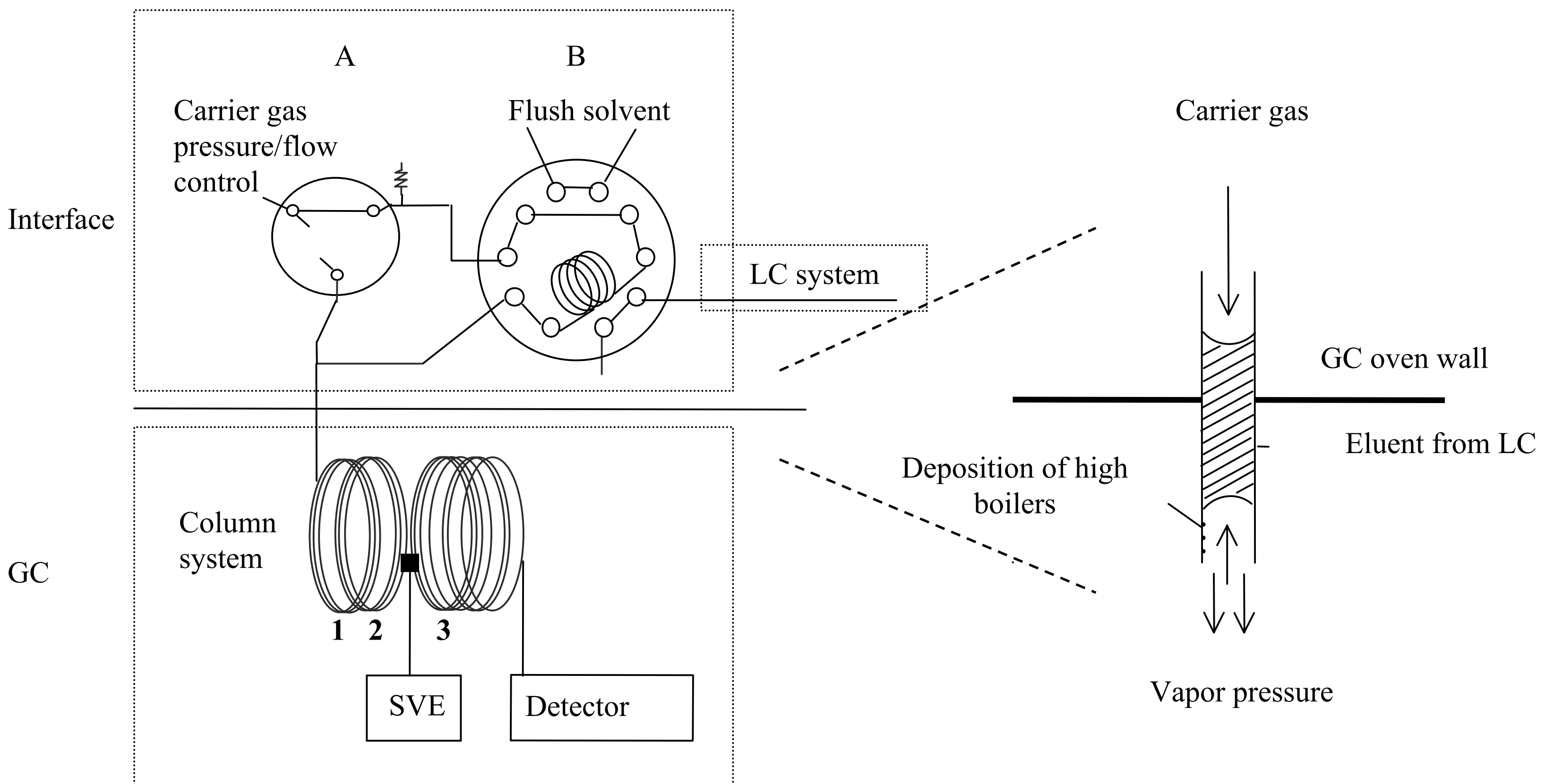


Figure 7.

Scheme of the loop-type interface which consists of two switching valves (carrier gas/pressure control valve A, flushing valve including transfer loop B, solvent vapor exit (SVE), and a column system comprising: uncoated pre-column **1**, retaining pre-column **2**, analytical column **3**; configuration shown in transfer position.

3 MATERIALS AND METHODS

3.1 Materials

Cottonseed and rapeseed oil were obtained from India (Nestlé, Moga). Extra virgin olive oil was obtained from Olio Sasso, Nestlé Italiana, Italy. Milk fat was obtained after treatment of milk from a local dairy company (Canton Vaud, Switzerland) with sulfuric acid and centrifugation of the organic phase (according to the determination of total fat using the *Gerber method*) (International Organization for Standardization, 1983).

Green beans of Coffea canephora (Robusta) and Coffea arabica (Arabica) were obtained from Africa (Uganda, Togo) and Latin America (Brazil and Costa Rica) respectively. The beans were ground using a laboratory mill (Laboratory mill 3303, Falling Number AB, Huddinge, Sweden). During grinding the beans were chilled using solid carbon dioxide to avoid pastiness. To rule out cross-contamination between different coffee samples, the mill was carefully cleaned in between and furthermore, the first batch of a new sample was discarded. The ground material was stored under nitrogen in glass jars at –20°C. For the evaluation of the method, an industrial coffee oil, obtained in-house, was used without further treatment.

Cocoa butter samples of four different origins (South America, Asia, Africa, African/Asian mix) were obtained from commercial suppliers (Nord Cacao, Gravelines, France; Archer Daniels Midland, Koog Aan De Zaan, The Netherlands; Barry Callebaut, Lebbeke-Wieze, Belgium; SACO, Abidjan, Côte d'Ivoire; Gerkens Cacao, Wormer, The Netherlands; Aarhus Olie, Aarhus, Denmark).

3.2 Chemicals

Stigmasterol (96%), β-sitosterol (98%), campesterol (65%), 5α-cholestan-3β-ol (95%), cholesteryl laurate (98%), stigmasterol (96%) and *tert*-butyl methyl ether for liquid chromatography (gradient grade) were purchased from Sigma (Buchs, Switzerland). Palmitoyl chloride, stearoyl chloride, oleoyl chloride and linoleoyl chloride (all at least 99% pure) were supplied by Nu-chek-Prep (Elysian, Minnesota, USA). N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS), BSTFA (99% puriss.), TMCS (99% puriss.), molecular sieve (4Å) and 4-dimethyl-aminopyridine (DMAP) were obtained from Fluka (Buchs, Switzerland). Analytical grade pyridine, dichloromethane, chloroform, methanol, sodium hydrogencarbonate, sodium sulfate, hydrochloric acid (0.1 mol/l), acetonitrile, *n*-hexane for liquid chromatography (gradient grade), 2-propanol, water (LiChrosolv), sodium methylate in methanol (30%), citric acid monohydrate, 2,7-dichlorofluoroscein, chloroform, potassium hydroxide, sodium sulphate, acetone and diethyl ether were purchased from Merck Eurolab (Dietikon, Switzerland). BSA (*N,O*-bis (trimethylsilyl) acetamide) and HMDS (hexamethyldisilazane) were purchased from Supelco (Buchs, Switzerland). 16-*O*-methylcafestol (97%) was synthesized by C. Bertholet as described previously (Speer and Mischnick, 1989b).

3.3 Methods

3.3.1 Official method for the analysis of total sterols in olive oil

The applied method corresponds to the official method (DGF, 1991; EU, 1997) with minor modifications. 100 µl of the internal standard 5α-cholestan-3β-ol (160 mg in 25 ml chloroform = 6.4 mg/ml) were added to about 5 g of extra virgin olive oil. The oil was saponified with 50 ml of methanolic KOH (2 mol/l) under reflux until the

solution turned turbid (approximately 20 minutes). The warm soap solution was quantitatively transferred with a total volume of 100 ml of water into a 500 ml separation funnel. The neutrals were extracted at ambient temperature with 3 × 100 ml of diethyl ether. Emulsions were destroyed by adding a few ml of methanol. The combined ether phases were then repeatedly washed with 50 ml of water until the aqueous phase became neutral, usually three washings were sufficient. The ether phase was dried over sodium sulphate, filtered through a paper filter and collected in a round bottom flask. The solvent was removed using a rotary evaporator at reduced pressure. The remaining material was dried at 105°C in an oven for about 15 minutes, cooled in a desiccator and weighed. Drying and weighing was repeated until two successive weighings did not differ by more than 0.001 g. The unsaponifiable material was dissolved in chloroform (about 5%) and subjected to thin layer chromatography (TLC) on silica plates (Si60, 20 × 20 cm, 25 µm, Merck Eurolab, Dietikon, Switzerland) to separate the different classes of minor components: desmethylsterols, 4-α-methylsterols, 4,4'-dimethylsterols and hydrocarbons. The TLC plates were pre-treated by immersion in methanolic KOH (0.5 mol/l) for 10 s and heated for 1 h at 100°C. The plate was developed in *n*-hexane/diethylether (60:40, v/v), with saturation of the chamber. Detection of the compounds was performed by spraying with an ethanolic fluorescein solution (0.2% v/v) and the resulting spots were observed under UV light at 254 nm. After scraping off the fraction of interest, the desmethylsterols were quantitatively transferred using a sintered glass filter on an Erlenmeyer flask containing 2-3 boiling stones. The glass filter was washed using 10 ml of chloroform and 2 × 10 ml of diethylether. The solvent was concentrated to a volume of about 4 ml and transferred to a derivatization vial. The solvent was removed under a stream of nitrogen and after adding few drops of acetone the vial was heated for 10 minutes at

105°C in an oven. The residue was silylated using a standard silylation agent (pyridine/ hexamethyldisilazane/trimethyl-chlorosilane 9:3:1 v/v/v) for 15 minutes followed by an addition of 2 ml of BSA solution (5% v/v in hexane). A 1:10 dilution in n-hexane was prepared and 1 µl was injected into the gas chromatographic capillary column (J&W Sci., MSP Friedli, Koeniz, Switzerland, 30 m × 0.32 mm i.d., 0.25 µm film thickness, with 5% phenyl dimethylpolysiloxane coating) using an on-column injector. GC analysis was performed on a Fractovap Series 4160 GC with FID detection and hydrogen as carrier gas (2 ml/min, measured at 75°C). A programmed temperature increase of 40°C/min from 75°C (held for 1 min) to 260°C, held for 25 min and a final increase of 10°C/min to 300°C (held for 5 minutes) was used. The detector was heated at 320°C and data were processed using the Turbochrom (version 6) software (Perkin Elmer, Rotkreuz, Switzerland) and a personal computer.

3.3.2 LC-GC analysis of total sterols

3.3.2.1 Olive oil

Sample preparation

Sample preparation was based on the procedure described by Biedermann *et al.* (1993). About 100 mg of the extra virgin olive oil were weighed into a centrifuge tube containing 100 µl of 5α-cholestan-3β-ol solution in *n*-hexane (2 mg/ml), used as internal standard (IS). One ml of a solution of sodium methylate (13 ml of 30% sodium methylate in methanol and 27 ml methanol made up to 100 ml with *tert*-butyl methyl ether) was added. The mixture was homogenized by vortexing. After 20 minutes at room temperature 1 ml of water and 8 ml of *n*-hexane were added and the mixture was gently shaken. The bottom phase was removed by means of a Pasteur

pipette, then one ml of water containing 1 mg/ml citric acid was added and this washing step was repeated. The *n*-hexane phase was analyzed by on-line LC-GC.

Instrumentation

The analysis was performed using a fully automated on-line LC-GC instrument (Dualchrom 3000, C.E. Instruments, ThermoQuest, Rodano, Italy). The HPLC part of this instrument included two 20 ml syringe pumps (Phoenix 20), one used as the master pump for eluent delivery and one used as slave pump for backflushing the HPLC column. Detection was performed with a variable wavelength detector (Micro UVIS 20, Linear Instruments, Reno, Nevada, USA). The separation column was thermostated using a column oven (Jetstream Plus, Advanced Seperation Technologies Inc., USA). The GC was equipped with a fully automated interface valve system. A loop-type interface with a 250 µl sample loop and a solvent vapor exit was used for the transfer of the fraction of interest from LC. A flame ionization detector was used for detection. All components were controlled by the Dualchrom software. Data aquisistion was performed by the Chromcard Software (C.E. Instruments, ThermoQuest, Rodano, Italy).

LC-GC conditions

HPLC pre-separation of the sterols from the excess of fatty acid methyl esters and other constituents was performed with a silica column (HPLC Hypersil, 2.1 × 100 mm, 60 Å, 5 µm particle size, Agilent, Geneva, Switzerland) using a mixture of 0.8% (v/v) of 2-propanol in *n*-hexane as eluent. The separation column was kept at 15°C, the flow rate was set to 200 µl/min and UV detection was at 205 nm. Backflush of the column was performed with *tert*-butyl methyl ether. The latter was

delivered by the slave pump at a flow rate of 200 µl/min during backflush and at 10 µl/min during stand-by. The backflush was started after 25 minutes and returned to stand-by 5 minutes later.

Transfer conditions

The transfer of the sterol fraction started 7.25 min after injection of 10 µl of the sample solution and occurred by concurrent eluent evaporation at 120°C. With a delay of 40 s on the reduction of the inlet pressure by 40 kPa at the end of the transfer, the solvent vapor exit (0.2 m × 0.25 mm i.d. fused silica) was switched to a restrictor (1 m × 0.05 mm i.d. fused silica, BGB Analytik, Anwil, Switzerland) leaving a small purge flow during analysis.

GC analysis

Prior to routine analysis for total sterols, GC conditions and GC columns of different polarities were evaluated: SE 54 (5% phenyl containing polysiloxane) from Alltech, Hoogeveen, Netherlands; SAC 5 (5% phenyl) from Supelco, Buchs, Switzerland; Optima 5 (5% phenyl) and Optima δ3 (15% phenyl) from Macherey Nagel, Düren, Switzerland; CP-Sil8 (5% phenyl), CP-Sil 19 (14% cyanopropyl-phenyl) and TAP-CB (65% phenyl) from Chrompack, Stehelin, Basel, Switzerland and a column coated with a 5/1 mixture (w/w) of SE 54 and OV-61-OH, resulting in a phenyl content of 10%. The latter column was kindly prepared by Dr. Schmarr, TU München, Germany according to procedures described by Biedermann *et al.* (Biedermann *et al.*, 1993).
Finally, the CP-Sil8 (5% phenyl) fused silica capillary with 25 m × 0.25 mm i.d. and a film thickness of 0.4 µm was chosen as separation column for the analysis. This analytical column was connected in series after a 3 m × 0.25 mm i.d. uncoated

phenyldimethyl silylated fused silica capillary (BGB Analytik, Anwil, Switzerland) and a coated pre-column (2 m × 0.25 mm i.d.) having the same coating as the analytical column (Chrompack, Stehelin, Basel, Switzerland).

The analytical conditions used in the LC-GC analysis were: 120°C (5 min hold; "transfer temperature") then programmed at 40°/min to 220°C (0.5 min hold) and then to 300°C at 5°/min, which was held for 15 min. The flame ionization detector (FID) was set to 320°C. The carrier gas inlet pressure behind the flow regulator was set to 200 kPa and the resulting gas flow was measured at 2.0 ml/min at a temperature of 120°C (measured on the FID outlet).

Quantification

The amounts of total sterols were determined by comparing the GC peak areas to those obtained for the internal standard 5α-cholestan-3β-ol (128 µg/g olive oil). For the calculation a relative response factor of 1.0 was used.

3.3.2.2 Milk fat

Sample preparation

Milk fat and mixtures of milk fat with rapeseed and cottonseed oil (5%, w/w) were analyzed as outlined above (chapter 3.3.2.1). For this analysis, around 70 mg of the fat was weighed into a centrifuge tube containing 150 µl of a stigmasterol solution (0.6 mg/ml *n*-hexane) used as internal standard (IS).

LC-GC conditions

Instrumentation and analytical conditions used for the LC-GC analysis were identical to those described in chapter 3.3.2.1, except for the following minor modifications: The transfer of the 250 µl fraction containing the sterols started 7.2 min after injection of 10 µl of the sample solution and occurred by concurrent eluent evaporation at 120°C.

Quantification

The amount of β-sitosterol was determined by comparing the GC peak area to that obtained for the internal standard stigmasterol (1.5 mg/g milk fat). For the calculation a relative response factor of 1.0 was used.

3.3.2.3 Coffee oil

Sample preparation

Green beans of Coffea canephora (Robusta) and Coffea arabica (Arabica) were obtained from Africa (Uganda, Togo) and Latin America (Brazil and Costa Rica) respectively. About 15 g of ground green coffee beans were extracted with *tert*-butyl methyl ether for 8 h using the Büchi extraction system (B-811, Büchi, Flawil, Switzerland). The solvent was evaporated with nitrogen and the residue was dried at 105°C. About 100 mg of the coffee oil obtained were weighed into a centrifuge tube containing 100 µl of 5α-cholestan-3β-ol solution in *n*-hexane (2 mg/ml), used as internal standard (IS). Further transesterification followed the procedure described in chapter 3.3.2.1.

LC-GC

HPLC pre-separation of the sterol fraction of the coffee oil from the excess of fatty acid methyl esters was performed using the conditions already described for olive oil in chapter 3.3.2.1. With respect to the instrument set-up for the analysis of 16-OMC (see chapter 3.3.3.1), a transfer-loop of 750 µl was used. Considering the 250 µl fraction window for the sterols (3.3.2.1), this was "over-sized", but was used for simplicity as it enabled both transfer options without changing the instrument set-up. However, this was only possible as no interfering material was transferred within this enlarged transfer window in the case of the analysis of coffee oil. The transferred fraction containing the sterols started 13.5 min after injection of 5 µl of the sample solution and occurred by concurrent eluent evaporation at 120°C. The backflush was started after 16 minutes and returned to stand-by 5 minutes later.

LC-GC conditions

HPLC pre-separation of the sterol and 16-OMC fractions from the excess of fatty acid methyl esters was performed with a silica column (HPLC Hypersil, 2.1 × 100 mm, 60 Å, 5 µm particle size, Agilent, Geneva, Switzerland) using a mixture of 0.8 % 2-propanol in *n*-hexane as eluent. The separation column was kept at 15°C, the flow rate was set to 200 µl/min and UV detection was at 205 nm. Backflush of the column was performed with *tert*-butyl methyl ether. The latter was delivered by the slave pump at a flow rate of 200 µl/min during backflush and at 10 µl/min during stand-by. The backflush was started after 16 minutes and returned to stand-by 5 minutes later.

GC separation was performed on a 25 m × 0.25 mm i.d. fused silica capillary coated with a film thickness of 0.4 µm (CP-Sil8CB, Stehelin, Basel), connected in series with 2 m × 0.25 mm i.d. of an uncoated phenyldimethyl silylated fused silica capillary and

a coated pre-column (1 m × 0.25 mm i.d.) having the same coating as the analytical column. An early solvent vapour exit (0.2 m × 0.25 mm i.d. fused silica) was installed between the coated pre-column and the separation column via a Y-piece press-fit connector (BGB Analytik, Anwil, Switzerland). Hydrogen was used as carrier gas at a flow rate of 2.0 ml/min, measured at 120°C. The carrier gas inlet pressure behind the flow regulator was set to 200 kPa.

The transfer of the 750 µl sterol fraction started 9.5 min after injection of 5 µl of the sample solution and occurred by concurrent eluent evaporation at 120°C. With a delay of 40 s on the reduction of the inlet pressure by 40 kPa at the end of the transfer, the solvent vapour exit was switched to a restrictor (1 m × 0.05 mm i.d. fused silica), leaving a small purge flow during analysis. After holding the transfer temperature of 120°C for 5 min, the column temperature was programmed to 220°C at 40°C/min, and then to 300°C at 5°C/min, which was held for 15 min. The flame ionization detector (FID) was set to 320°C.

The transfer of the 16-OMC fraction (750 µl) started 13.5 min after injection and was performed using the conditions already described for the sterol fration. Quantitative analysis of 16-OMC was performed within the HPLC pre-separation run using the conditions described above.

Quantification

The amounts of total sterols were determined by comparing the GC peak areas to those obtained for the internal standard 5α-cholestan-3β-ol (378 µg/g coffee oil). For the calculation a relative response factor of 1.0 was used.

3.3.3 Analysis of 16-*O*-methylcafestol in coffee oil

3.3.3.1 LC-GC

HPLC pre-separation of the 16-*O*-methylcafestol fraction of the coffee oil from other constituents was performed under the conditions described in chapter 3.3.2.1 Instrumentation and analytical conditions used for the LC-GC analysis were only slightly modified: The transfer of a 750 µl fraction containing 16-OMC started 13.5 min after injection of 5 µl of the sample solution and occurred by concurrent eluent evaporation at 120°C.

3.3.3.2 GC-MS

16-OMC fractions obtained after pre-separation by HPLC were collected manually and the eluent was removed under a stream of nitrogen. Trimethylsilylation was performed using a mixture of BSTFA/TMCS (4/1; v/v) and heating for 30 min at 60°C. GC-MS analysis of this solution was performed on a Finnigan SSQ 7000 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) working in electron impact mode at 70 eV. Samples were introduced via a gas chromatograph (Hewlett-Packard 5890, Agilent, Geneva, Switzerland) equipped with an HP-7673 autosampler using the following conditions: cold on-column injection, fused silica capillary column DB-5 (J&W Scientific, Folsom, CA, USA) 30 m × 0.32 mm i.d., film thickness 0.25 µm. The carrier gas used was helium at a constant inlet pressure of 69 kPa. The temperature program was 60 °C (1 min) programmed to 200°C (20 min) at 30°/min, then to 250°C (10 min) at 20° C/min and finally to 300°C (20 min) at 3°/min. The transfer line and the source were kept at 250°C and 180°C, respectively. The analysis of 16-OMC was carried out in full scan mode at unit resolution from 20 to 500 Da.

Data were processed using MassLib v8.6 software package (MSP Friedli, Koeniz, Switzerland).

3.3.3.3 Quantification of 16-*O*-methylcafestol

The amount of 16-OMC was determined using an external calibration curve.

3.3.3.4 Recovery of 16-*O*-methylcafestol

The recovery of 16-OMC was evaluated by spiking an industrial coffee oil with a known amount of the reference compound (1.87 mg/g oil) and comparing the results to at obtained for the unspiked coffee oil.

3.3.4 Analysis of steryl esters in cocoa butter

3.3.4.1 Sample preparation

Cocoa butter samples were liquified by heating in an oven (40°C). About 100 mg of the liquified sample were weighed into an 11 ml vial containing 50 µl of a cholesteryl laurate solution (1 mg/ml) used as internal standard (IS). After adding 100 µl of the silylating agent (BSTFA + 1% TMCS) and 20 µl of pyridine as catalyst, the mixture was homogenized by vortexing. The solution was heated for 20 minutes at 80°C using a heating block. After heating, 8 ml of *n*-hexane was added and the mixture was gently shaken. This solution was used for analysis by LC-GC.

3.3.4.2 Synthesis of steryl esters

In accordance with the procedure described by Höfle *et al*. (1978), 1 ml of a DMAP solution (10 mg/ml in anhydrous pyridine) was added to a solution of 150 mg of the fatty acid chlorides (palmitoyl chloride, stearoyl chloride, oleoyl chloride, linoleoyl

chloride) in 1 ml of *n*-hexane. The mixture was vortexed until the solution became clear. About 200 mg of the sterol were added together with 30 ml of chloroform and the mixture was stirred for 2 h at room temperature in the absence of light. Upon stirring, the initially turbid mixture became clear, otherwise an additional ml of dichloromethane was added dropwise. The solution was washed 3 times with hydrochloric acid (0.1 mol/l) and the aqueous phase containing the excess of the acyl pyridinium salt was discarded. To neutralize the excess of acid, the solution was washed with a sodium hydrogencarbonate solution. The organic phase was dried with sodium sulfate and the solvent was evaporated using a rotary evaporator. For the separation of non-reacted sterols, solid phase extraction (SPE) on a reversed phase silica gel column (Chromabond C18ec, 45 µm, 500 mg, Macherey-Nagel, Oensingen, Switzerland) was applied. The cartridges were conditioned with about 4 ml of methanol. The solid residue was transferred from the flask onto the cartridge, the flask was rinsed with about 0.1 ml chloroform. Elution was carried out with 8 ml methanol (fraction was discarded) and 8 ml of *n*-hexane/*tert*-butyl methyl ether (9/1, v/v). The steryl esters (purities >80%) were obtained after removal of the solvent of the latter fraction with a stream of nitrogen.

3.3.4.3 GC-MS

GC-MS analyses of steryl esters were performed on a Finnigan SSQ 7000 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) working in positive chemical ionization mode at 150 eV with ammonia as ionization gas. Samples were introduced via a gas chromatograph (Hewlett-Packard 5890, Agilent, Geneva, Switzerland) equipped with an HP-7673 autosampler using the following conditions: cold on-column injection, fused silica capillary column DB-5ht (J&W Sci, MSP

Friedli, Koeniz, Switzerland) 15 m × 0.25 mm i.d., film thickness 0.1 µm. The carrier gas used was helium at a constant inlet pressure of 186 kPa. The temperature program was 60°C (1 min), then programmed with 30°C/min up to 270°C (5 min), then 10°/min up to 340°C (1 min). The transfer line and the source were heated to 300°C and 180°C, respectively. The analysis of steryl esters was carried out in full scan mode at unit resolution from 100 to 800 Da (scan time: 0.5 sec, inter-scan delay: 0.1 sec).

3.3.4.4 LC-GC

HPLC pre-separation of the fraction containing the silylated sterols and the steryl esters from the di- and triglycerides was performed with a cyano bonded silica column Zorbax-SB, 150 × 2.1 mm, 80 Å pore size, 5 µm particle size (Agilent, Geneva, Switzerland) using a mixture of 2% of dichloromethane and 0.1% of acetonitrile in *n*-hexane as eluent. The separation column was kept at 30°C, the flow rate was set to 200 µl/min and UV detection was at 215 nm. Backflush of the column was performed with *tert*-butyl methyl ether. The latter was delivered by the slave pump at a flow rate of 200 µl/min during backflush and at 10 µl/min during stand-by. The backflush was started after 10 minutes and returned to stand-by 5 minutes later.

GC separation was performed on a 15 m × 0.25 mm i.d. fused silica capillary coated with a film thickness of 0.1 µm (DB-5ht), connected in series with 3 m × 0.25 mm i.d. of an uncoated phenyldimethyl silylated fused silica capillary and a coated pre-column (2 m × 0.25 mm i.d.) having the same coating as the analytical column. An early solvent vapor exit (0.2 m × 0.25 mm i.d. fused silica) was installed between the coated pre-column and the separation column via a Y-piece press-fit connector (BGB Analytik, Anwil, Switzerland). Hydrogen was used as carrier gas at a flow rate of

2.5 ml/min, measured at 120°C. Carrier gas inlet pressure behind the flow regulator was set to 250 kPa.

The transfer of the non-retained apolar LC fraction (250 µl) started 3 min after injection of 5 µl of the analytical sample and occurred by concurrent eluent evaporation at 120°C. With a delay of 40 s on the reduction of the inlet pressure by 40 kPa at the end of the transfer, the solvent vapor exit was switched to a restrictor (1 m × 0.05 mm i.d. fused silica), leaving a small purge flow during analysis.

After holding the transfer temperature of 120°C for 5 min, the column temperature was programmed to 260°C at 30°/min, and then to 340°C at 15°/min, which was held for 15 min. The flame ionization detector (FID) was set to 360°C.

3.3.4.5 Quantification of steryl esters

The amounts of steryl esters were determined by comparing the GC peak areas to those obtained for the internal standard cholesteryl laurate (378 µg/g cocoa butter). For the calculation, a relative response factor of 1.0 was used for all steryl ester homologues.

3.3.4.6 Linearity

Linearity of the response in the concentration range of interest was checked using synthesized stigmasteryl palmitate as reference substance and cholesteryl laurate as internal standard. Different concentration levels of stigmasteryl palmitate (1.1 - 13.6 µg/ml) and a constant amount of cholesteryl laurate (10.5 µg/ml) were analyzed in triplicate by LC-GC.

3.3.4.7 Recovery

The recovery was evaluated by spiking cocoa butter with a known amount of stigmasteryl palmitate. Two portions of a cocoa butter sample were mixed with a solution containing the internal standard cholesteryl laurate as described above. One of the portions was spiked with a stigmasteryl palmitate solution in *n*-hexane, resulting in an additional amount of 252 µg/g cocoa butter. The recovery was calculated on the basis of the GC peak areas obtained for the internal standard and for stigmasteryl palmitate in the spiked and the unspiked portion.

4 RESULTS AND DISCUSSION

4.1 Establishment of LC-GC technology

4.1.1 Parameters for total sterol analysis in olive oil

The determination of total sterols in olive oil as described by Grob *et al.* (1989a,d) and Biedermann *et al*. (1993) was used as benchmark to set up the on-line LC-GC technology and to optimize critical parameters in the LC- as well in the GC-part.

Pre-separation by liquid chromatography

The goal of sample preparation by liquid chromatography is the clean-up of the desired fraction containing the 4-desmethylsterols. They have to be separated from the large amounts of fatty acid methyl esters (derived from the transesterification of the triglycerides), wax alcohols and other minor components, such as 4,4-dimethylsterols or 4α-methylsterols.

For the on-line LC-GC transfer, transfer volumes should be kept moderate, in order to fascilitate the transfer conditions. Therefore, columns used in LC-GC often are of 2-3 mm i.d. and 10-15 cm in length. Flow rates on such micro- or mini-bore columns are about 150-500 µl/min and resulting fraction volumes often range from 200-800 µl only. In the present study, a flow rate of 200 µl/min was applied for the analysis.

On the other hand, sample capacity is an issue as sufficient material has to be injected into the HPLC in order to enable a sensitive detection by GC. As HPLC only serves as a sample preparation step and not as analytical tool, overloading of the HPLC column (*e.g.* with the sample matrix) is routinely applied. Therefore, concentrated sample solutions were injected and the fatty acid methyl esters formed during transesterification of the fat or oil were removed by backflushing the HPLC column to

avoid contamination/deactiviation of the fused silica particles. For example, in the case of extra virgin olive oil, around 100 mg of fat was dissolved in 8 ml of *n*-hexane, resulting in a 12.5 µg/µl oil solution and using a 10 µl injection loop, the sample load was 125 µg oil per injection.

The HPLC conditions were adjusted in order to obtain an optimized separation for the fraction of interest, the 4-desmethylsterols. A silica column (Hypersil, 2.1 × 100 mm, 60 Å, 5 µm particle size) was chosen and a mixture of 0.8% of 2-propanol in *n*-hexane was used as eluent, which kept the transfer volume reasonably small to 250 µl. The transfer window for the Δ^5-sterols fraction was determined using standard compounds (cholesterol, campesterol, stigmasterol, β-sitosterol) and the elution order for Δ^7-sterols was determined according to published literature (Grob 1989d; Grob *et al.*, 1990a; Biedermann *et al.*, 1993).

A representative HPLC analysis of extra virgin olive oil is shown in Figure 8. A good separation of the 4-desmethyl sterols from other constituents could be achieved. The transfer of the sterol containing fraction (250 µl) is indicated as (I**)**.

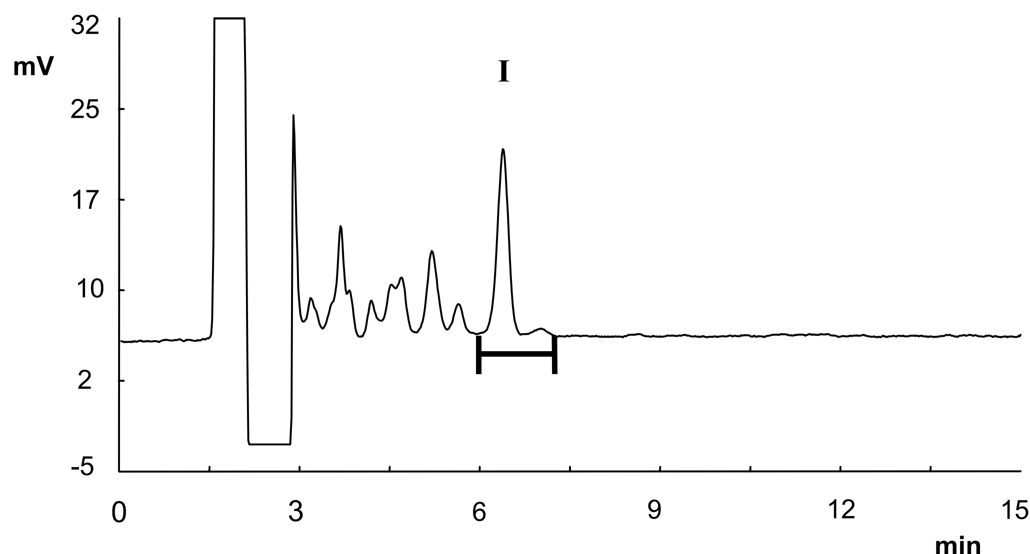


Figure 8.
Analysis of an extra virgin olive oil after transesterification. Separation on normal phase LC with indication of the transferred fraction containing Δ^5-desmethylsterols and Δ^7-desmethylsterols; conditions as described in chapter 3.3.2.1.

Analysis of free sterols by capillary GC

Whereas chromatography of silylated sterols is a procedure often applied, care has to be taken to analyze free sterols (non-derivatized) by GC. First, elution temperatures are relatively high (250-300°C) on most stationary phases. Secondly, adsorptive surfaces easily cause tailing of sterol peaks, thus ruining GC separation efficiency and increasing problems with quantification. Therefore, well deactivated columns have to be used in GC and column in-line filters should be installed before the LC-GC transfer valve, in order to avoid penetration of *e.g.* “small particles” from the silica gel often used as stationary phase in the HPLC separation column.

GC analysis of sterols in olive oil as part of an on-line LC-GC procedure requires special consideration of the relationship between the three basic parameters in chromatography: capacity, efficiency and selectivity (Figure 9) to obtain optimum resolution of critical pairs.

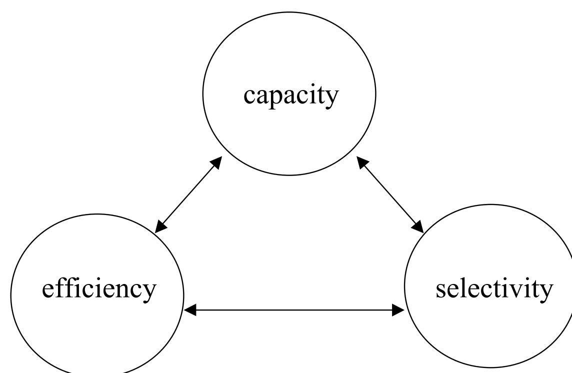


Figure 9.
Basic parameters to be considered in the GC analysis as part of an on-line LC-GC approach.

The analysis of the predominant sterols calls for a considerable capacity of the separation column to prevent overloading which could possibly suppress smaller peaks of minor components. Capacity primarily depends on the amount of stationary phase present (*i.e.* the film thickness at a given column diameter) but is also influenced by the chemistry – "polarity" – of the stationary phase used. On the other hand, considering the chemical similarity of the different sterols, one needs a high separation "power" of the column. The latter can be expressed both by the terms "selectivity" and "efficiency". Unfortunately one has to compromise, as the optimization in one direction often leads to reduction of the quality of another one of these "basics". For instance, increasing the selectivity can usually be achieved by increasing the polarity of the stationary phase, *e.g.* increasing the amount of phenyl groups or introducing other polar functional groups. However, elution temperatures of

polar components will also be increased, which might become a problem for labile compounds, which can decompose (*e.g.* steryl esters). In addition, higher elution temperatures can reduce selectivity, as the interactions of analytes and stationary phase are dependent on the Gibbs-Helmholtz-equation, and the entropy term might become excessive. The geometry (length and diameter) of a column has a great influence on separation efficiency, as the number of theoretical plates will be influenced directly. Long columns with small internal diameters would be preferable for optimization of theoretical plates, but drawbacks in analysis time, increased elution temperatures with longer columns and reduced capacities due to the small diameters would result. The capacity (film thickness) will also have a strong influence on the efficiency, as thinner films (presupposing chemically equal coating) exhibit higher efficiencies than thicker films. Furthermore, the chemistry of the stationary phase (polarity) is often crucial for efficiency, as “coating efficiencies”, a term to describe the “quality” or uniformity of a film, are lower for more polar stationary phases due to wettability problems (Grob 1995d).

The evaluation of GC columns available for this study was based on parameters such as (i) separation of critical peaks, *e.g.* the separation of β-sitosterol and Δ^5-avenasterol, (ii) column stability (maximum operation temperature, baseline drift, “bleed level”), (iii) separation of the minor sitostanol along with the major β-sitosterol, and (iv) peak shape of free sterols (excessive tailing reduces efficiency and causes problems in quantification). The results of an evaluation of the columns according to these criteria are listed in Table 1.

Table 1.
Evaluation of different gas chromatography columns for sterol analysis.

Column	Dimensions	Stationary phase Chemistry	Thickness µm	Thermal stability T_{max} °C	Thermal stability Bleed[a]	Chromatography Tailing[b]	Chromatography Separation[c]	Detection of sitostanol[d]
SE 54	20 m × 0.32 mm	5% diphenyl	0.2	325	++	+	++	-
SAC 5	15 m × 0.25 mm	5% diphenyl	0.25	325	+	+	++	-
Optima 5	25 m × 0.25 mm	5% diphenyl	0.2	325	++	+	++	-
Optima δ3	20 m × 0.25 mm	15% diphenyl	0.2	340	+++	++	++	-
CP-Sil 8	25 m × 0.25 mm	5% diphenyl	0.4	325	+	+	++	+
CP-Sil 19	25 m × 0.25 mm	14% cyano-propylphenyl	0.2	280	++	++	++	-
TAP-CB	25 m × 0.25 mm	65% diphenyl	0.1	320	+++	++	-	-
SE 54/OV61-OH (5/1, w/w)	20 m × 0.25 mm	10% diphenyl	0.25	300	+	+	++	-

[a] Column bleed: +++ strong bleed, ++ medium bleed, + moderate bleed; [b] Peak tailing: ++ medium tailing, + moderate tailing; [c] Separation of β-sitosterol from Δ^5-avenasterol: ++ well separated, - not separated; [d] Separation of sitostanol from β-sitosterol and Δ^5-avenasterol: + detected, - not detected.

The "ideal" GC column for this kind of analysis should have an increased loading capacity, a low to medium polarity, and/or a small film thickness to reduce elution temperatures, hence increasing the selectivity. Small internal diameters (even below 0.25 mm i.d.) would be preferable with respect to plate numbers, but have adverse capacity properties.

A good compromise was found with a film thickness of 0.4 µm and a 5% diphenyl-95% dimethyl polysiloxane stationary phase and a geometry of 25 m x 0.25 mm i.d. (CP-Sil8). This capillary column was the only one investigated which enabled the detection of sitostanol which was otherwise co-eluted between β-sitoserol and Δ^5-avenasterol. The other columns showed further deficiencies with respect to bleed, tailing and maximum operating temperatures.

A representative GC separation of the sterol fraction (250 µl) from extra virgine olive oil after on-line LC-GC transfer is shown in Figure 10.

The sterols found after LC-GC analysis were identified by analyzing standard solutions and/or comparison of the elution patterns to results published in literature (Biedermann *et al*., 1993; Lanuzza *et al.*, 1995, 1996; Jekel *et al.*, 1998; Schuhmann and Schneller, 1996).

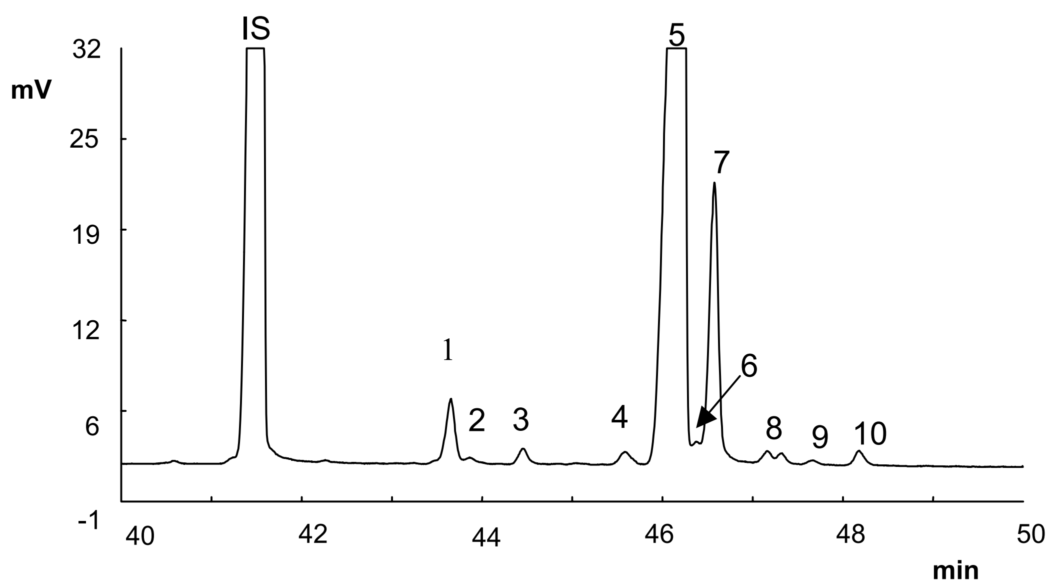


Figure 10.
Gas chromatogram of the transferred 4-desmethylsterol fraction (**I** from Figure 8): (I.S.) 5α–cholestane–3β–ol, (1) campesterol, (2) campestanol, (3) stigmasterol, (4) clerosterol, (5) β-sitosterol, (6) sitostanol, (7) Δ^5-avenasterol, (8) $\Delta^{5,24}$-stigmastadienol, (9) Δ^7-stigmastenol, (10) Δ^7-avenasterol; (conditions described in 3.3.2.1)

4.1.2 Comparison of the LC-GC approach to the offical EU method

Total sterols were analysed in extra virgin olive oil by using (i) the official EU-method involving several steps such as saponification, extraction of the neutrals from the soap solution, pre-separation by preparative thin-layer chromatography, silylation and GC-FID analysis (DGF, 1991; EU, 1997) and (ii) the described on-line LC-GC technique (Biedermann *et al.*, 1993).

Results obtained after repeated sixfold analysis according to both procedures are summarized in Table 2. The two methods yielded comparable results as regards the relative proportions of total sterols. The findings are in agreement with previous

studies demonstrating the reproducibility and comparability of the LC-GC procedure with the official method (Biedermann *et al.*, 1993; Lanuzza *et al.*, 1995).

Table 2.

Total sterols analyzed in extra virgin olive oil obtained by the EU method (EC, 1991) and the LC-GC method (chapter 3.3.2).

	EU method[a]	LC-GC method[a]
Sterols	rel. %	rel. %
Campesterol	2.8 ± 0.2	2.7 ± 0.2
Campestanol	0.2 ± 0.1	0.2 ± 0.1
Stigmasterol	0.5 ± 0.1	0.6 ± 0.3
Clerosterol	0.8 ± 0.1	0.8 ± 0.3
β-Sitosterol	80.0 ± 0.7	80.9 ± 2.1
Δ^5-Avenasterol	12.2 ± 0.6	12.8 ± 0.5

[a] mean values of six repeated analyses
± confidence limit at a 5% error probability

It is noteworthy that a six fold analysis by LC-GC takes about half an hour for sample preparation (six samples prepared in paralell) and the rest is up to the automated LC-GC instrument using the autosampler, (one LC-GC analysis cycle about 60 minutes). On the other hand, sample workup (three samples in paralell) according to the official

method for total sterol analysis (EU, 1991) takes one day. Therefore, the main drawback of the official EU method is the time consuming and manual work involved in these steps. Only few samples can be analyzed at a time and there are additional risks for losses or contaminations during the individual sample preparation steps.

Conclusion

The results obtained using the described on-line LC-GC method with considerably reduced sample preparation steps are in agreement with results obtained using the EU method for total sterol analysis. Using a transesterification step as sample preparation and using the on-line LC-GC technique, a reliable alternative to the official method for the analysis of total sterols in fats and oils is available.

4.2 Applications of on-line LC-GC for authenticity assessment

4.2.1 Adulteration of milk fat with vegetable oil

4.2.1.1 Introduction

Milk fat is a valuable fat for human nutrition and represents a fairly expensive raw material, especially in Asian countries. The determination of admixtures, such as vegetable fats in milk fat, is therefore of interest.

Analytical methods used for authenticity assessment of milk fat have a long tradition and are often based on the fact that the presence of butyric acid is rather unique to milk fat. For example, the amount of butyric acid or the ratio between butyric and oleic acid determined by analysis of fatty acid methyl esters, have been used as parameters to analyse admixtures of vegetable fats to milk fat (Fox *et al.*, 1988; Molkentin and Precht *et al.*, 2000). However, low percentage (<10%) admixtures of vegetable oils with fatty acid composition similar to milk fat render standard fatty acid methyl ester analysis difficult. This approach is additionally limited by the natural variability of the fatty acid composition of milk fat. In general, the fatty acid composition of milk fat is influenced by variations in feeding depending on season and region (Precht *et al.*, 1985; Żegarska and Kuzdzal-Savoie, 1988; Palmquist *et al.*, 1993; Contarini *et al.*, 1996), by genetic factors (Gibson *et al.*, 1989) and by the nutritional status of the cattle (Precht *et al.*, 1985). Thus, statistical evaluation of a large data sets, *e.g.* by linear discriminant analysis is often necessary (Ulberth, 1994).

Alternatively, the analysis of intact triacylglycerols (TAGs) is regarded as advantageous because the genetically controlled specific distribution of the fatty acid moieties on the glycerol backbone is preserved, and thus the information content is higher. However, the milk fat triglyceride composition is also influenced by seasonal effects (Frede and Thiele, 1987; Hinrichs *et al.*, 1992) and depends on the nutritional

status and diet of the cow (Precht *et al.*, 1985; Frede and Thiele, 1987; Contarini *et al.*, 1996). Taking into account the natural variability of the TAG profile, a multilinear regression method was necessary to detect the presence of extraneous fats in milk and butter (Timms, 1980; Precht, 1991; Collomb *et al.*, 1998). The method was officially accepted by the legislative bodies of the European Union for the determination of the purity of milk fat (EC, 1999, 2001) and limits are given to assess the presence of extraneous fat (Van Renterghem, 1997). Furthermore, additional information can be obtained by analyzing the regiospecific distribution of the fatty acids in the triglycerides using lipase-catalyzed hydrolysis of the triglycerides prior to the fatty acid analysis (Soliman and Younes, 1986). Recently, a review on the analysis of the triglycerides in milk fat has been published (Lipp, 1995).

Other methods suitable for the detection of an adulteration of milk fat are based on the analysis of minor lipid constituents, *e.g.* tocopherols (Keeney *et al.*, 1971; Coors and Montag, 1985), diglycerides (Mariani *et al.*, 1990), sterols (Collomb and Spahni, 1991) and steradienes (degradation products of sterols) (Mariani *et al.*, 1994). The determination of the sterol composition is the most sensitive method to differentiate vegetable and animal fat. Animal fats such as milk fat primarily contain cholesterol; phytosterols are not detectable or present only at trace levels (Parodi, 1973; Homberg and Bielefeld, 1979; Younes and Soliman, 1987; Alonso *et al.*, 1997). Accordingly, the analysis of the sterol fraction is a widely used approach to detect vegetable oils in milk fat. Among the different sterols present in vegetable oils, β-sitosterol usually is the main constituent and therefore a suitable marker for the detection of the addition of vegetable oil to milk fat (Homberg and Bielefeld, 1979; Younes and Soliman, 1987). Its analysis is usually based on the official methods developed for determining total sterols in fats and oils (EU, 1991; DGF, 1991; A.O.C.S., 1991). They involve

saponification of the lipids, extraction of the unsaponifiables, pre-separation by thin-layer chromatography, derivatization of the sterols and subsequent gas chromatographic analysis. In recent years, on-line coupling of high pressure liquid chromatography and capillary gas chromatography (LC-GC) has been described as a useful alternative to the laborious sample preparation steps involved in the official methods (Grob *et al.*, 1989a, 1989d, 1990a; Biedermann *et al.*, 1993; Lanuzza *et al.*, 1995). This technique has been successfully applied to the analysis of sterols in plant-derived oils, such as olive oil, and was shown to yield results comparable to the official method (Biedermann *et al.*, 1993; Lanuzza *et al.*, 1995).

The objective of this study was to establish an on-line LC-GC methodology for the analysis of β-sitosterol and thus to lay the basis for rapid analysis of this sterol as marker for the detection of adulterations in milk fat. The potential of the procedure will be demonstrated by investigations of model mixtures of milk fat with vegetable oils, using rapeseed oil and cottonseed oil as examples.

4.2.1.2 On-line LC-GC sterol analysis

The sterol analysis of milk fat or admixtures of vegetable oils to milk fat by on-line LC-GC is based on the following steps: (i) transesterification of the fat, (ii) pre-separation of the sterol fraction from other lipid constituents by HPLC, and (iii) on-line transfer to GC.

Cottonseed and rapeseed oil were chosen as examples for admixtures to milk fat and analyzed for their total sterol compositions. In accordance with the procedure described for olive oil (Biedermann *et al.*, 1993), normal phase HPLC on silica gel was used to separate the sterol fraction from the rest of the transmethylated sample. Campesterol, stigmasterol, and ß-sitosterol were identified by analysis of the

respective reference compounds. Other sterols present in much lower amounts, such as 24-methylenecholesterol, campestanol, clerosterol, sitostanol, Δ^5-avenasterol, $\Delta^{5,24}$-stigmastadienol, Δ^7-stigmastanol and Δ^7-avenasterol were assigned on the basis of results obtained from other vegetable oils analyzed under comparable conditions (Biedermann *et al.*, 1993; Lanuzza *et al.*, 1995). The results obtained for the sterol compositions of the cottonseed and rapeseed oils used in this study (Table 3) are in accordance with previously reported data for vegetable oils determined by classical procedures (Kamm *et al.*, 2001b). This confirms the reliability of the on-line LC-GC analysis of sterols in oils on the basis of the described procedure and the good comparability with the classical saponification method as demonstrated previously (Biedermann *et al.*, 1993; Lanuzza *et al.*, 1995; Grob and Lanfranchi, 1989a).

Table 3.

Total sterol compositions of cottonseed and rapeseed oil determined by on-line LC-GC.

Sterols	Cottonseed mg/kg 1	Cottonseed mg/kg 2	Cottonseed rel. %	Rapeseed mg/kg 1	Rapeseed mg/kg 2	Rapeseed rel. %
Brassicasterol	7	7	0.3	788	829	10.2
24-Methylenecholesterol	n.d.[a]	n.d.	-	34	50	0.5
Campesterol	115	176	7.3	2613	2739	33.9
Campestanol	6	6	0.2	21	14	0.2
Stigmasterol	16	17	0.7	26	24	0.3
Clerosterol	46	53	0.7	55	64	0.3
β-Sitosterol	1991	2064	84.8	3871	4074	50.3
Sitostanol	32	23	1.2	12	11	0.1
Δ^5Avenasterol	35	36	1.5	250	261	3.2
$\Delta^{5,24}$-Stigmastadienol	30	30	1.3	43	36	0.5
Δ^7-Stigmastenol	8	11	0.4	19	16	0.2
Δ^7-Avenasterol	3	6	5	14	12	0.2
Total	2349	2430		7710	8081	

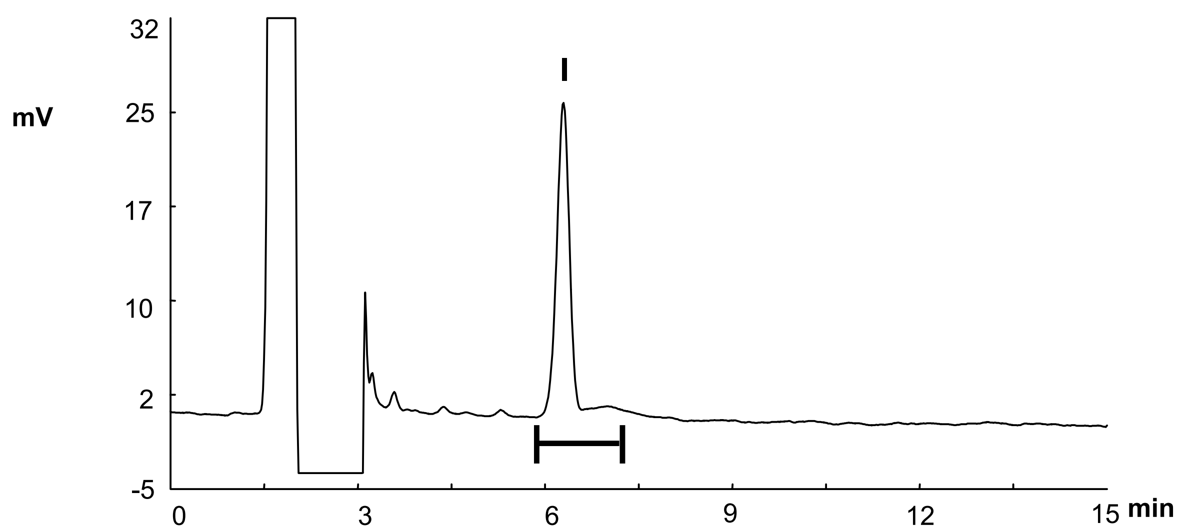
[a] n.d.: not detected.

4.2.1.3 Analysis of model mixtures

Taking into account levels relevant for potential adulterations in practice, 5% (w/w) admixtures of cottonseed and rapeseed oil, respectively, to milk fat were analysed via on-line LC-GC. The HPLC pre-separation of the mixture of milk fat with rapeseed oil is shown in Figure 11a. The determination of the transfer window was based on previously published results (Biedermann *et al.*, 1993) and verified with olive oil and reference sterols.

The GC chromatogram obtained after on-line transfer of the sterol fraction is presented in Figure 11b. The peaks dominating in the chromatogram represent cholesterol (**1**) from milk fat and stigmasterol (IS) as internal standard. β-Sitosterol (**4**) as the marker sterol can easily be detected and quantified. Furthermore, brassicasterol (**2**) (a sterol typically found in rapeseed) and campesterol (**3**) could be assigned as other prominent peaks in the chromatogram.

(a)



(b)

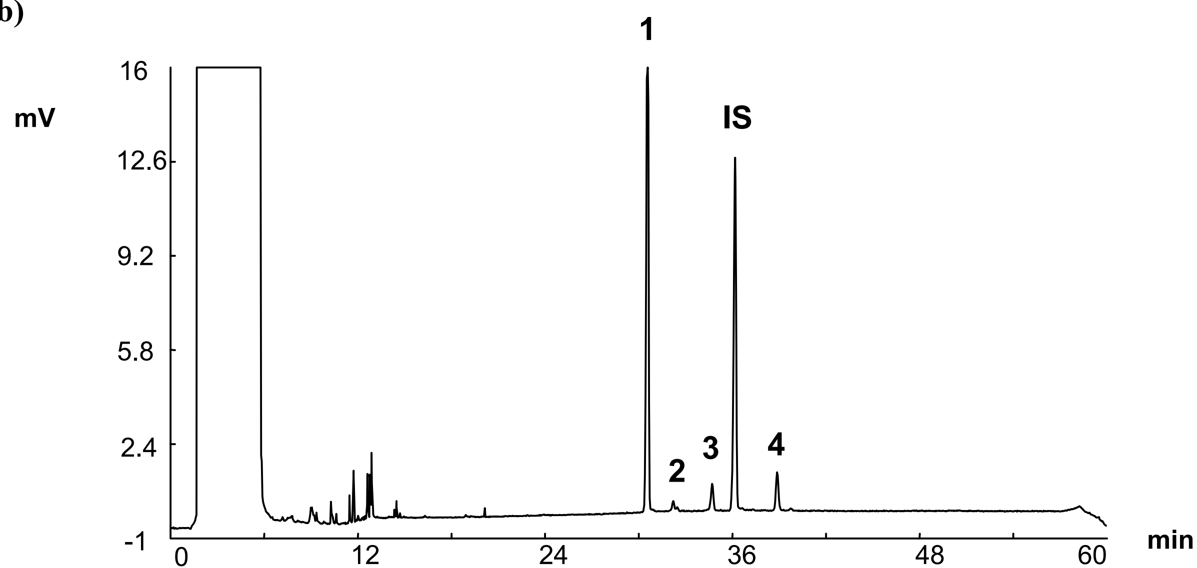


Figure 11a,b.

Analysis of a milk fat sample adulterated with rapeseed oil (5% w/w level). **(a)** Separation on normal phase LC; transferred fractions: (I) sterols; **(b)** GC separation of fraction (I): (1) cholesterol, (2) brassicasterol, (3) campesterol, (I.S.) stigmasterol, (4) β-sitosterol; conditions see Materials and Methods (3.3.2.2).

Quantitative analysis revealed a β-sitosterol content of 193 mg/kg milk fat. This is in good accordance with the amount expected due to the addition of rapeseed oil (Table 4). In the case of the admixture of cottonseed oil, the determined amount of β-sitosterol was also in excellent agreement with the expected value. Regarding the internal standard, 5α-cholestan-3β-ol is often used for the analysis of sterols in vegetable oils (EU, 1991; DGF, 1991; Lanuzza *et al.*, 1995). However, it cannot be used for the analysis of admixtures of vegetable oils to milk fat, because it coelutes with cholesterol, the major sterol in animal fat. For the present model experiments, stigmasterol was applied because it was available in a purity (96%) sufficient for its use as standard. It is present in many vegetable oils, however, its concentration is usually low compared to that of the target compound β-sitosterol in adulterated milk fat (Kamm *et al.*, 2001; Lees, 1998). This error arising from the natural occurrence of stigmasterol could be eliminated by using compounds such as betulinol, ergosterol or epicoprostanol which are not present in common vegetable oils or milk fat, as internal standards.

Table 4.

β-Sitosterol amounts in milk fat expected and determined after addition of rapeseed and cottonseed oil (5% w/w), respectively.

	β-sitosterol	
	expected[a]	determined
	mg/kg	mg/kg
Milk + 5% rapeseed (w/w)	199	193
Milk + 5% cottonseed (w/w)	101	102

[a]based on mean values of β-sitosterol found in the corresponding oils (see Table 3).

Using olive oil as matrix, the detection limit for the determination of β-sitosterol via on-line LC-GC was found to be 2 mg/kg fat (measured as three times the standard deviation of the background noise). This is considerably lower than detection limits of 40 mg/kg and 10 mg/kg, respectively, reported for the convential procedures (Homberg and Bielefeld, 1979). Considering the amount of β-sitosterol present in rapeseed oil (Table 3), an addition of about 0.05% rapeseed oil to milk fat would be detectable. However, an adulteration at such a low level is of no practical concern and usually, detection limits of 1-2% should be acceptable. Even for palm oil, an example for a vegetable oil exhibiting only a relatively low content of β-sitosterol (211-389 mg/kg) (Lees, 1998), the resulting detection limit (0.5-1%) would be sufficient.

Another interesting aspect is the fact, that cholesterol is included in the sterol fraction transferred from LC to GC. This allows to use the described method for the quantification of cholesterol in milk fat or mixtures thereof. The quantification of the cholesterol peak in the pure milk fat (using stigmasterol as internal standard) yielded 253 mg/100g fat. This is in excellent agreement with the recently published concentration of 258.5 ± 19,9 mg/100g milk fat determined as average of 165 samples from 12 EU-countries (Precht, 2001).

4.2.1.4 Summary

On-line liquid chromatography-gas chromatography (LC-GC) has been applied to the detection of vegetable oils in milk fat using β-sitosterol as marker. The method involves transesterification of the fat, pre-separation of the sterol fraction from other lipid constituents and on-line transfer to the capillary GC system. The on-line approach avoids time-consuming sample preparation steps prior to GC analysis. The suitability of this analytical approach was tested with model mixtures of milk fat with

cotton and rapeseed oil. The method allows the detection of adulterations at low levels. Simultaneous quantification of cholesterol in milk fat is also possible. Considering the rapid sample preparation, the described method can be applied for screening of large sample numbers.

4.2.2 Authenticity of coffee beans

4.2.2.1 Introduction

The two coffee species *Coffea canephora* and *Coffea arabica* (commonly called Robusta and Arabica) are of major importance for commercial coffee production. Arabica coffees are generally considered superior in quality. This leads to price differences on the market and the potential for adulteration or misrepresentation by a dishonest trader. It is important to have appropriate methods for the discrimination of the two coffee species and chemical analysis of different components of coffee beans is often applied as a useful tool.

Analytical approaches described in literature for the discrimination of Arabica and Robusta coffees comprise the investigation of volatile compounds (Tressl *et al.*, 1978), alkaloids (Casal *et al.*, 2000), the use of infrared spectroscopy (Briandet *et al.*, 1996; Suchánek *et al.*, 1996; Martín *et al.*, 1999) or DNA analysis via polymerase chain reaction (Remler and Pfannhauser, 1999). Lipid constitutents, especially minor components such as the diterpene 16-*O*-methylcafestol (16-OMC) (Pettitt, 1987; Speer, 1989d; Speer and Mischnick-Lübbecke, 1989c; Speer *et al.*, 1991), tocopherols (González *et al.*, 2001) or sterols (Duplatre *et al.*, 1984; Picard *et al.*, 1984; Carrera *et al.*, 1998; Valdenebro *et al.*, 1999) have also been described as useful analytical parameters.

The use of 16-O-methylcafestol (16-OMC) as a marker component is based on its exclusive presence in Robusta coffee (Speer and Mischnick, 1989b; Pettitt, 1987) and its stability during roasting of coffee beans or deodorization of coffee oils (Speer and Montag, 1989d; Speer *et al.*, 1991). The amount of 16-OMC is usually determined by extraction of the lipid fraction, saponification, liquid-liquid extraction and subsequent analysis by reversed phase HPLC (DIN, 1999). Variations of this method use column

chromatography on silica gel rather than liquid-liquid extraction for clean up of the extracts (Speer, 1989a; Trouche *et al.*, 1997) or direct HPLC analysis of the saponified material (Dionisi *et al.*, 1999). Lipid extraction has been avoided by direct saponification of the ground coffee beans, extraction of the unsaponifiable matter, derivatization and subsequent gas chromatographic analysis (Urgert *et al.*, 1995).

Within the class of sterols, Δ^5-avenasterol is another marker proposed for discrimination of Arabica and Robusta (Duplatre *et al.*, 1984; Picard *et al.*, 1984; Carrera *et al.*, 1998; Valdenebro *et al.*, 1999). Its analysis is usually based on the official methods developed for determining total sterols in animal and vegetable fats and oils (EU, 1991; DGF, 1991; A.O.C.S., 1991). Such analyses involve saponification of the lipids, extraction of the unsaponifiables, pre-separation by thin-layer chromatography, derivatization and subsequent gas chromatographic analysis. On-line coupling of high pressure liquid chromatography and capillary gas chromatography (LC-GC) (Grob *et al.*, 1989a, 1989d, 1990a; Biedermann *et al.*, 1993; Lanuzza *et al.*, 1995) can be a useful alternative to this tedious procedure. This technique has been successfully applied to the analysis of sterols in plant-derived oils, such as olive oils (Grob *et al.*, 1989a, 1990a; Biedermann *et al.*, 1993; Lanuzza *et al.*, 1995).

The objective of this study was to establish such an LC-GC methodology for the investigation of sterols in coffee oil and to extend this approach to the analysis of 16-*O*-methylcafestol. This should result in a rapid and simultaneous determination of two marker substances suitable for the differentiation of Robusta and Arabica coffees.

4.2.2.2 Method development

LC-GC analysis of coffee oil was based on the following steps: (i) transesterification of the oil, (ii) pre-separation of the fractions of interest from other lipid constituents by HPLC, and (iii) on-line transfer to the GC.

Results obtained for coffee oil from Robusta are shown in Figure 12. In accordance with the procedure described for olive oil (Biedermann *et al.*, 1993), normal phase HPLC on silica gel was used to separate the sterol fraction (**I**) from the rest of the transmethylated sample (Figure 12a). Robusta coffee oil showed an additional, well separated peak (**II**) which was also cut out and transferred on-line to the GC.

GC analysis of fraction (**I**) confirmed the transfer of the sterols via HPLC. Campesterol (1), stigmasterol (2), and ß-sitosterol (4) were identified by analysis of the respective reference compounds. Clerosterol (3) and Δ^5-avenasterol (5) were assigned on the basis of results obtained from extra virgin oil analyzed under comparable conditions (Biedermann *et al.*, 1993; Lanuzza *et al.*, 1995). The pattern obtained by this procedure is in accordance with previously reported distributions of total sterols in Robusta coffee determined by the classical procedures (Tiscornia *et al.*, 1979; Picard *et al.*, 1984; Duplatre *et al.*, 1984; Lercker *et al.*, 1995; Carrera et al., 1998; Valdenebro *et al.*, 1999).

Precision data on the on-line transfer of sterols from transesterified oils on the basis of the same procedure as described in this study have been reported (Table 3) and the good comparability with the classical saponification method has been demonstrated (Biedermann *et al.*, 1993; Lanuzza *et al.*, 1995).

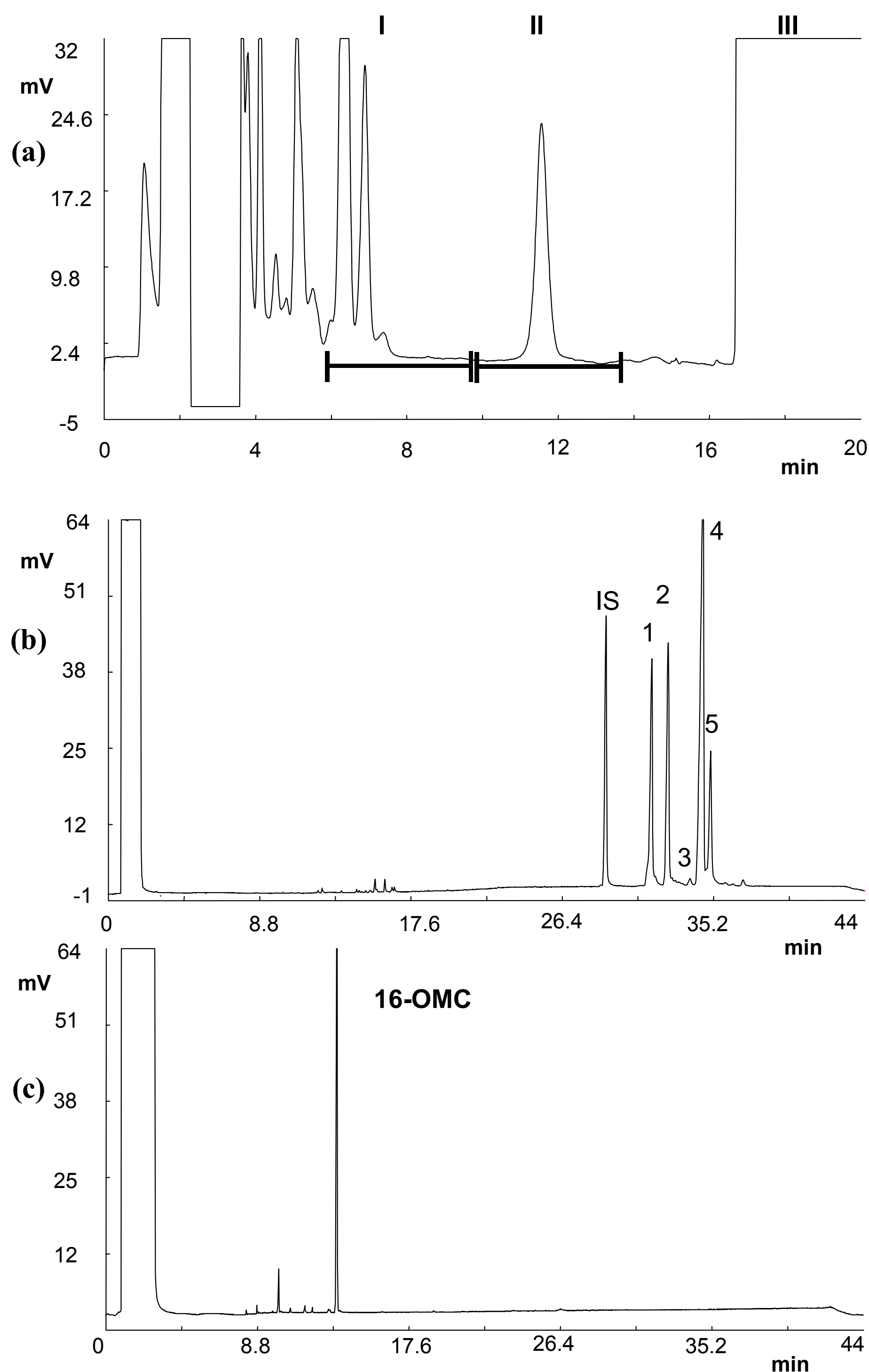


Figure 12.

Analysis of a Robusta coffee sample. **(a)** Separation on normal phase LC with indication of the transferred fraction containing Δ^5-sterols and Δ^7-sterols (I); fraction of 16-OMC (**II**); backflush with MTBE (**III**); GC chromatogram of fraction (**I**) **(b)**: (I.S.) 5α–cholestane–3β–ol, (1) campesterol, (2) stigmasterol, (3) clerosterol, (4) β-sitosterol, (5) Δ^5-avenasterol and fraction (**II**) **(c)**; conditions see chapter 3.3.3.

GC analysis revealed a high purity of HPLC fraction (**II**) (Figure 12c). Subjection of the respective reference compound to the on-line LC-GC procedure demonstrated the transferred lipid constituent to be 16-*O*-methylcafestol. Unambiguous confirmation of the identity of this peak was achieved by repeated manual sampling of HPLC- fraction (**II**), silylation and subsequent GC-MS. The mass spectrum obtained is shown in Figure 13 and was identical to previously reported data (Speer and Mischnick 1989b).

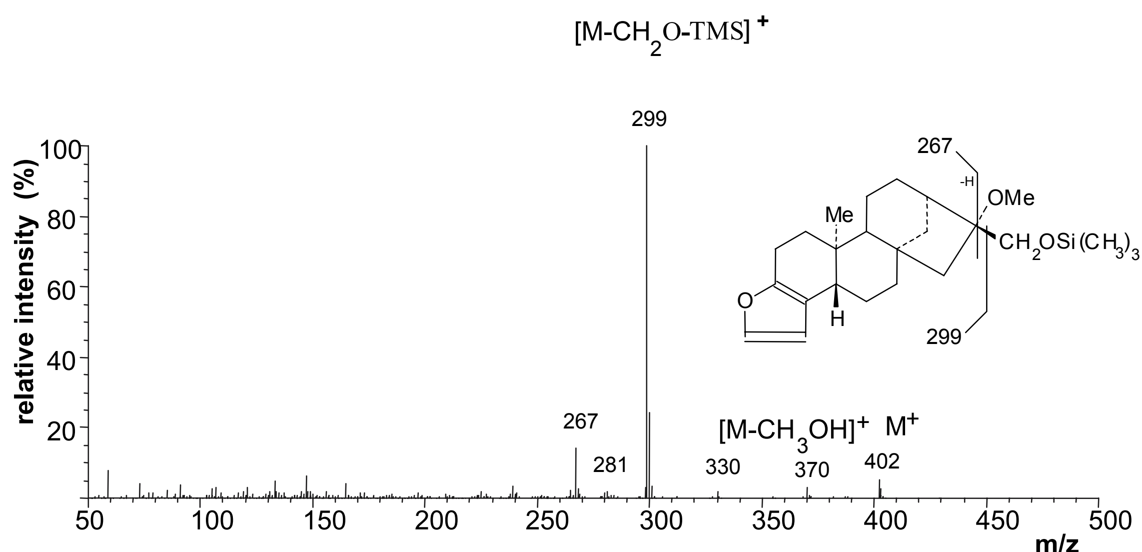


Figure 13.
Electron impact ionization mass spectrum of silylated 16-*O*-methylcafestol in a Robusta coffee sample; conditions as described in chapter 3.3.3.2.

If two individual transfers are performed the described procedure allows the on-line LC-GC analysis of both total sterols and 16-OMC. In principle, “scanning” of an HPLC run is possible using “stopped flow” conditions and was described for the analysis of essential oils (Munari *et al.*, 1990).

Considering the excellent HPLC separation of 16-OMC from the other components of the transesterified oil, the possibility to quantify this compound directly in the HPLC pre-separation step and thus to avoid a second transfer was further investigated. The repeatability of the analysis of 16-OMC by HPLC was evaluated with industrial coffee oil; data obtained from six analyses of the same sample showed excellent repeatability (Table 5). A recovery of 96.2 ± 3.3 % was determined by spiking (in triplicate) an industrial coffee oil with 16-OMC (1.87 mg/g). The detection limit was determined as 0.01 mg/kg coffee oil.

Table 5.
Test for repeatability of the HPLC quantification of 16-OMC in the course of LC-GC analysis of coffee oil.

Analysis	16-OMC (mg/kg)
1	2.25
2	2.24
3	2.27
4	1.96
5	2.11
6	2.22
Mean	2.18
(SD)	(0.12)

4.2.2.3 Application of the LC-GC method to the analysis of green coffee beans

Robusta and Arabica coffees of different geographical origin were analyzed according to the described LC-GC procedure. The sequence of chromatograms obtained for Arabica coffees is exemplarily shown in Figure 14 for the sample from Brazil.

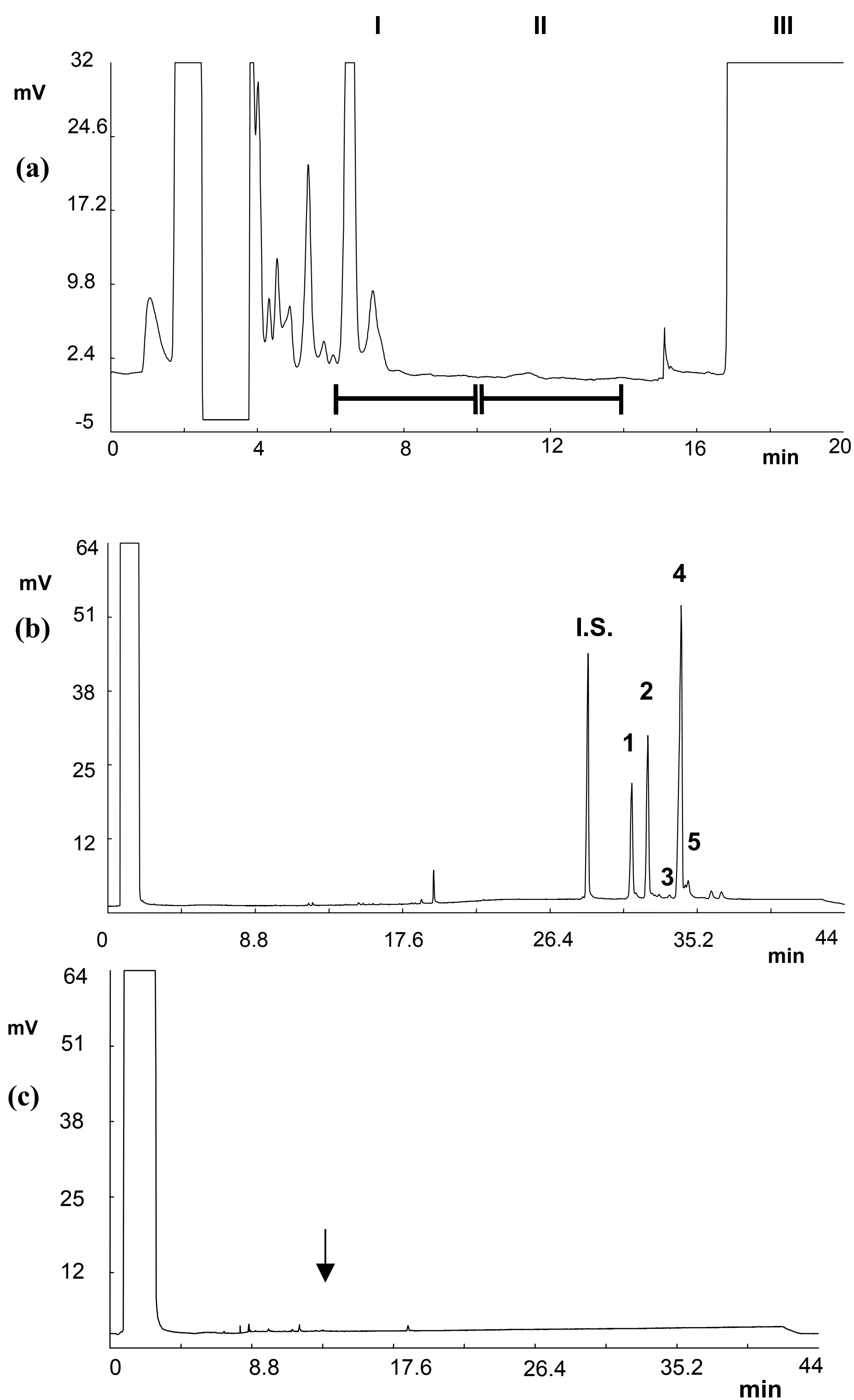


Figure 14.

Analysis of an Arabica coffee sample. **(a)** Separation on normal phase LC with indication of the transferred fraction containing Δ^5-sterols and Δ^7-sterols (**I**), fraction of 16-OMC (**II**), backflush with MTBE (**III**); **(b)** GC chromatogram of fraction (**I**): 5α–cholestane–3β–ol (I.S.), campesterol (1), stigmasterol (2), clerosterol (3), β-sitosterol (4), Δ^5-avenasterol (5); **(c)** GC chromatogram of fraction (**II**).

The absence of 16-OMC in Arabica coffee oil demonstrated in the HPLC run (Figure 14a) and confirmed by the on-line GC analysis run (Figure 14c) underlines the role of this constituent as marker to discriminate Arabica and Robusta species (Pettitt, 1987; Speer, 1989d; Speer and Mischnick-Lübbecke, 1989c; Speer *et al.*, 1991). The concentrations of 16-OMC determined in the Robusta samples from Togo and Uganda via quantification in the HPLC step were 0.69 and 0.67 g/kg coffee beans, respectively. These levels are within the range reported in literature (0.6-1.3 g/kg) (Speer and Montag, 1989d).

The analysis of the sterol fractions (Table 6) confirmed the reported differences in the Δ^5-avenasterol contents of Arabica and Robusta coffees. The relative percentage amounts determined in Arabica (2.5 and 2.6%) and Robusta (10.2 and 10.8%) are in excellent agreement with the literature (Duplatre *et al.*, 1984; Picard *et al.*, 1984; Valdenebro *et al.*, 1999).

Table 6.

Distribution of sterols determined in lipid extract from Arabica and Robusta green coffee beans.

	Robusta		Arabica	
	Togo	Uganda	Costa Rica	Brazil
Sterols	*mg/kg*		*mg/kg*	
Campesterol	306	341	244	260
Stigmasterol	296	328	318	332
Clerosterol	9	10	8	6
β-Sitosterol	796	855	719	765
Δ^5-Avenasterol	171	175	35	35
Total	*1642*		*1361*	

The ratios between the average contents of β-sitosterol and Δ^5-avenasterol in Robusta (4.8) and Arabica (21.4) show good accordance with the respective ranges calculated from data previously determined in green and roasted coffee beans by the classical analytical procedures (Table 7).

Table 7.

Ratios between the average contents of β-sitosterol and Δ^5-avenasterol calculated from data previously determined in green and roasted coffee beans by the classical analytical procedures.

	Robusta		Arabica	
	green	roasted	green	roasted
(1)	4.0 (3.6-4.6)[a]	4.0 (3.5-4.8)	26.3 (20.1-31.8)	24.4 (22.1-28.1)
	-[b]	4.3 (3.4-5.7)	-	23.7 (17.5-29.2)
(*2*)	6.5 (3.7-10.4)	-	19.3 (14.2-21.0)	-
(3)	-	4.3[c]	-	18.6

(1) Duplatre *et al.*, 1984; *(2)* Carrera *et al.*, 1998; *(3)* Valdenebro *et al.*, 1999; [a] mean (min.-max.); [b] not investigated.

4.2.2.4 Summary

An on-line LC-GC method for analysis of 16-*O*-methylcafestol (16-OMC) and total sterols in oils obtained from green beans of *Coffea canephora* (Robusta) and *Coffea arabica* (Arabica) has been developed. HPLC pre-separation of the transesterified lipids allows quantitation of the content of 16-OMC. The fraction containing the sterols is transferred to on-line GC analysis. The results obtained for Arabica and Robusta samples were in good agreement with literature data obtained by classical

analytical procedures. The method allows rapid and simultaneous determination of two markers (16-OMC and Δ^5-avenasterol) proposed for authenticity assessment of coffee beans and overcomes time-consuming sample pre-treatment steps. The rapid and convenient on-line LC-GC procedure fulfills major prerequisites to be applied as high throughput-analysis.

4.3 Analysis of steryl esters in cocoa butter

4.3.1 Introduction

As mentioned in the general introduction, the investigation of minor lipid constituents plays an important role in the assessment of quality and authenticity of fats and oils (Lercker and Rodriguez-Estrada, 2000; Cert *et al.*, 2000; Kamm *et al.*, 2001b). Among the diverse classes of compounds, sterols proved to be suitable to determine the genuineness of vegetable oils. Sterols are present as free alcohols as well as in esterified form (Johansson, 1978a; Johansson and Appelqvist, 1978b; Boskou and Vlachopoulou, 1986; Gordon and Griffith, 1992a) and besides the analysis of the total sterol content, analysis of the steryl esters has been proposed as a tool for the characterization of fats and oils (Grob *et al.*, 1990a; Gordon and Griffith, 1992b; Gordon and Miller, 1997).

The most routine approach to the analysis of steryl esters is based on isolation by thin layer chromatography (TLC) and subsequent gas chromatographic (GC) or liquid chromatographic separation (LC) (Evershed *et al.*, 1987b). The similar polarities of steryl esters and the abundant triglycerides require highly efficient separation by TLC and/or the application of steps such as column chromatography (Ferrari *et al.*, 1997), low-temperature crystallization (Johansson and Appelqvist, 1978) or lipase-catalyzed hydrolysis of the triglycerides (Miller and Gordon, 1996).

The separation of intact steryl esters by HPLC can be achieved by reversed-phase chromatography (Gordon and Griffith, 1992a). However, the application of this technique is limited by the lack of resolution of different steryl esters and the relatively poor detection limit (Evershed *et al.*, 1987b). The scope of the approach might be broadened by the use of LC/MS as described for the analysis of steryl esters in blood plasma (Kuksis *et al.*, 1986). An alternative, overcoming some of the

difficulties encountered with HPLC, is the analysis of steryl esters by GC and GC-MS. However, due to their relatively low volatility, the GC analysis of steryl esters calls for high temperature GC applications with demands on thermal stability of the separation columns and injection techniques used. Therefore, saponification is often applied prior to GC analysis followed by analysis of the sterols as trimethylsilyl derivatives and of the fatty acids as methyl esters (Johansson and Appelqvist, 1978). This approach prevents the recognition of the individual steryl esters and thus valuable information is lost.

There have been approaches to combine HPLC and GC off-line for analysis of steryl esters and sterols (Mariani *et al.*, 1991; Schuhmann and Schneller, 1996). An elegant and very efficient solution based on the use of HPLC as pre-separation step and GC for final resolution has been realized by on-line LC-GC (Grob, 1991). The two methods developed for the simultaneous determination of sterols and steryl esters involve reaction of the sample with pivalic acid anhydride (Grob *et al.*, 1989b, 1990a) or a silylating reagent (Artho *et al.*, 1993) and subsequent on-line transfer of the HPLC fraction containing the derivatized hydroxy compounds and the steryl esters onto the GC column. This combination allows the direct GC analysis of the intact steryl esters and has been applied to the analysis of various fats and oils (Plank and Lorbeer, 1994; Lechner *et al.*, 1999).

This chapter reports the application of the LC-GC procedure involving silylation of the sample to the analysis of steryl esters in cocoa butter (Artho *et al.*, 1993). The sterol composition of cocoa butter has been investigated in detail (Staphylakis and Gegiou, 1985a). Although esterified sterols have been shown to amount to 11.5% of total sterols (Staphylakis and Gegiou, 1985b), knowledge on identities and amounts of individual members of this class of compounds is limited. The only information

available is an assignment of the gas chromatographic peak pattern obtained from a steryl ester rich fraction of cocoa butter to GC and HPLC retention data of synthesized references (Gordon and Griffith, 1992b).

The objective of this study was to identify and quantify individual steryl esters in cocoa butter and thus to lay the analytical basis for potential use of these constituents in authenticity assessment.

4.3.2 Steryl esters – synthesis of reference materials

Prior to analysis, steryl esters of the most important representatives were synthesized as described in materials and methods because reference substances were not commercially available. The identities of the synthesized steryl esters (palmitate, stearate, oleate and linoleate of campesterol, stigmasterol and β-sitosterol) were confirmed by GC-MS using positive chemical ionization with ammonia as ionization gas. The mass spectra of sitosteryl palmitate and sitosteryl stearate are shown as typical examples in Figure 15. All spectra are characterized by intense $[M+NH_4]^+$ adduct ions, in general base peaks of the spectra, and two fragment ions corresponding to the loss of the fatty acid moiety ($[M+NH_4-RCO]^+$) and of one water molecule ($[M+NH_4-RCO-H_2O]^+$). It should be noticed that positive chemical ionization with ammonia as ionization gas was the only ionization method able to generate intense ions indicative of the molecular weight of the steryl esters. Neither electron impact nor positive chemical ionization (with methane) or negative chemical ionization (with ammonia or methane) were found to produce molecular or protonated molecular ions, respectively. These observations are in agreement with data published by Lusby *et al.* (1984) and Evershed and Goad (1987a). Moreover, positive chemical ionization with ammonia as ionization gas allows to determine the sterol moiety of the steryl ester as the two distinctive fragments $[M+NH_4-RCO]^+$ and $[M+NH_4-RCO-H_2O]^+$ and thus enables determination of the molecular weight of the sterol.

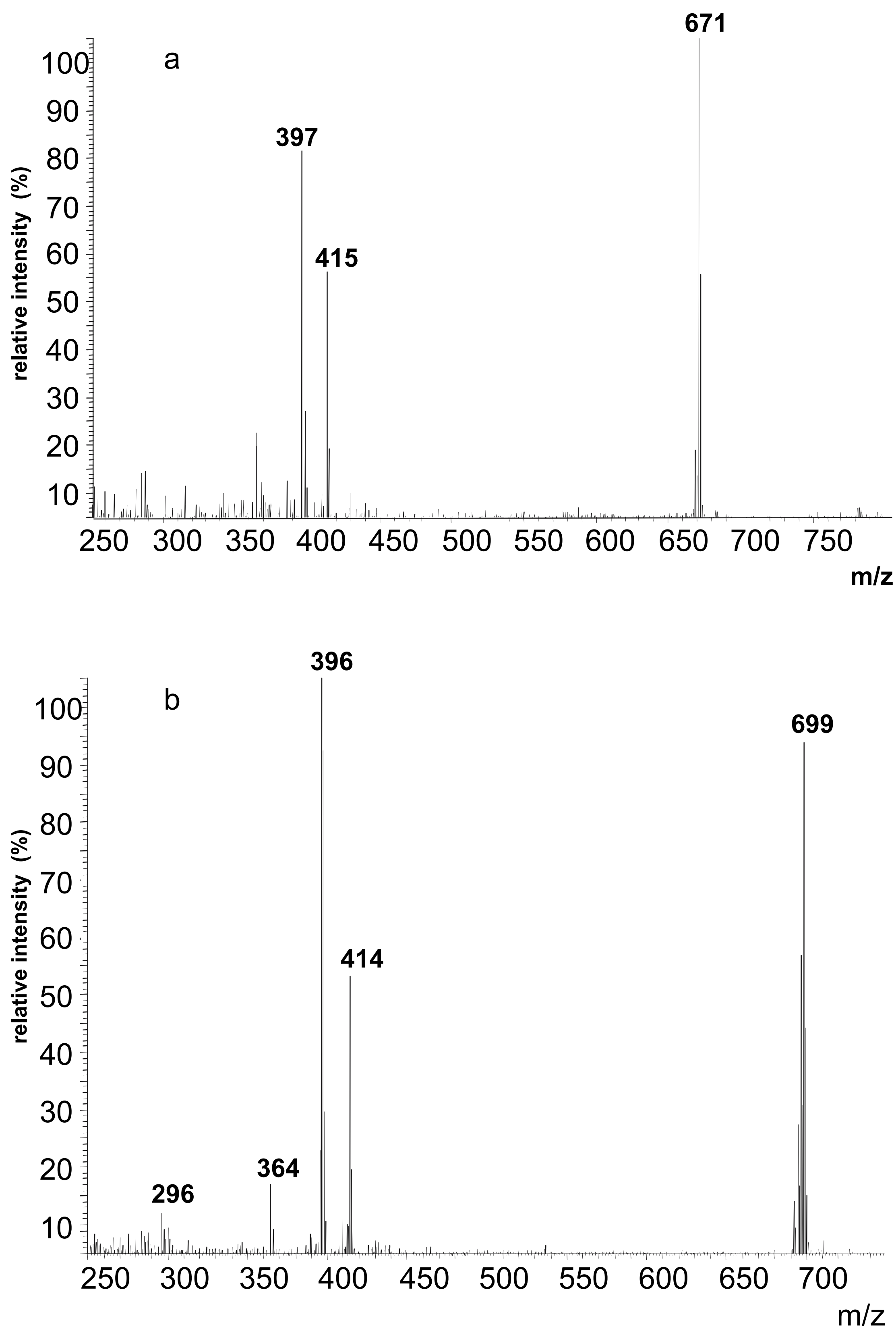


Figure 15.

Positive chemical ionization (ammonia) mass spectra of sitosteryl palmitate (**a**) and sitosteryl stearate (**b**); conditions see chapter 3.3.3.2.

4.3.3 On-line LC-GC analysis of steryl esters

Reference Standards

The transfer conditions of the LC-GC method described in 3.3.4.4 was applied to the analysis of the synthesized steryl esters. The capillary gas chromatographic separation of the steryl ester standards after on-line LC-GC transfer is shown in Figure 16.

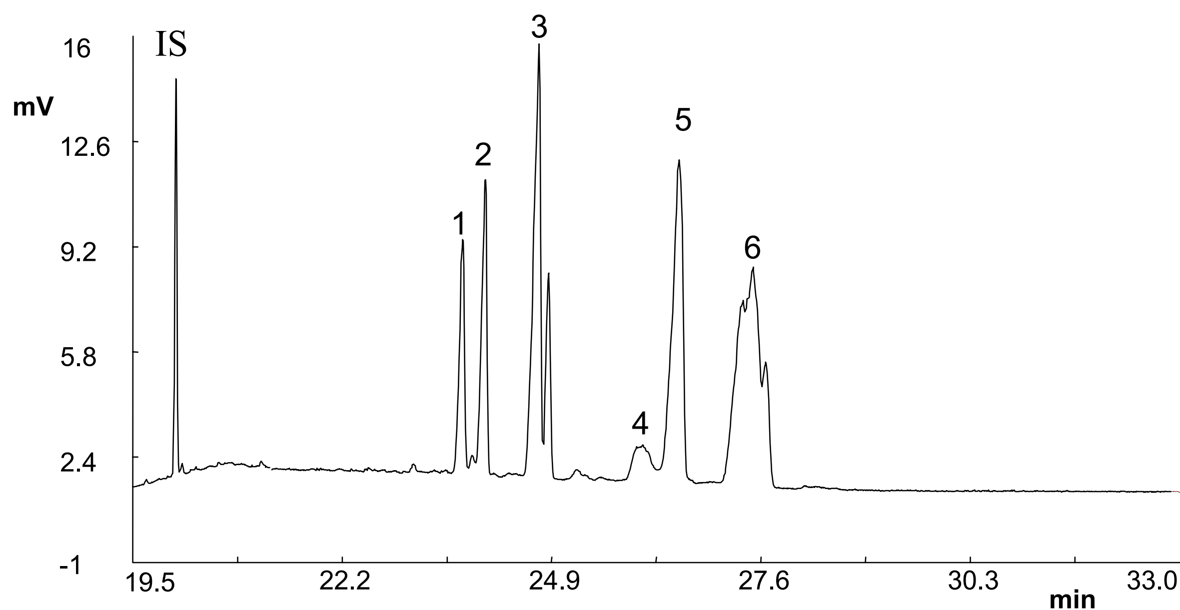


Figure 16.
Gas chromatogram of synthesized steryl ester standards after on-line LC-GC transfer: campesteryl palmitate (**1**), stigmasteryl palmitate (**2**), sitosteryl palmitate (**3**), campesteryl stearate, -oleate, -linoleate (**4**), stigmasteryl stearate, -oleate, -linoleate (**5**), sitosteryl stearate, -oleate, -linoleate (**6**); conditions see 3.3.4.4.

On the employed apolar GC column (DB 5ht) the steryl palmitates (**1**-**3**) are base line resolved, whereas steryl esters of the unsaturated C_{18} fatty acids cannot be resolved according to their degree of unsaturation. The peaks for steryl stearates (18:0), oleates (18:1), linoleates (18:2) form merged peaks only (**4**-**6**). Therefore, no individual quantification for these components was possible and the sum is reported.

This elution behaviour, *i.e.* forming merged peaks on an apolar stationary phase, is in agreement with results obtained for cholesteryl esters (Evershed *et al.*, 1987b; Smith, 1983).

Cocoa butter

The complete analysis of a cocoa butter sample by on-line LC-GC is shown in Figure 17. The separation of the silylated cocoa butter sample achieved by normal phase HPLC is presented in Figure 17a.

Steryl esters and components of similar polarity (*e.g.* silylated sterols) elute prior to di- and triglycerides and were transferred in one fraction. The GC chromatogram of the transferred fraction is shown in Figure 17b. The peaks in the first part of the chromatogram are apolar components (*e.g.* silylated sterols and tocopherols, hydrocarbons) (Grob *et al.*, 1994). As the focus of this study was on the determination of the steryl esters, optimum resolution and identification of the compounds in this region of the chromatogram were not pursued.

The identities of the steryl esters were further confirmed by off-line LC-GC-MS. The apolar fraction (indicated as “a” in Figure 17a) was collected manually and analyzed by GC-MS with positive chemical ionization. The chromatogram was recorded in the full scan mode and ion chromatograms for the steryl esters (**1**-**6**) are given in Figure 18a and 18b; identities and molecular mass of the steryl esters are given in Table 8.

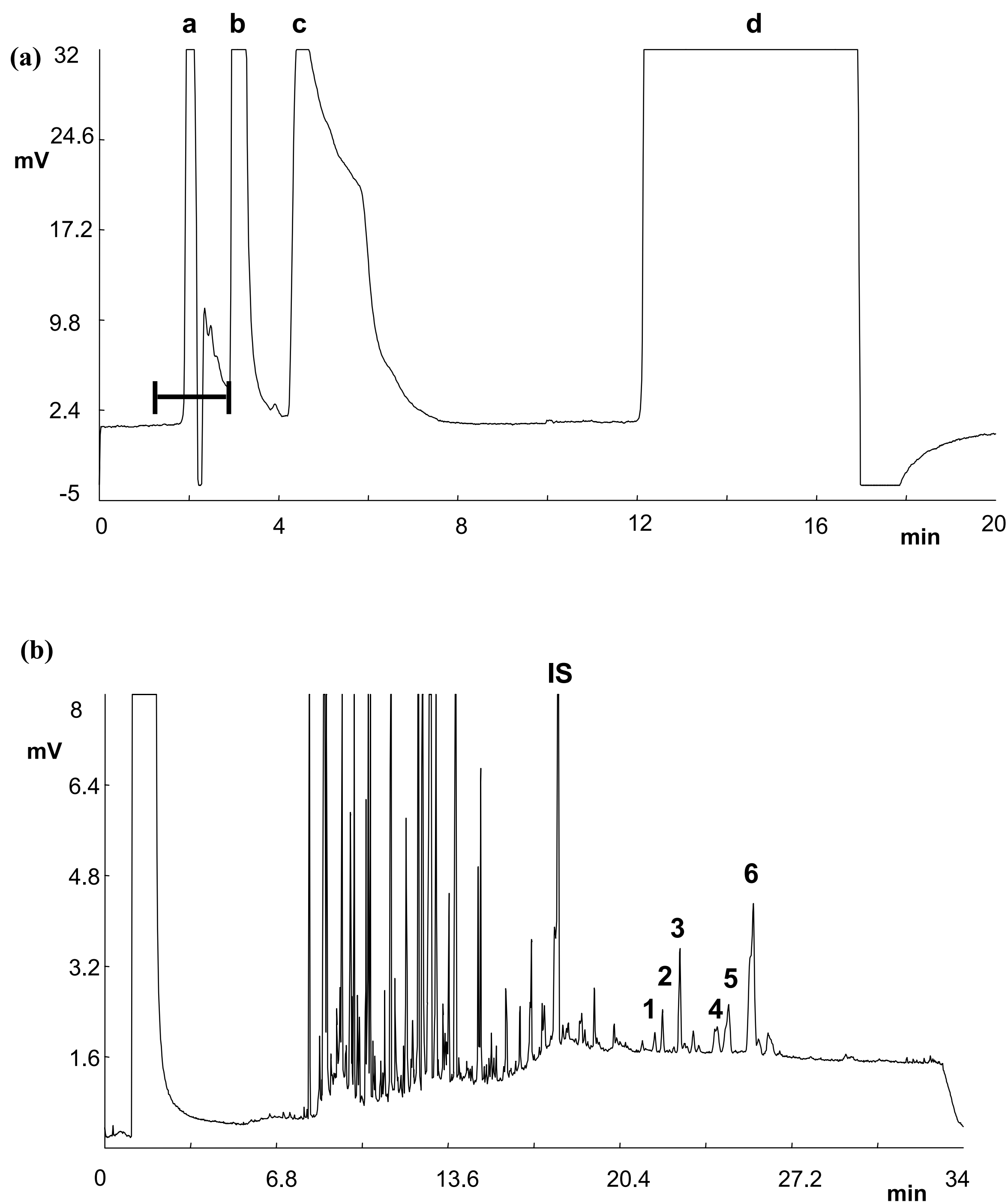


Figure 17.

Analysis of steryl esters in a cocoa butter sample. (**a**) Separation on normal phase LC with indication of apolar fraction (a) containing silylated sterols and steryl esters and range of transfer window. Other fractions represent the elution range of diglycerides (b) and triglycerides (c); backflush with MTBE (d); (**b)** GC chromatogram after on-line LC-GC of fraction a; condition see chapter 3.3.4.4.

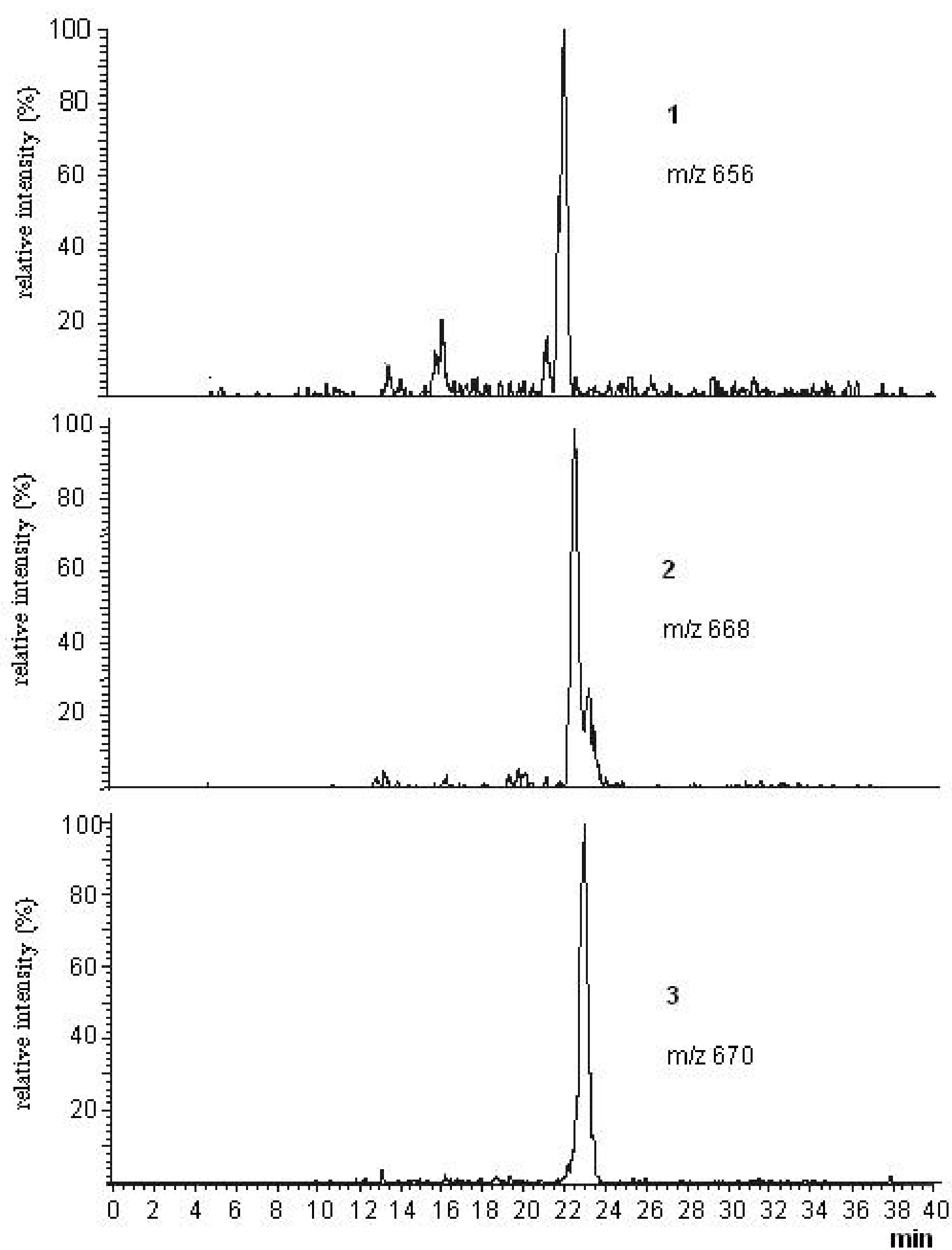


Figure 18a.

Positive chemical ionization (ammonia) mass spectra of steryl esters in a cocoa butter sample after off-line LC-GC-MS. Confirmation of the individual steryl esters (**1-3)** with ion chromatograms of the individual $[M+NH_4]^+$ ions; conditions as described in chapter 3.3.4.3.

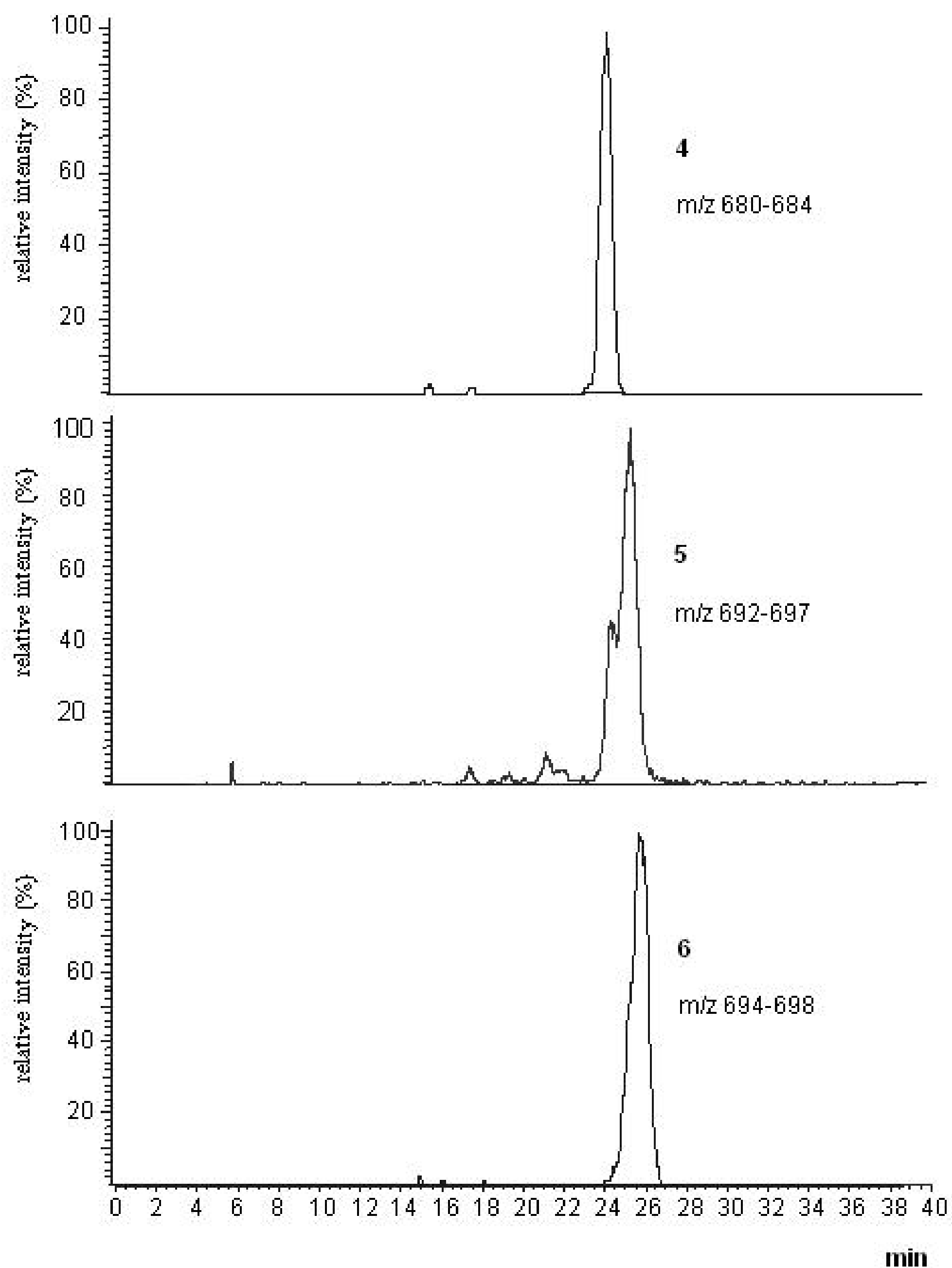


Figure 18b.

Positive chemical ionization (ammonia) mass spectra of steryl esters in a cocoa butter sample after off-line LC-GC-MS. Confirmation of the individual steryl esters (**4-6)** with ion chromatograms of the individual $[M+NH_4]^+$ ions; conditions as described in chapter 3.3.4.3.

Table 8.

Identities and molecular mass of the steryl esters of interest.

Identity	Steryl esters	Mass selected for the $[M+NH_4]^+$ ion m/z
1	campesteryl palmitate	656
2	stigmasteryl palmitate	668
3	sitosteryl palmitate	670
4	campesteryl stearate, -oleate, -linoleate	680-684
5	stigmasteryl stearate, -oleate, -linoleate	692-697
6	sitosteryl stearate-, -oleate, -linoleate	694-698

4.3.4 Method evaluation

Linearity of the response

The linearity of the response in the concentration range of interest was checked using synthesized stigmasteryl palmitate as reference substance and cholesteryl laurate as internal standard. The calibration function ($y = 0.940\ x + 0.099$) showed a good linearity, illustrated by a high correlation coefficient (0.997) (Figure 19).

Repeatability

The repeatability of the on-line LC-GC analysis of the steryl esters was evalutated by complete analysis of six cocoa butter samples. As shown in Table 9 low standard deviations were determined proving the good repeatability of the on-line LC-GC method.

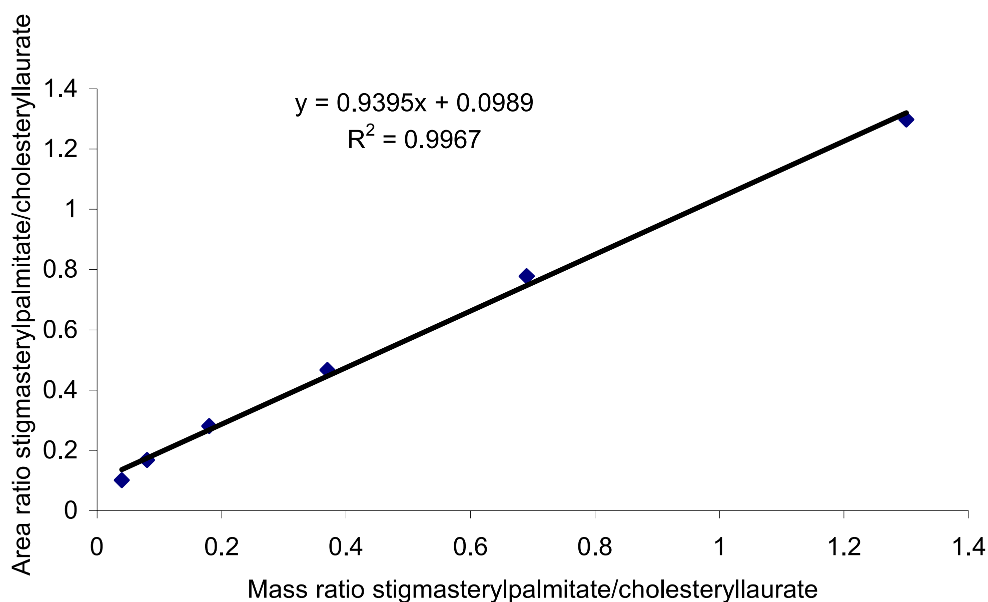


Figure 19.

Linearity of the response of stigmasteryl palmitate.

Table 9.

Repeated LC-GC analysis (n = 6) of a cocoa butter sample.

	Steryl esters (mg/kg)
Campesteryl palmitate	14 (2)
Stigmasteryl palmitate	28 (1)
Sitosteryl palmitate	90 (5)
Campesteryl stearate, -oleate, -linoleate	40 (1)
Stigmasteryl stearate, -oleate, -linoleate	66 (2)
Sitosteryl stearate, -oleate, -linoleate	253 (3)
Sum	491 (8)

[a] mean values (standard deviations)

Recovery and detection limit

The recovery of the compounds of interest was investigated by the additon of a known amount of stigmasteryl palmitate to cocoa butter and was found to be 98%. The limit of detection (defined as the peak area being three times the standard deviation of the noise of the blank) was 3 mg/kg and the limit of quantification (defined as the peak area being ten times the standard deviation of the noise of the blank) was 10 mg/kg.

4.3.5 Steryl ester analysis in cocoa butter samples of different origin

The data obtained for the analysis of steryl esters in commercial cocoa butter samples of different geographical origin are listed in Table 10.

With respect to the geographical origin of the material, only minor differences in the absolute amounts of steryl esters in cocoa butter were observed. According to the suggested classification of vegetable oils on the basis of their steryl ester content (Gordon and Miller, 1997), cocoa butter belongs to the group with a low content (< 1200 mg/kg). The average amount of 548 mg/kg determined in the material available for analysis is in the range reported for soybean oil (Gordon and Miller, 1997).

Table 10.

Distribution of steryl esters in commercial cocoa butter samples.

	South America[a]		Asia[a]		Africa[b]		African/Asian[b]	
Steryl esters	mg/kg	rel. %	mg/kg	rel. %	mg/kg	rel. %	mg/kg	rel. %
Campesteryl palmitate	16	2.7	16	3.1	18 (5)	3.1 (0.2)	14 (1)	2.9 (0.5)
Stigmasteryl palmitate	43	7.1	31	5.9	42 (16)	7.0 (1.2)	32 (11)	6.5 (0.7)
Sitosteryl palmitate	115	19.3	96	18.3	119 (28)	20.2 (1.2)	94 (27)	19.1 (1.1)
Campesteryl stearate, -oleate, -linoleate	41	6.8	45	8.6	44 (10)	7.5 (0.6)	37 (7)	7.7 (0.4)
Stigmasteryl stearate, -oleate, -linoleate	87	14.5	69	13.1	82 (19)	14.0 (1.0)	69 (18)	14.1 (0.7)
Sitosteryl stearate, -oleate, -linoleate	296	49.5	267	51.0	278 (38)	48.2 (3.2)	241 (45)	49.5 (1.9)
Total	598		524		583 (109)		487 (111)	

[a] mean values of two samples; [b] mean values (standard deviations) of seven samples

The relative proportions of the steryl esters were also consistent for all samples. The percentage distributions of campesteryl, stigmasteryl and sitosteryl esters as well as of C_{16} and C_{18} esters are in good agreement with the fatty acid and sterol compositions reported for cocoa butter (Firestone, 1999). This is interesting as others (Gordon and Miller, 1997) found in the case of rapeseed oil differences in fatty acid and sterol composition between those isolated from the oil or the steryl esters. However, with the data available so far, a conclusion whether steryl ester biosynthesis in cocoa, represents the "pool" of naturally available sterols and fatty acids, remains speculative.

A comparison of data obtained for three pairs of deodorized and non-deodorized samples revealed this refining step to have no influence on the pattern and on the amounts of steryl esters in cocoa butter (Table 11). This is in agreement with previous publications reporting that normal refining conditions have only a minor impact (5-10% loss) on the amounts of steryl esters in fats and oils (Ferrari *et al.*, 1996, 1997).

Table 11.

Distribution of steryl esters in non-deodorized (1a - 3a) and deodorized cocoa butter samples (1b - 3b) of african/asian origin.

	Steryl esters (mg/kg)					
	1		2		3	
	a	**b**	**a**	**b**	**a**	**b**
Campesteryl palmitate	13	14	15	16	12	13
Stigmasteryl palmitate	28	29	33	34	25	24
Sitosteryl palmitate	84	90	100	103	75	76
Campesteryl stearate, -oleate, -linoleate	33	36	37	37	34	33
Stigmasteryl stearate, -oleate, -linoleate	61	65	71	71	57	62
Sitosteryl stearate, -oleate, -linoleate	211	232	244	243	219	216
Sum	430	466	500	504	422	424

4.3.6 Summary

On-line liquid chromatography-gas chromatography (LC-GC) has been applied to the analysis of steryl esters in cocoa butter. Separation of the steryl esters was achieved after on-line transfer to capillary GC. HPLC removes the large amount of triglycerides and pre-separates the components of interest, thus avoiding time consuming sample preparation prior to GC analysis. The identities of the compounds were confirmed by GC/MS investigation of the collected HPLC fraction and by comparison of the mass spectra (chemical ionization using ammonia as ionization gas) to those of synthesized reference compounds. Using cholesteryl laurate as internal standard, steryl esters were quantified in commercial cocoa butter samples, the detection limit being 3 mg/kg and the quantification limit 10 mg/kg respectively. Only slight differences in percentage distributions of steryl esters depending on the geographical origin of the material were observed. The patterns were shown to remain unchanged after deodorization. The method described might be a valuable tool for authenticity assessment of cocoa butter.

5 OUTLOOK

Authenticity assessment of foods will be an issue of increasing importance for both the production companies and the control authorities as more and more products designed for special purposes (functional foods) will enter the international market. The introduction of new crop varieties with altered traits, genetically engineered plants, new production techniques etc. will become more important in food production and will call for both an improved quality and safety assessment of such foodstuffs. Furthermore, new regulations by the legislation as *e.g.* the EU Directive (EC, 2000b), which describes the composition and/or labelling of chocolade products, allows the addition of vegetable fats other than cocoa butter in chocolate products and again, calls for analytical methods assessing these products. New methods such as the described analysis of the steryl esters might be helpful in this respect. However, combination of approaches rather than a single technique will probably be required (Lipp 1998a,b; Simoneau *et al.*, 2000; Barcarolo and Anklam, 2001; Spangenberg and Dionisi, 2001).

The methodology described for the authenticity assessment of green coffee beans offers the possibility to screen two markers simultaneously and thus to increase confidence in the conclusions drawn in terms of authenticity assessment of green coffee beans.

In developed countries there is a strong emphasis on the role of dietary fats in health and nutrition and a substantial interest to introduce functional foods reducing important risk factors for common diseases like diabetes, hypertension, and hypercholesterolaemia. Minor components such as steroidal alcohols recently have attracted much attention because of their possible role as bioactive components in the

prevention of heart diseases. For instance, phytosterols are considered as bioactive ingredients because they are similar in structure to cholesterol, and are capable of interfering with the absorption of dietary cholesterol in the body. Intake of phytosterols from edible oils can lower serum cholesterol concentrations in humans (Piironen *et al.*, 2000; Vissers *et al.*, 2000; Wester, 2000) and have been recently incorporated at high levels in some yellow fat spreads (EC, 2000a), which are now commercialized. The analysis of such minor components in fats and oils is therefore of general interest and analysis of target compounds being responsible for a specific function is important for the development of new foods or for the improvement of traditional ones.

The approach of using the hyphenated “on-line LC-GC” technique for the analysis of such minor components therefore deserves special attention as it overcomes many drawbacks of traditional methods. On the other hand both nutritional evaluation and the assessment of authenticty of foods involve usually the analysis of more than one “marker” or parameter in general. Hence a combination of different analytical methods will often be necessary for an extensive evaluation and/or assessment of foodstuffs. The rapid and convenient on-line LC-GC procedure fulfills major prerequisites to be applied as high throughput-analysis.

6 SUMMARY

The suitability of on-line LC-GC as analytical approach for the authenticity assessment of fats and oils was investigated. Flexibility and usefulness of the technique were demonstrated by the analysis of several minor lipid constituents (sterols, steryl esters, diterpenes) in various matrices (milk fat, cocoa butter and coffee oil). The fully automated technique was shown to be advantageous compared to conventional, alternative techniques involving time consuming manual sample pre-separation steps. Minimization of sample preparation and on-line conditions resulted in drastically reduced time required for analysis, avoidance of losses and low detection limits. The results obtained by on-line LC-GC methods were in agreement with those determined by traditional methods.

The applicability of the on-line LC-GC technique for the detection of vegetable oil in milk fat was evaluated by investigating model admixtures of cottonseed and rapeseed oil to milk fat (5%). Based on the analysis of the total sterols, β−sitosterol as the dominant phytosterol present in vegetable oils, was chosen as marker substance. The results showed that the on-line LC-GC method is suitable for the detection of an addition of vegetable oil to milk fat. A detection limit of 2 mg/kg of β−sitosterol in fat was assessed. Considering the amount of β-sitosterol present in rapeseed oil, an addition of about 0.05% rapeseed oil to milk fat would be detectable. However, an adulteration at such a low level is of no practical concern and usually, detection limits of 1-2% should be acceptable. Sample preparation is reduced to a minimum by the on-line approach.

An on-line LC-GC method for analysis of 16-*O*-methylcafestol (16-OMC) and total sterols in oils obtained from green beans of *Coffea canephora* (Robusta) and *Coffea arabica* (Arabica) has been developed. HPLC pre-separation of the transesterified lipids allows quantitation of the content of 16-OMC. The fraction containing the sterols is transferred to on-line GC analysis. The results obtained for Arabica and Robusta samples were in good agreement with literature data obtained by classical analytical procedures. The method allows rapid and simultaneous determination of two markers (16-OMC and Δ^5-avenasterol) proposed for authenticity assessment of coffee beans and overcomes time-consuming sample pre-treatment steps.

The described on-line LC-GC method for the analysis of steryl esters shows distinct advantages compared to the commonly applied analysis of steryl esters involving saponification and investigation of the cleavage products. The LC-GC method yields information on the composition of intact steryl esters and allows their quantification at a detection limit of 3 mg/kg and a quantification limit of 10 mg/kg, respectively. This low detection limit is of importance as only low amounts of steryl esters are present in the case of cocoa butter. Furthermore, as the applied LC-GC procedure is based on the approach of a silylation of the sample, it can easily be extended to the simultaneous determination of steryl esters and free sterols in cocoa butter.

On-line LC-GC is predestinated for routine analysis of many samples, especially when automated commercial equipment is used. However, method optimization for LC-GC is more demanding than for traditional methods and considerable knowledge of chromatographic techniques is required. Considering the advantages of such an on-line approach, analysts should be encouraged to incorporate LC-GC techniques into their analytical portfolio.

7 ZUSAMMENFASSUNG

Die Eignung der Kopplung von Hochdruckflüssigkeitschromatographie und Kapillargaschromatographie (on-line LC-GC) für die Authentizitätskontrolle von Fetten und Ölen wurde untersucht. Anhand der Analyse von Minorbestandteilen (Sterole, Sterylester, Diterpenderivate) in unterschiedlichen Matrizes (Milchfett, Kakaobutter, Kaffeeöl) wurden Flexibilität und Nützlichkeit der Technik demonstriert.

Die voll automatisierte Technik zeigte Vorteile gegenüber konventionellen Methoden. Durch on-line Kopplung von LC mit GC wurden die Probenvorbereitung minimiert, die Analysenzeiten deutlich reduziert, Ausbeuteverluste vermieden und niedrige Bestimmungsgrenzen erreicht. Die mit dieser einfachen und schnellen Methode erhaltenen quantitativen Ergebnisse stimmten mit Literaturdaten überein, die unter Anwendung traditioneller Methoden erhalten wurden.

Die Anwendbarkeit der on-line LC-GC zum Nachweis einer Zumischung von Pflanzenfetten zu Milchfett wurde mit Modellmischungen von Ölen (Baumwollsaatöl, Rapsöl) mit Milchfett (5%) evaluiert. Basierend auf der Analyse von Gesamtsterolen wurde β-Sitosterol als dominierendes Phytosterol in Pflanzenfetten als Marker ausgewählt. Die Ergebnisse zeigen, dass die Methode geeignet ist, eine Zumischung von Pflanzenfett zu Milchfett nachzuweisen. Die Nachweisgrenze von β-Sitosterol lag bei 2 mg/kg Fett. Unter Berücksichtigung des Gehalts an β-Sitosterol in Rapsöl kann somit eine 0,05%ige Zumischung zu Milchfett nachgewiesen werden. Damit konnte gezeigt werden, dass die on-line LC-GC Methode geeignet ist, in der Praxis vorkommende Verfälschungen von 1-2% bei minimaler Probenvorbereitung sicher zu erkennen.

Es wurde eine on-line LC-GC Methode zur Analyse von 16-*O*-Methylcafestol (16-OMC) und Gesamtsterolen in Kaffeeölen von *Coffea canephora* (Robusta) und *Coffea arabica* (Arabica) entwickelt. Die HPLC-Vortrennung der umgeesterten Kaffeelipide erlaubt die Quantifizierung von 16-OMC. Die HPLC Fraktion der Kaffeesterole wurde zur weiteren Auftrennung on-line in das GC System transferiert. Die Ergebnisse der Untersuchungen von Arabica- und Robustaproben stimmten gut mit Daten aus der Literatur, welche mittels klassischer analytischer Verfahren erhalten wurden, überein. Die entwickelte on-line LC-GC Methode erlaubt eine schnelle und simultane Bestimmung von zwei für die Authentizitätskontrolle vorgeschlagenen Markersubstanzen (16-OMC und Δ^5-Avenasterol) unter Vermeidung zeitaufwendiger Probenvorbereitungsschritte.

Die beschriebene on-line LC-GC Methode für die Analyse von intakten Sterylestern zeigt deutliche Vorteile gegenüber den üblicherweise angewandten Verfahren, die eine Verseifung mit anschließender Bestimmung der Spaltprodukte beinhalten. Sie erlaubt die Ermittlung der Sterylesterverteilung bei einer Nachweisgrenze von 3 mg/kg und einer Bestimmungsgrenze von 10 mg/kg Fett. Damit ist das Verfahren zur Analyse von Sterylestern als Minorkomponenten in Kakaobutter einsetzbar. Ferner kann es leicht auf die simultane Bestimmung der freien Sterole in Kakaobutter ausgedehnt werden.

On-line LC-GC ist prädestiniert für den Routinebetrieb und hohen Probendurchsatz. Die Entwicklung einer on-line LC-GC Methode ist anspruchsvoller als die von off-line Verfahren und erfordert ein beträchtliches Verständnis chromatographischer Grundlagen. Betrachtet man jedoch die Vorteile einer solchen on-line Kopplung, sollten Analytiker ermutigt werden, die LC-GC Technik in ihr analytisches Repertoire aufzunehmen.

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