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The contents and effects of polyphenols in chocolate

Qualitative and quantitative analyses of polyphenols in chocolate and chocolate raw products as well as evaluation of potential implications of chocolate consumption in human health

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0 Introduction and objectives

In 2001, chronic conditions, including cardiovascular diseases (CVD), diabetes, obesity, cancers and respiratory diseases, account for 59% of the 56.5 million deaths annually and 45.9% of the global burden of disease. It has been projected that, by 2020, chronic diseases will account for almost three-quarters of all deaths worldwide (WHO, 2002).

An estimated 16.6 million - or one-third of total global deaths - result from the various forms of CVD. By 2010, CVD will be the leading cause of death in developing countries. The major CVD include coronary (or ischaemic) heart disease (heart attack), cerebrovascular disease (stroke), hypertension (high blood pressure), heart failure, and rheumatic heart disease, many of which are preventable by action on the major primary risk factors: unhealthy diet, physical inactivity, and smoking. Cancer accounts for 7.1 million deaths annually (12.6% of the global total). Dietary factors are responsible for about 30% of all cancers in Western countries and approximately up to 20% in developing countries. Thus, diet is second only to tobacco as a preventable cause. Approximately 20 million people suffer from cancer; a figure projected to rise to 30 million within 20 years. The number of new cases annually is estimated to rise from 10 million to 15 million by 2020 (WHO, 2002).

A diet low in energy-dense foods that are high in saturated fats and sugars, and abundant in fruit and vegetables, together with an active lifestyle are among the key measures to combat chronic disease recommended in an independent expert report prepared for two UN agencies, the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) (WHO and FAO, 2003). Thus, at least in the industrialised world, nutrition concepts are progressing from ‘adequate nutrition’ to ‘optimal nutrition’ (Bellisle et al., 1998). Accumulating evidence suggests that people with high dietary intakes of fruits and vegetables are less likely to develop CVD and/or cancer than people who have low dietary intakes of these foods (Kris-Etherton et al., 2002; Temple and Gladwin, 2003). Block et al. (1992) have reviewed approximately 200 epidemiological studies that examined the relation between fruit and vegetable intake and several cancers, and their results are remarkably consistent. Low intake of fruits and vegetables is estimated to cause about 19% of gastrointestinal cancer, and about 31% of ischaemic heart disease and 11% of strokes worldwide. 2.7 million deaths

are attributable to low fruit and vegetable intake. Of the disease burden attributable to low fruit and vegetable intake, more than four fifths is from heart diseases and the balance from cancers (WHO and FAO, 2003). As a consequence, on 11 November 2003, the World Health Organization (WHO) and the UN Food and Agriculture Organization (FAO) announced a unified approach to promote greater consumption of fruits and vegetables.

There are several mechanisms by which these protective effects may be mediated, such as reduced plasma cholesterol, modulation of lipid and lipoprotein metabolism, estrogenic effects, modulation of enzymes, such as Phase I and Phase II enzymes of the detoxification pathway, or modulation of apoptosis (Schramm and German, 1998; Go et al., 2001). However, one major hypothesis is involving the protection against radical oxygen species (ROS) modulated damage of biological molecules, including DNA, lipids, proteins and carbohydrates by antioxidant micronutrients including vitamin C, vitamin E, beta-carotene, selenium or non-nutrient phytochemicals, such as phytoestrogens, other carotenoids than beta-carotene, and polyphenols.

In fact, the wide range of food products available to today's consumer offers a wide variety of complex food components, both nutritive and non-nutritive. In 1995, the American Dietetic Association (ADA) formulated in a position statement that specific substances in foods (e.g. phytochemicals as naturally occurring components and functional food components) may have a beneficial role in health as part of a varied diet (Bloch and Thomson, 1995). They are produced in the secondary metabolism of many plants and play a role for instance in the defence against micro-organisms, as signalling compounds, etc. Therefore they are called "secondary plant products" (Watzl and Leitzmann, 1995), "phytochemicals" (Agarwal and Mukhtar, 1996; Newmark, 1996; Yang et al., 1996), or "chemo-preventers" (Zumbé, 1998). Such chemo-preventers are believed to have the potential to delay, prevent, or even reverse many conditions from cancer to dental caries. They have the potential for inclusion into manufactured foods as an added ingredient, or exist as an intrinsic ingredient of the food in question (Zumbé, 1998).

Among phytochemicals, polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic

structures currently known (Bravo, 1998). They occur in a variety of fruits, vegetables, nuts, seeds, flowers, bark, beverages, and even some manufactured food as a component of the natural ingredients used. Polyphenols have become an intense focus of research interest because of their perceived health-beneficial effects in the treatment and prevention of cancer, cardiovascular disease, and other pathologies. The suggested beneficial effects include anticarcinogenic, antiatherogenic, antiulcer, antithrombotic, antiinflammatory, antiallergenic, immunemodulating, antimicrobial, vasodilatory, and analgesic activities (reviewed in Wollgast and Anklam, 2000a and 2000b).

Cocoa is particularly rich in polyphenols, the total polyphenol content of the bean being estimated to be 6% to 8% by weight of the dry bean (Zumbé, 1998). However, portions in chocolate, the most commonly consumed cocoa product, are significantly lower with levels of 1.7-8.4 mg/g reported in dark chocolate and even lower in milk chocolate with levels of 0.7-5 mg/g, respectively. Firstly, part of this apparent discrepancy can be explained by the chocolate recipe, i.e., chocolate contains not only processed cocoa beans but also added polyphenol-free sugar, milk solids and cocoa butter, respectively. Secondly, the lower polyphenol portions in chocolate compared to the raw cocoa beans are believed to be caused by the conditions during cocoa bean processing and chocolate making resulting in polyphenol degradation (Wollgast and Anklam, 2000a). Nevertheless, chocolate and other cocoa products are widely consumed in Western countries. In 1994, the per head consumption of chocolate and chocolate confectionery in the European Union ranged from 1.3 kg/year in Portugal to 8.8 kg/year in Germany with people in the Northern countries in average consuming more than people in the South (Stutzer, 1998). Not surprisingly therefore, catechins from chocolate contributed 20% of the total catechin intake in a representative sample of the Dutch population (Arts et al., 1999). Thus, chocolate can be seen as a relevant source for phenolic antioxidants for the European population.

Does this imply that people may benefit from the consumption of increased amounts of chocolate as they do from fruits and vegetables and that chocolate should be considered a functional food among other polyphenol-rich foodstuff? Whereas on the one hand the market for functional foods grows (Bellisle et al., 1998) according to Waterhouse et al. (1999) there is on the other hand a risk of premature or even aggressively drawn conclusions regarding phytochemicals and functional foods and their relation to human

health due to commercial interest. Obviously there must be sound scientific evidence before any claimed health effect of a food or food component can be substantiated.

The assessment of such scientific evidence is critical for the scientific community giving advice to health policy but also for regulatory bodies approving or rejecting applications for health claims by food producers. Therefore, leading scientists in the field of nutrition and (functional) food science are currently developing and establishing science-based approaches for the emerging concepts in functional food development as well as guidelines and principles for assessing the scientific support for health-related claims for food and food components within a WHO/FAO expert consultation (WHO and FAO, 2003), the Codex Alimentarius Commission (Codex Alimentarius, 2003) as well as a European Concerted Action "Process for the Assessment of Scientific Support for Claims on Foods" (PASSCLAIM). Very recently, a set of interim criteria to scientifically substantiate claims on foods has been published resulting from the PASSCLAIM consortium (Cummings et al., 2003; Richardson et al., 2003). It is suggested that in evidence-based science which is also the basis of dietary reference values including recommended daily intakes, as well as of dietary guidelines data for scientific substantiation of health claims should include all relevant human intervention, human observational, animal, and *in-vitro* studies as well as other pertinent data. 'Significant scientific agreement' or even consensus on a health claim under discussion finally derives from a body of consistent, relevant evidence from well-designed studies, critical reviews of evidence by experts as well as nationally and internationally accepted evidence from expert bodies and health professional organisations which forms the basis of public health recommendations (Richardson et al., 2003).

Product-specific health claims should be based primarily on the results of well-designed human studies that are consistent with generally recognised scientific procedures and principles. The research should assess the effects of foods or food components on the health status of human subjects. In other words, the outcome – measured in clinical/observational, epidemiological and, where possible, nutrition intervention studies – should be the improvement in some indicators of health or well-being or the reduction of risk of diseases. It is acceptable and even desirable to provide evidence for the effects of the food(s) or food component(s) on appropriately identified, characterised and validated biomarkers. The development of validated and predictive

biomarkers is an essential research objective. Biomarkers must be both biologically and methodologically valid and should reflect a future health outcome at a stage when dietary intervention will be effective (Richardson et al., 2003).

Both in the Codex Alimentarius work on guidelines for the use of nutrition and health claims (Codex Alimentarius, 2003) and in a European Commission proposal for a EU regulation on nutrition and health claims made on foods (European Commission, 2003) there are several restrictions for health claims themselves, including the prohibition of claims that are not clear, accurate and meaningful and cannot be substantiated but also for the foods bearing the claim. The latter restrictions are considered necessary because foods promoted with claims may be perceived by consumers as "good" foods having a nutritional, physiological or other health advantage over similar or other products without such constituents added. This may encourage consumers to make choices which directly influence their total intake of individual nutrients or other substances in a way which would run counter to scientific advice. It is thus seen appropriate to prohibit claims made for foods that contain nutrients or constituents in amounts that increase the risk of disease or an adverse health-related condition (Codex Alimentarius, 2003). In this context, factors such as the presence of certain substances, such as the alcohol content of the product or the nutrient profile of the product, in particular nutrient contents of those such as fat, saturated fat, trans-fatty acids, salt/sodium and sugars whose excessive intakes in the overall diet are not recommended and those such as poly- and monounsaturated fats, available carbohydrates other than sugars, vitamins, minerals, protein and fibre that are elements of a healthy diet are appropriate criteria for determining whether the product can bear claims (European Commission, 2003).

Lastly but not less importantly, if the claimed effect is attributed to a constituent of the food, there must be a validated method to quantify the food constituent that forms the basis of the claim (Codex Alimentarius, 2003).

All this illustrates that the evaluation of the health-protective potential of polyphenols present in chocolate as well as chocolate itself as a food rich in potentially health-beneficial polyphenols is a complex problem and needs an integrated approach.

Therefore and in line with the proposed guidelines described above, the objectives of this Ph.D. work were to:

- assess the current scientific evidence of health-beneficial effects of polyphenols, in particular those present in cocoa and chocolate (literature study)

- evaluate the nutrient profile of chocolate with respect to its possible implications on disease risk and adverse health-related conditions (literature study)

- characterise the polyphenol profile and contents in chocolate and chocolate raw products such as cocoa beans and cocoa liquor by selection, implementation and validation of appropriate state of the art methodologies (experimental work)

- study the influence of selected process parameters in the chocolate manufacturing procedure on the profile and content of the polyphenols present in order to identify key process conditions responsible for polyphenol degradation and by this identify possible ways of preserving them or producing chocolates having enhanced polyphenol levels (experimental work)

- investigate on the bioavailability of cocoa polyphenols as well as identify their metabolites in human blood plasma and urine thereby determining the actual bioactive agents *in vivo*, which in turn, is important for prioritising the accurate analytical determination of the most relevant polyphenols in chocolate and chocolate raw products (experimental work)

- identify, select and characterise appropriate biomarkers for studying the proposed protection against oxidative damage by antioxidant cocoa polyphenols (literature study) as well as develop and validate fast state of the art methodologies suitable for application in large human intervention studies (experimental work)

1 Literature survey

1.1 Theobroma cacao and chocolate

1.1.1 History

Chocolate and cocoa are products derived from cacao beans, the seeds of the *Theobroma cacao* tree. This tree is native to South America, from where it naturally spread into Central America (Bearden et al., 2000). Both the Mayans of the Yucatán and the Aztecs of Mexico prepared, roasted and ground beans into a drink, named "xoxocoatl", "cacahuatl", or "chocolatl" by the Aztecs, among whom it was reserved for the highest social classes (Lupien, 1999; Dillinger et al., 2000). Until recently evidence was dating back to the first and second centuries AD of cacao usage by the Olmec Indians in the Gulf Coast regions of Mexico. However, Hurst et al. (2002) have suggested the usage of cacao in the Preclassic Maya civilisation as early as 600 BC detecting residues of theobromine in several spouted ceramic vessels from the archaeological site at Colha in northern Belize, Central America. Based on epigraphic analysis of such vessels and historic documents written at the time of the Spanish conquest, liquid chocolate was frothed to produce a foam considered by the Maya and Aztecs to be the most desirable part of the drink (Hurst et al., 2002). Cocoa beans also comprised a kind of unified monetary system in the middle Americas of the Aztecs and Mayas and ancient records chronicle price lists at that time (Lupien, 1999). Although the first Latin name of the tree – *Amygdalae pecuniariae* – meant "money almond" in recognition of its status as currency, it was the Swedish botanist Linnaeus who named the genus *Theobroma*, which translates as "food of the gods" (Lupien, 1999; Dillinger et al., 2000). This was prompted by the natives' belief that the cocoa tree was of divine origin.

Following the Spanish conquest of Central and South America during the 16th century, Hernando Cortés took samples of cacao beans back to Spain along with the tools and instructions to make a chocolate beverage and by this introduced chocolate to the Royal Court in Spain. It was described and recommended by European physicians as a nutritious drink that allowed one to travel without any further food in hot weather (Bearden et al., 2000). Owing to the expense of both the cacao and the sugar that soon

came to be added to it, consumption of this drink was originally confined to royalty and the aristocracy and many claims were made about it, one of which was its being aphrodisiac (Borchers et al., 2000). Hernando Cortés reported that the Aztec emperor Montezuma regularly consumed a preparation of roasted cocoa nibs, maize, water and spice particularly before visiting his wives (Lupien, 1999; Dillinger et al., 2000).

Evidence for the use of cacao or chocolate for medicinal purposes can be traced to ancient Aztecs sources. This ancient knowledge was transmitted to Europe through extensive collection of information on medicinal use of cacao mainly in the second half of the 1500s and resulted in important documents including the Badianus Manuscript (1552) and the Florentine Codex (1590) as well as the Princeton Codex, a Mayan language codex discovered only in 1914 in Yucatán (Dillinger et al., 2000). Additionally, numerous English, French and Spanish medical texts of the 17th to the beginning of the 20th century relate to the presumed merits and medicinal properties of cacao and chocolate. In reviewing these historic manuscripts Dillinger et al. (2000) found more than 100 reported medicinal uses for cacao and chocolate. Three consistent uses over the centuries were described as to treat emaciated patients to gain weight, to stimulate nervous systems of apathetic, exhausted or feeble patients as well as to improve digestion and elimination where cacao or chocolate countered effects of stagnant or weak stomachs, stimulated kidneys and improved bowel function. Other conditions treated have included anaemia, poor appetite, mental fatigue, poor breast milk production, tuberculosis, fever, gout, kidney stones, reduced longevity, and poor sexual appetite or low virility.

Eventually cultivation of *T. cacao* lowered the production cost of cacao beans, and consumption of the chocolate beverage spread into all segments of society. Cocoa powder was not developed until two centuries after Cortés imported cacao to Spain by Conrad van Houten of the Netherlands. Solid chocolate soon followed, and finally milk chocolate was created by Daniel Peters of Vevey, Switzerland (Borchers et al., 2000). It is said that chocolate contains sugar and fat in exactly right proportions and is rich in complex flavours to provide a sensory experience like no other. Cocoa butter with its special characteristics of being solid at room temperature, yet melting at slightly less than body temperature results in the unique combination of a “snappy” texture in its solid form with a smooth, fluid form in the mouth (Lupien, 1999). Nevertheless,

consumer perception of chocolate in the Western societies is that of a rather sinful luxury item. Yuker (1997) reported on the view of chocolate by U.S. college students as sweet (91%), fattening (81%), energising (60%), unhealthy (54%), and good (50%).

1.1.2 Cocoa bean

Cocoa beans are derived from cocoa trees which are found in warm, moist climates in areas about 20 degrees latitude north and south of the equator. In general, the seeds of the *Theobroma cacao* (of the order Sterculiacae) are known chiefly in two varieties: Criollo and Forastero, with Forastero divided into several subvarieties. A third group called Trinitario is essentially a cross between Criollo and Forastero and is not found in the wild. The cocoa bean is comprised of an inner nib portion covered by an outer shell (Lange and Fincke, 1970; Minifie, 1989).

1.1.3 Fermentation and drying

The correct fermentation and drying of cocoa beans, which is carried out in the countries of origin is essential to the development of suitable flavours and/or flavour precursors. After the pods are cut from the trees the beans with the adhering pulp are removed and transferred to heaps, boxes, or baskets for fermentation to take place. Fermentation lasts from five to six days with Forastero beans taking rather longer than Criollo. During the first day the adhering pulp becomes liquid and drains away, with the temperature rising steadily. Under anaerobic conditions micro-organisms produce acetic acid and ethanol. These processes inhibit germination of seeds and contribute to structural changes in fermented beans such as the removal of the compartimentation of enzymes and substrates. Cell liquids move across cell walls and are spread all over the cocoa nib. This occurs generally after 24-48 h of bean fermentation. By the third day the mass of beans will have been fairly evenly heated to 45°C and will remain between this temperature and 50°C until the fermentation is complete. It is necessary to mix the beans occasionally for aeration and to ensure that those being initially in the outside of the heap are exposed to the temperature in the interior (Lange and Fincke, 1970; Kim and Keeney, 1984; Kealey et al., 1998).

After fermentation the beans are placed in shallow trays to dry. In some growing areas where the main harvest coincides with the dry season sun drying is adequate. In areas

where rainfall and humidity do not permit sun drying artificial drying becomes necessary (Kim and Keeney, 1984; Minifie, 1989).

After fermentation and drying the cocoa beans should have moisture content of ca. 5-7%. This is of great importance for a correct storage and transport as above a critical moisture content of 8% moulds are likely to develop (Kim and Keeney, 1984; Kealey et al., 1998).

1.1.4 Chocolate making

The first process in user countries that must precede the manufacture of chocolate or cocoa is that of raw-bean cleaning. The machinery consists of a series of operations which remove fibre (from the jute sacks), stones and grit, metal, bean clusters, and immature beans (Minifie, 1989).

Roasting of the whole bean or nib is an essential step in the manufacture of chocolate liquor or partially defatted cocoa solids. Cocoa beans are roasted to develop further the chocolate flavour which should already exist in the form of precursors arising from the correct fermentation and drying of the original beans. Whole bean roasting also loosens the shell so that it can be readily removed during the winnowing process. The degree of cocoa roast is a time/temperature dependent relationship, where the time can vary from 5 to 120 minutes and the temperature of whole bean can typically vary from 120 to 150°C. Lower-temperature roasts are usual for milk-chocolate and for some dark chocolates. In the pre-roasting of whole beans an initial heating step can be performed at just below 100°C followed by roasting of the nibs at elevated temperatures up to about 130°C. Other heat pre-treatment steps to loosen the shell can be a thermal shock of the beans given by hot air, steam or infra-red heat (Lange and Fincke, 1970; Minifie, 1989; Kealey et al., 1998).

The next step in conventional cocoa processing involves nib grinding. Nib grinding is typically performed in two stages, an initial grinding stage to convert the solid nibs into a fluid paste and a final grinding stage to achieve the desired particle size. During the grinding the nib is ground, for instance by milling, into a fluid, dark brown “liquor”. The fluidity is due to the breakdown of the cell walls and the release of the cocoa butter during the processing (Lange and Fincke, 1970; Minifie, 1989; Kealey et al., 1998).

Other conventional cocoa processing includes the separation of cocoa butter from the liquor by either hydraulic presses or screw presses. For chocolate manufacturing this has importance for the cocoa butter that is added when mixing all ingredients for chocolate and/or in the end of the conching process (described later) (Lange and Fincke, 1970; Minifie, 1989; Kealey et al., 1998).

Another step may involve alkalising of the beans, liquor, nibs, or powder with solutions or suspensions of alkali, mainly to change colour. Alkalisation also affects flavour but it is dubious whether there is any improvement. However, alkalising is not an indispensable step in chocolate manufacture but more common for other cocoa products such as dark cocoa powder, cocoa drinks or as an ingredient in a coating or a cookie (Lange and Fincke, 1970; Minifie, 1989).

The basic ingredients required for the manufacture of chocolate are cocoa nibs, cocoa liquor, sugar, other sweeteners, cocoa butter, butter fat (oil), milk powder, milk crumb, and emulsifiers. These ingredients have to be mixed first either continuously or in batch mixers. This should produce a chocolate paste of somewhat rough texture and plastic consistency (Minifie, 1989).

The subsequent refining of chocolate paste is an important operation and produces the smooth texture being desirable in modern chocolate confectionery. Today, the refining is conducted on multi-step refining systems using roll refiners. In modern refiners the pressure between the rolls is controlled and each roll is cooled internally so that the desired temperature can be achieved. Temperatures on the rolls usually vary from 25 to 50°C (Lange and Fincke, 1970; Minifie, 1989; Kealey et al., 1998).

The refined chocolate paste is stored for 24 hours at 45-50°C (“ripening”) getting a doughy texture. It may be used as baker’s chocolate but for fine chocolate conching is required (Minifie, 1989).

Conching may be regarded as the last process in the manufacture of bulk chocolate, whether dark or milk chocolate. It is certainly an essential process for the development of the final texture and flavour. It is usually a two-step process with the first to decrease moisture, drive out volatile substances and distribute the fat equally so that all particles are dispersed in a continuous fat phase. In the second step cocoa butter is added and

finally lecithin to get a liquid homogenised paste. The time/temperature conditions during conching can vary reasonably for the type of chocolate to be processed. With crumb milk chocolate 10-16 hours at 49-52°C is frequently be used, 16-24 hours at 60°C is more likely with milk powder chocolates and dark chocolate is generally conched at higher temperatures, 70°C, sometimes up to 82°C (Lange and Fincke, 1970; Minifie, 1989; Kealey et al., 1998).

Conching conditions may be modified (shortened) by pre-treatment of the chocolate liquor, e.g. in a petzomat, a thin-layer roaster applying pressurised water vapour at over 100 °C being a rather gentle procedure with a very short residence time of the cocoa liquor (Minifie, 1989).

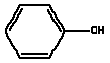
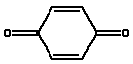
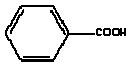
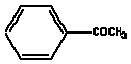
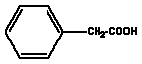
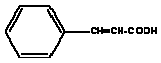
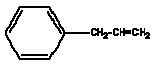
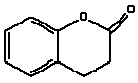
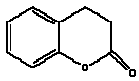
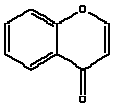
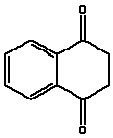
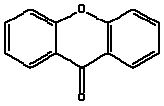
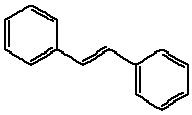
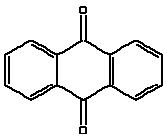
Before filling into forms, the chocolate paste has to be cooled down to 10°C and reheated several times to 29-31°C for good crystallisation (Lange and Fincke, 1970; Minifie, 1989).

1.2 Polyphenols – chemistry and biosynthesis

Phenolic compounds or polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom with more than 8000 phenolic structures currently known. They are products of the secondary metabolism of plants and arise biogenetically from two main primary synthetic pathways: the shikimate pathway and the acetate pathway (Bravo, 1998). Both acetic acid and shikimic acid are derived from glucose metabolism (Schwarze, 1958; Formica and Regelson, 1995). Acetic acid in its active form acetyl-CoA or later in the pathway as malonyl-CoA is the starting point of fatty acid synthesis in a primary pathway but is also the starting point in a secondary pathway of the synthesis of the A ring of the flavonoids. Products of the primary shikimate pathway are the aromatic amino acids (phenylalanine, tyrosine) but their degradation leads also into the phenylpropanoid pathway considered as a secondary pathway. However, the phenylpropanoid pathway is ubiquitously present in higher plants forming the core of a series of related pathways leading to diverse products including flavonoids and stilbens. The phenylpropanoid pathway appears essential to the survival of terrestrial plants providing plant constituents such as lignin with its important mechanical and structural role. Furthermore, phenylpropanoid-derived compounds have distinct roles in the physiology of plants (e.g., as signalling compounds within the plant and as factors controlling male sterility and regulating hormonal activity) and their inter-relations with other organisms (e.g., as defensive compounds against micro-organisms and signalling compounds between plants and other organisms) (Rhodes, 1998). The accumulation of compounds derived from the phenylpropanoid pathway is controlled in a way sensitive to the plant environment involving a hierarchy of controls at the genomic level and regulation of a highly specific set of enzyme proteins. Moreover, a spacial compartition allows for a series of parallel pathways leading to specific products and for different regulatory systems to operate. Thus, the distinction that is drawn between a primary and secondary metabolism is somewhat arbitrary and Rhodes (1998) suggested to think in terms of an integrated metabolism which is unique and characteristic in plants.

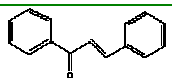
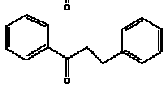
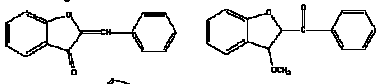
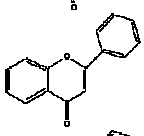
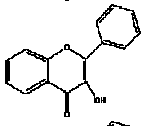
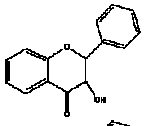
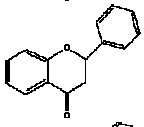
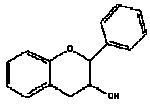
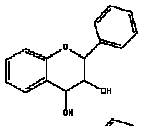
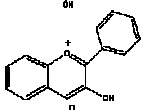
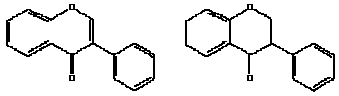
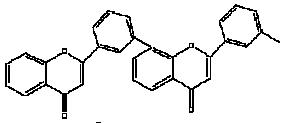
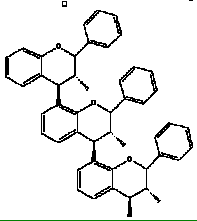
Polyphenols can be divided into at least 10 different classes depending on their basic structure. Table 1.1 illustrates the basic chemical structure of the main polyphenolic compounds.

Table 1.1 Main Classes of Polyphenolic Compounds (Bravo, 1998)

Class	Basic Skeleton	Basic Structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6-C_1	
Acetophenones	C_6-C_2	
Phenylacetic acids	C_6-C_2	
Hydoxycinnamic acids	C_6-C_3	
Phenylpropenes	C_6-C_3	
Coumarines, isocoumarines	C_6-C_3	 
Chromones	C_6-C_3	
Naphtoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthraquinones	C_6-C_2-C_6	
Flavonoids	C_6-C_3-C_6	
Lignans, neolignans	$(C_6$-$C_3)_2$	
Lignins	$(C_6$-$C_3)_n$	

Flavonoids, which constitute the most important single group, can be further divided into 13 classes with more than 5000 compounds described by 1990 (Table 1.2).

Table 1.2 Classification of Food Flavonoids (Bravo, 1998)

Flavonoid	Basic Structure
Chalcones	
Dihydrochalcones	
Aurones	
Flavones	
Flavonols	
Dihydroflavonols	
Flavanones	
Flavanols	
Flavandiol or leucoanthocyanidin	
Anthocyanidin	
Isoflavonoids	
Biflavonoids	
Proanthocyanidins or condensed tannins	

Their common structure is that of diphenylpropanes (C_6-C_3-C_6) and consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (figure 1.1).

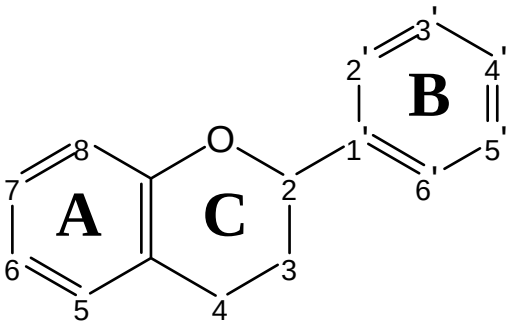


Figure 1.1 Basic structure and numbering system of flavonoids (Cook and Samman, 1996; Bravo, 1998; Vinson, 1998).

As mentioned above the A ring is biosynthesised by the condensation of three moles of malonyl-CoA derived from the metabolism of glucose. The C and B rings are also derived from glucose mechanism by way of the shikimate pathway and phenylpropanoid pathway, respectively to yield C-9 acids (e.g., cinnamic acid, hydroxycinnamic acid, and coumaric acid). As CoA derivatives these C-9 acids condense with the C-6 product from malonate to form a C-15 chalcone. Subsequent ring closure and hydratation give rise to the diverse flavonoids (Formica and Regelson, 1995).

In summary figure 1.2 shows a representation of the flavonoid biosynthesis according to Schwarz (1958), Formica and Regelson (1995), Rice-Evans et al (1996), and Rhodes (1998).

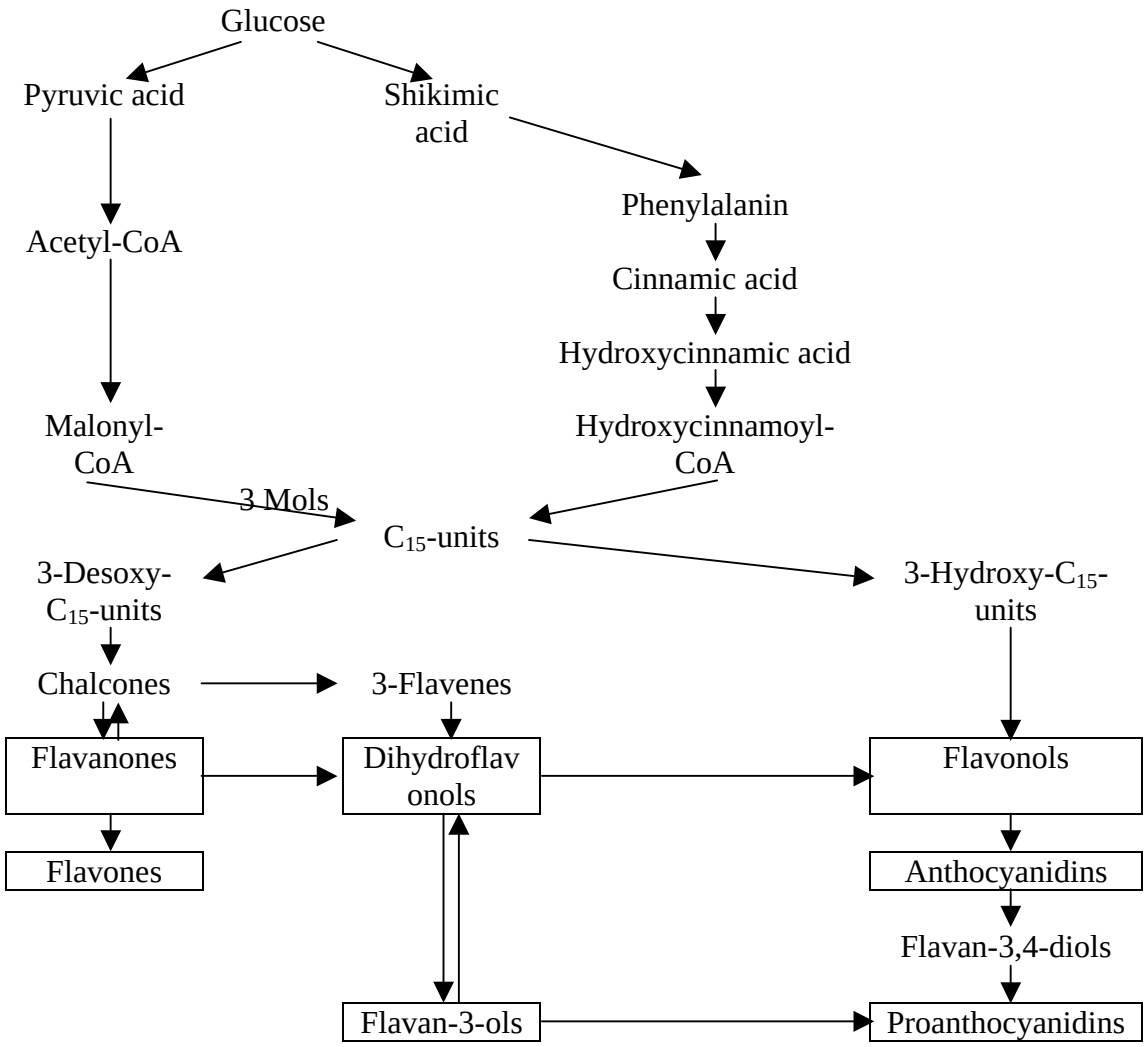


Figure 1.2 Schematic outline of the biosynthesis and interconnections of flavonoids (according to: Schwarz, 1958; Formica and Regelson, 1995; Rice-Evans et al., 1996; Rhodes, 1998).

Along with phenylpropanoids or hydroxycinnamic acid derivatives flavonols and to a lesser extent flavones are found in almost every plant. While flavanones and flavones are often found together (e.g., in citrus fruits) and are connected by specific enzymes, there is a certain mutual exclusion between flavones and flavonols in many plant families and anthocyanins are almost absent in flavanone-rich plants (Rice-Evans et al., 1996).

Flavonoids occasionally occur in plants as aglycones, although they are most commonly found as glycoside derivatives (Shahidi and Naczk, 1995; Bravo, 1998). The preferred glycosylation site is the 3 position and less frequently the 7 position. Glucose is the most usual sugar residue but others include galactose, rhamnose, and xylose (Rice-Evans et al., 1996). Furthermore, individual differences within each group of flavonoids result from the variation in number and arrangement of the hydroxyl groups with the most commonly occurring being those with dihydroxylation in the 3' and 4' positions (Rice-Evans et al., 1996).

Flavonoids, especially the flavan-3-ols catechin, epicatechin, gallocatechin, and epigallocatechin, are the monomeric constituents of the condensed tannins, although they are also very common as free monomers (Herrmann, 1995; Bravo, 1998; Kealey et al., 1998).

Anthocyanins are the most important group of water-soluble plant pigments and are responsible for the colour of flowers and fruits of higher plants. The term anthocyanin refers to the glycosides of anthocyanidin (e.g. malvidin, cyanidin). Anthocyanins and polymeric pigments formed from anthocyanins by condensation with other flavonoids are responsible for the colour of red wine (Bravo, 1998).

Unlike previously described groups of plant phenolics, tannins are compounds of intermediate to high molecular weight. Tannins with a molecular mass up to 30,000 Da have been found in carob pods. Tannins are highly hydroxylated molecules and can form insoluble complexes with carbohydrates and protein. This function of plant tannins is responsible for the astringency of tannin-rich foods, because of the precipitation of salivary proteins. The term "tannin" derives from the tanning capacity of these compounds in transforming animal hides into leather by forming stable tannin-protein complexes with skin collagen (Makkar et al., 1987; Makkar, 1989; Herrmann, 1994; Bravo 1998; Chung et al., 1998).

Plant tannins can be subdivided into two major groups: hydrolysable and condensed tannins. Hydrolysable tannins consist of gallic acid and its dimeric condensation product, hexahydroxydiphenic acid, esterfied to a polyol, which is mainly glucose. As their name indicates, these tannins are easily hydrolysed with acid, alkali, and hot water

and by enzymatic action yielding to polyhydric alcohol and phenylcarboxic acid (Herrmann, 1994; Bravo, 1998).

Condensed tannins or proanthocyanidins are high-molecular-weight polymers. The monomeric unit is a flavan-3-ol (e.g., catechin, and epicatechin) with a flavan-3,4-diol or leucoanthocyanidin molecule as its precursor. Oxidative condensation occurs between carbon C-4 of the heterocycle and carbons C-6 or C-8 of adjacent units. Most of the literature on the condensed tannin content refers only to oligomeric proanthocyanidins (dimers, trimers, and tetramers) because of the difficulty in analysing highly polymerised molecules. Proanthocyanidins, however, can occur as polymers with a degree of polymerisation of 50 and more. Autooxidative or enzymatic polymerisation of flavan-3-ol and flavan-3,4-diol units have been suggested as the process leading to the formation of condensed tannins (Herrmann, 1994; Romanczyk et al., 1997; Bravo, 1998; Kealey et al., 1998).

Interflavanoid linkages are acid labile and yield to anthocyanidins during acid hydrolysis in alcoholic solutions (e.g., HCl-butanol). This reaction is used for determination of proanthocyanidin molecules. If the sub-units consist only of catechin and epicatechin, cyanidin is the only resulting product of hydrolysis. Those proanthocyanidins are then called specifically procyanidins, however, this differentiation is not followed consistently in the literature. Phlobaphene-like substances are also formed when condensed catechins are heated in mineral acid solutions from the further polymerisation of these compounds (Rigaud et al., 1991; Escribano-Bailon et al., 1992; Prieur et al., 1995; Romanczyk et al., 1997; Bravo, 1998; Pascual Teresa et al., 1998).

Oligomeric proanthocyanidins are soluble in different aqueous and organic solvents, such as acetone and methanol. However, high-molecular-weight condensed tannins are insoluble. In addition, when tannins form complexes with protein or cell wall polysaccharides, they remain insoluble. This insolubility is responsible for significant errors in the quantification of the polyphenolic content of plants, because polyphenols are usually analysed in extracts, often omitting the quantification of insoluble or non-extractable tannins (Bravo, 1998).

1.3 Polyphenols in cocoa

Considerable investigation on cocoa polyphenols has been conducted since the early 50s due to their importance as flavour components (Forsyth, 1955). Only anthocyanins, catechins, procyanidins, and some phenolic acids have been positively identified in the unfermented violet seedlings of the fruit of *theobroma cacao*. The polyphenols in cocoa beans are stored in the pigment cells of the cotyledons. Depending on the amount of anthocyanins those pigment cells, also called polyphenol-storage cells, are white to deep purpur (Belitz and Grosch, 1992). The total amount of soluble polyphenols in the dried fat-free mass of fresh cocoa beans is 15 to 20% (equals approx. 6% in air-dried cocoa beans, containing 54% fat and 6% water) and approx. 5% in fermented beans, respectively (10% and more being considered a sign of a bad fermentation). These values are valid for Forastero beans, Criollo cocoa beans have approximately 2/3 of that amount of polyphenols (Lange and Fincke, 1970).

Since the 1950s it is well known that cocoa polyphenols consist mainly of the flavan-3-ols epicatechin, catechin and oligomeric and polymeric procyanidins (earlier: leucocyanidins) build-up of (epi)catechin subunits (figure 1.3) (Forsyth, 1955). Porter et al. (1991) and later Hammerstone et al. (1999) identified oligomeric procyanidins built up mainly of epicatechin sub-units. Hammerstone et al. (1999) described procyanidins with up to twelve subunits (dodecamer) in unfermented cocoa.

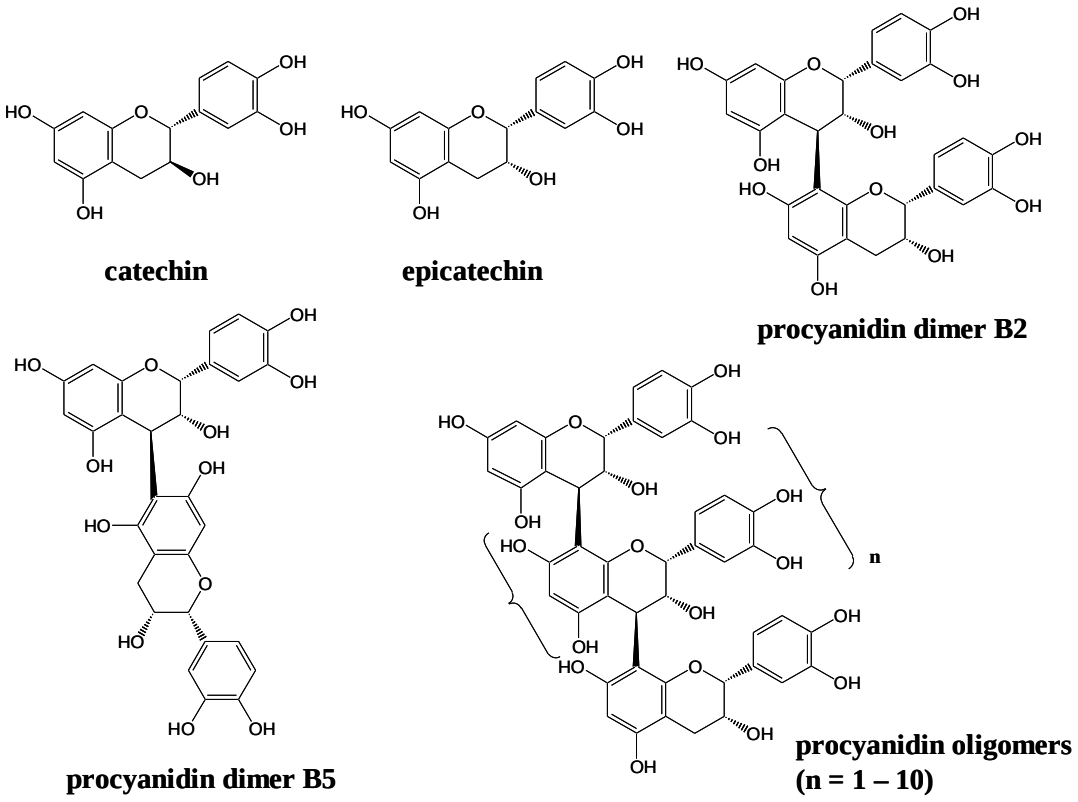


Figure 1.3 Major polyphenols found in *Theobroma cacao*.

Occurrence of trace amounts of gallocatechin and epigallocatechin (figure 1.4), two antioxidant catechins that are present in significant amounts in tea (Zeeb et al., 2000), has been proposed for cocoa as well (Forsyth, 1955).

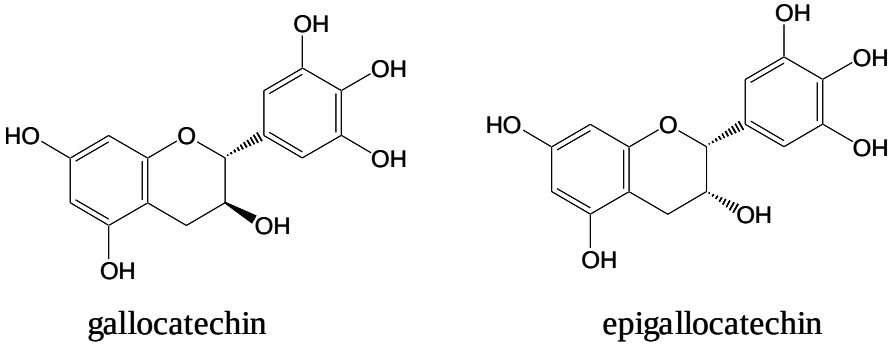
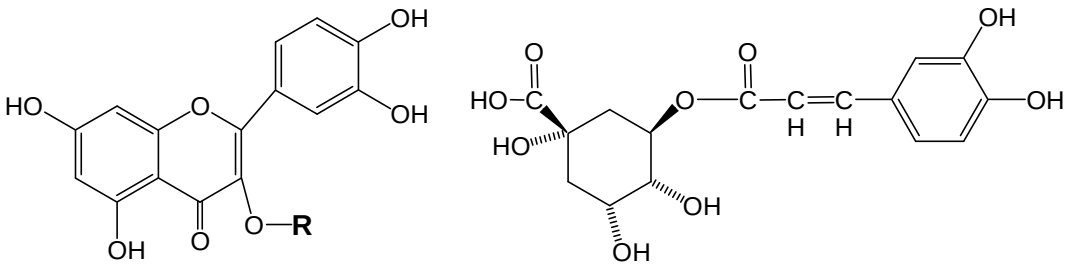


Figure 1.4 Additional catechins found in minor quantities in cocoa.

Jalal and Collin (1977) reported for the first time the presence of minor amounts of additional flavonoid glycosides being mainly quercetin derivatives and hydroxycinnamic acids, in particular chlorogenic acid (figure 1.5 right) in the seedling cotyledons. More than two decades later, quercetin aglykon as well as quercetin bound to arabinose, glucose and galactose (figure 1.5 left) have also been described in cocoa liquor and cocoa powder, respectively (Sanbongi et al., 1998; Lamuela-Raventos, 2001; Sanchez-Rabaneda et al., 2003). In addition, the presence of quercetin-glucuronide has been suggested by Andres-Lacueva et al. (2000).



quercetin chlorogenic acid

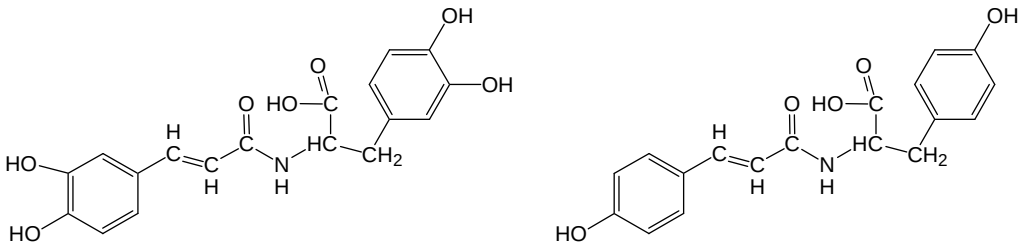
R = H, arabinose, glucose,
galactose, glucuronic acid

Figure 1.5 Other non-flavan-3-ol polyphenols found in cocoa.

Very recently, Sanchez-Rabaneda et al. (2003) reported some additional flavonoids so far never described in cocoa powder including naringenin, luteolin, apigenin and some glucosides of these compounds. Nevertheless, most of these compounds have been already isolated from the cotyledons of the cacao seedlings by Jalal and Collin (1977). Moreover, Sanchez-Rabaneda et al. (2003) needed to prepare highly concentrated and enriched fractions of extracts from cocoa powder, the more or less fat-free part of cocoa liquor containing the phenolic compounds, for having sufficient amounts of theses flavonoids in order to be able to detect them by liquid chromatography-tandem mass spectrometry, a highly specific and sensitive analytic technique. Thus, there are grounds

for the assumption that these compounds are present only in negligible amounts compared to the earlier described quercetin compounds.

In contrast, Sanbongi et al (1998) have isolated two additional hydroxycinnamic acid derivates in amounts comparable to quercetin glycosides. Such hydroxycinnamic acids linked to an amine function are common types of hydroxycinnamic acid conjugates and have been implicated in inducible defence systems of plants (Tebayashi et al., 2000). However, hydroxycinnamic acid amides with aromatic amino acids such as the compounds described by Sanbongi et al. (1998) have been less frequently described. These rosmarinic acid derivates isolated from cocoa liquor show also *in vitro* antioxidant activity similar to those of catechin or quercetin. N-(3',4'-dihydroxy-trans-cinnamoyl)-3-(3,4-dihydroxyphenyl)-L-alanine also called caffeoyl-DOPA or clovamide (trivial name) has shown to have slightly stronger antioxidant activity than its dideoxy analogue N-(,4'-hydroxy-trans-cinnamoyl)-3-(4-hydroxyphenyl)-L-alanine also called p-coumaroyltyrosine or dideoxyclovamide (Sanbongi et al., 1998). These two compounds have been previously described in red clover and the trivial name "clovamide" has been proposed for this origin of the compounds (Tebayashi et al., 2000). Figure 1.6. shows the chemical structures of clovamide and dideoxyclovamide.

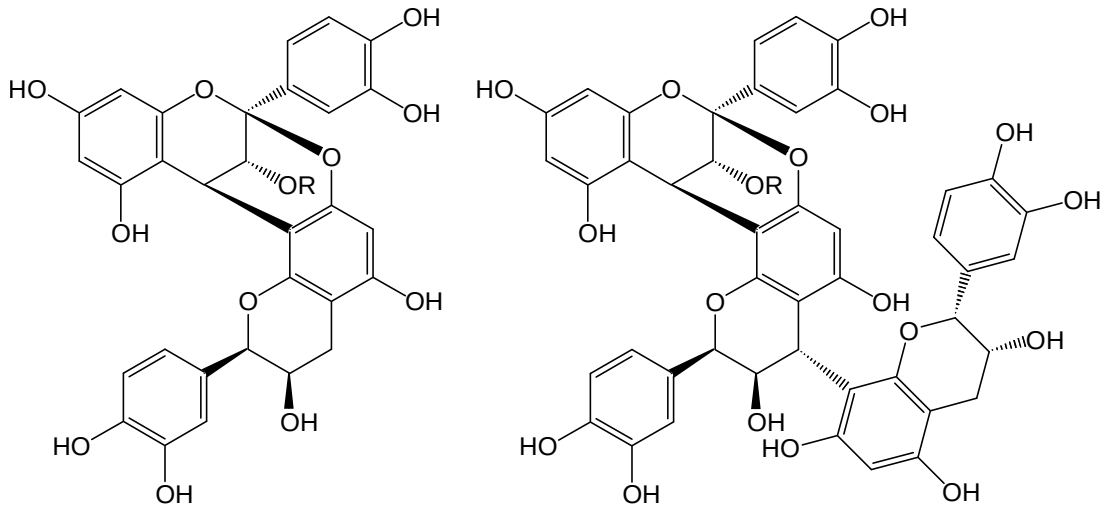


clovamide or caffeoyl-DOPA

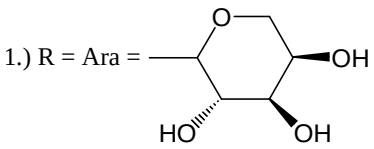
dideoxyclovamide or p-coumaroyltyrosine

Figure 1.6 Hydroxycinnamic acid amides isolated from cocoa liquor (Sanbongi et al., 1998).

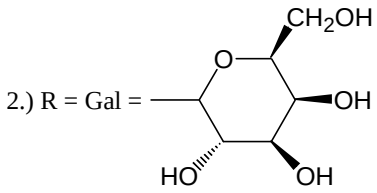
Finally, Porter et al. (1991) reported on three A-type procyanidin dimers two of which are linked to arabinose and galactose residuals, respectively, the third one being an aglykon. More recently, Hatano et al. (2002) isolated two trimeric procyanidin glycosides having each one A-type (double) and one B-type (single) linkage. As with the desribed A-type dimers one is linked to an arabinose moiety and the other to galactose. In addition to the dimeric A-type procyanidin arabinoside described by Porter et al., (1991), Hatano et al. (2002) propose a new epimeric isomer thereof.



R = H → aglykon



1.) R = Ara = → arabinoside



2.) R = Gal = → galactoside

Figure 1.7 Dimeric (left) and trimeric (right) A-type procyanidins identified in cocoa.

A-type procyanidins with their unusual second ether linkage between an A-ring hydroxyl function of the bottom unit to C-2 of the T-unit have received considerable attention recently. They are naturally occurring compounds that are proposed to derive from oxidative conversion of B-type procyanidins, both radical-mediated (Kondo et al., 2000) and catalysed by the enzyme polyphenol oxidase (Tanaka et al., 2000a). The conversion of flavan-3-ols and dimeric procyanidins from cocoa into A-type procyanidins has shown strong pH dependence with the original compounds being more stable under acidic conditions (Zhu et al., 2003). A-type procyanidins have shown antioxidant activity *in vitro* (Barreiros et al., 2000), inhibiting activity against hyaluronidase (Lou et al., 1999), an enzyme involved in chronic haemorrhagia and bronchitis as well as prevention of the adherence of Escherichia coli, the principal bacterial species responsible for urinary tract infection to uroepithelial cells (Ferreira and Slade, 2002).

The quantitative determination of polyphenols in chocolate and cocoa powder has been reported only very recently. These studies include the assessment of either a sum parameter for “total polyphenols” or the measurements of epicatechin and catechin, in some cases also B-type procyanidin contents. To the authors’ knowledge no quantitative values have been described for any of the other phenolic compounds isolated from cocoa and discussed above.

Using the colorimetric Folin-Ciocalteu method, Waterhouse et al. (1996) have found 8.4 mg polyphenols/g dark chocolate, 5.0 mg polyphenols/g milk chocolate and 20 mg polyphenols/g cocoa powder given as gallic acid equivalents whereas Vinson et al. (1999) found higher values (36.5 mg/g, 15mg/g, and 65 mg/g, respectively) using the same method but catechin as a standard and the results are referred to defatted dry weight of the samples. These values are high compared to the results obtained by Adamson et al. (1999) using high performance liquid chromatography (HPLC) for the quantification of catechins and procyanidins, milk chocolate having 0.7 mg polyphenols/g and dark chocolate having 1.7 mg polyphenols/g. Arts et al. (1999) using HPLC as well measured the content of catechins only and found dark chocolate having 0.5 mg/g and milk chocolate having about 0.2 mg/g, respectively. Richelle et al. (1999) reported higher levels of only epicatechin in dark chocolate (2 mg/g) but the employed

method has not been mentioned. Results from quantitative analysis of chocolates and cocoa powders are summarised in table 1.3.

Table 1.3 Polyphenol content (mg/g) in coca powder, dark chocolate, and milk chocolate according to the applied analytical procedure

Source	Extraction solvent	Analysis method	Quantity	Reference
Cocoa powder	95% aqueous methanol	Folin-Ciocalteau (gallic acid standard)	20 mg/g total polyphenols	Waterhouse et al. (1996)
Cocoa powders	Methanolic HCl	Folin-Ciocalteau (catechin standard)	65 ± 19 mg/g total polyphenols[a]	Vinson et al. (1999)
Cocoa powder	70% aqueous methanol	Folin-Ciocalteau (gallic acid standard)	Ca 58 mg/g total polyphenols	Serra Bonvehi et al. (1997)
Cocoa powders (instant)	70% aqueous methanol	Folin-Ciocalteau (gallic acid standard)	6.46 ± 2.44 mg/g total polyphenols	Serra Bonvehi et al. (1997)
Cocoa powders	Methanolic HCl	RP-HPLC (catechin standards)	2.96-3.27 mg/g catechin and epicatechin	Vinson et al. (1999)
Cocoa powder	75% aqueous acetone	RP-HPLC (epicatechin standard)	Ca 3 mg/g epicatechin	Serra Bonvehi et al. (1997)
Cocoa powder (instant)	75% aqueous acetone	RP-HPLC (epicatechin standard)	0.26 ± 0.05 mg/g epicatechin	Serra Bonvehi et al. (1997)
Dark chocolate	95% aqueous methanol	Folin-Ciocalteau (gallic acid standard)	8.4 mg/g total polyphenols	Waterhouse et al. (1996)
Dark chocolates	Methanolic HCl	Folin-Ciocalteau (catechin standard)	36.5 ± 5 mg/g total polyphenols[a]	Vinson et al. (1999)
Dark chocolate	70% aqueous acetone	Modified NP-HPLC (procyanidin standards)	1.7 ± 0.08 mg/g total procyanidins	Adamson et al. (1999)
Dark chocolate	70% aqueous methanol	RP-HPLC (catechin standards)	0.5 mg/g catechin and epicatechin	Arts et al. (1999)
Dark chocolate	Methanolic HCl	RP-HPLC (catechin standards)	0.48-1.37 mg/g catechin and epicatechin	Vinson et al. (1999)
Dark chocolate	70% aqueous acetone	Modified NP-HPLC (procyanidin standards)	0.8 ± 0.08 mg/g catechin and epicatechin	Adamson et al. (1999)
Dark chocolate	n.a.	n.a.	2 mg/g epicatechin	Richelle et al. (1999)
Milk chocolate	95% aqueous methanol	Folin-Ciocalteau (gallic acid standard)	5 mg/g total polyphenols	Waterhouse et al. (1996)
Milk chocolate	Methanolic HCl	Folin-Ciocalteau (catechin standard)	15 ± 5.8 mg/g total polyphenols[a]	Vinson et al. (1999)
Milk chocolate	70% aqueous acetone	Modified NP-HPLC (procyanidin standards)	0.7 ± 0.17 mg/g total procyanidins	Adamson et al. (1999)
Milk chocolate	70% aqueous methanol	RP-HPLC (catechin standards)	0.16 mg/g catechin and epicatechin	Arts et al. (1999)
Milk chocolate	Methanolic HCl	RP-HPLC (catechin standards)	0.15-0.16 mg/g catechin and epicatechin	Vinson et al. (1999)
Milk chocolate	70% aqueous acetone	Modified NP-HPLC (procyanidin standards)	0.2 ± 0.05 mg/g catechin and epicatechin	Adamson et al. (1999)

[a] results are referred to defatted dry samples

Chocolate is not a standardised product, however, considering the great discrepancy, it is obvious that the reported results depend very much on the employed method for quantification and that a standardised procedure would be desirable. It is striking that results obtained by the colourimetric method are generally much higher than results obtained by HPLC methods. Additionally, observed differences may be explained by different amounts of fat free cocoa solids, the polyphenol containing part of the cocoa beans in different types of cocoa products as well as in the recipe of the same product. Finally, the raw material may be subject to changes in polyphenol content and/or pattern during the various steps of processing that cocoa undergoes from the harvesting of the fruit to the packaging of the final products such as chocolate, cocoa powder or other chocolate confectioneries.

In fact it is well known that polyphenol composition changes during fermentation (Kim and Keeney, 1984; Pettipher, 1986). During fermentation of cocoa beans polyphenols diffuse with cell liquids from their storage cells and undergo oxidation to condensed high molecular mostly insoluble tannins. These reactions are both non-enzymatic and catalysed by the enzyme polyphenol oxidase, even though this enzyme is strongly inactivated during the first days of fermentation, remaining only 50% and 6% of enzyme activity after 1 and 2 days, respectively (Hansen et al., 1998). The occurrence of condensation reactions is confirmed by the sharp decrease of epicatechin content between the second and third day of fermentation. Epicatechin and soluble polyphenol content, respectively, is reduced to approx. 10% to 20% during fermentation. This is not only due to the oxidation process but also caused by diffusing of polyphenols into fermentation sweatings (Bracco et al., 1969; Biehl, 1973; Kim and Keeney, 1984; Herrmann, 1995; Hansen et al., 1998).

Polyphenol oxidase is also sensitive to drying so that remaining enzyme activity after fermentation and drying of beans is only about 2%. It is believed that also non-enzymatic oxidation of polyphenols could be important during the drying process (Hansen et al., 1998). It has been shown that 2 days of sun-drying of fresh unfermented cocoa beans alone (without fermentation) causes a 50% decrease in epicatechin content (ca 22 instead of approx. 40 mg/g defatted sample). Thus, to investigate the epicatechin content of fresh cocoa beans, these had to be freeze-dried immediately after removal from the pods. Most beans for chocolate manufacture are fermented, however, it is far

from being a standardised process throughout the world, or even within a region. This is evidenced by a 6-fold variation in epicatechin concentration of 10 samples of fermented cocoa beans from different regions (Kim and Keeney, 1984).

During the fermentation process anthocyanins are hydrolysed to anthocyanidins. The latter compounds polymerise along with simple catechins to form complex tannins. Anthocyanins usually disappear rapidly during fermentation process (93% loss after 4 days) and colour of the beans changes from slaty over purple to brown (Kealey et al., 1998). Thus, anthocyanin content has been considered as a good index for determination of the degree of cocoa bean fermentation (Lange and Fincke, 1970; Pettipher, 1986; Shahidi and Naczk, 1995).

After the shipment of fermented cocoa beans from the countries of origin to the user countries, alteration in content and composition of polyphenol compounds in the process of chocolate manufacturing, preferable during roasting, grinding, refining, and conching where rather high temperatures are achieved and air (oxygen) is present, must be anticipated due to the high redox-activity of polyphenols. However, information on such changes in the scientific literature is virtually absent (Wollgast and Anklam, 2000a). Only in a patent application (Kealey et al., 1998) such changes have been reported in relation to process parameters. Generally, higher processing temperatures and/or longer processing times reduce the amount of catechins and procyanidins available in cocoa components. If an alkalising step is present in the process, this also leads to a remarkable decrease in the contents of catechins and procyanidins.

1.4 Analytical methodologies for the analysis of polyphenols in chocolate and chocolate raw products

Recently, we published a review that included the available methodology for analysis, quantification, isolation, purification, and structure elucidation of polyphenols in cocoa components and other commodities (Wollgast and Anklam, 2000a). The aspects considered include sample preparation, extraction procedure and suitable solvent systems for extraction, simple quantitative screening assays including colorimetric and polyphenol-protein-precipitation methodologies, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) coupled to a variety of detection systems, capillary electrophoresis (CE), quantitative considerations in particular with respect to HPLC coupled to ultraviolet (UV) detection, polyphenol isolation procedures, as well as structure analysis of polyphenols by degradation techniques and instrumental techniques including UV, mass spectrometry, nuclear magnetic resonance (NMR) and infrared-red (IR) spectroscopy. Finally, an overview of the procedures used in the literature for analysis, isolation, purification, identification and quantification of polyphenols in cocoa products and in other sources with similar polyphenol composition, namely rich in procyanidins is given in a tabular form (Wollgast and Anklam, 2000a). In addition, other reviews have been recently published on the analysis of condensed tannins (Schofield et al., 2001), on the analysis of tea catechins (Dalluge and Nelson, 2000), and on polyphenols analysis in plant material in general (Merken and Beecher, 2000).

Thus, in this chapter, the focus is put on the techniques applied in the experimental part of this work to cocoa polyphenol identification and quantification in chocolate and chocolate raw products. These include: sample extraction techniques, techniques for isolation and purification of polyphenols from food or plant matrices, the three most widely applied colorimetric methods for fast quantitative estimation of total polyphenols and procyanidins, respectively, a methodology for estimation of fibre and protein bound polymeric proanthocyanidins, techniques for qualitative analysis including an updated and extended review on the less common application of normal-phase HPLC, HPLC coupled to mass spectrometry (HPLC-MS) along with considerations on HPLC-MS for quantitative analysis as well as CE, in particular in the

form of micellar electrokinetic (capillary) chromatography (MEKC or MECC) as emerging techniques for polyphenol analysis.

1.4.1 Sample extraction techniques

Extraction from solid plant material is almost exclusively done manually with some mechanical treatment, such as ultrasonication, maceration or other means of homogenisation with the extraction solvent (Wollgast and Anklam, 2000a). Although instrumental extraction techniques are available allowing reduced solvent consumption and extraction time including microwave assisted extraction (MAE), pressurised liquid extraction (PLE), and supercritical fluid extraction (SFE) these have hardly been used for extraction of polyphenols from foodstuffs or other plant material (Buldini et al., 2002; Tura and Robards, 2002).

In a comprehensive review of the applications of MAE (Eskilsson and Bjorklund, 2000) no papers relating to plant phenols were reported. MAE in closed vessels usually applies elevated temperature and pressure resulting in enhanced recoveries and reduced extraction time (Tura and Robards, 2002). However, polyphenols are thermolabile compounds and consequently degradation, especially of the very temperature sensitive procyanidin oligomers even at very short extraction times must be envisaged. Moreover, cocoa polyphenol extraction usually comprises two steps, which in case of chocolate and cocoa would have to be done in two separate runs on MAE: extraction of fat (cocoa butter) with hexane or petroleum ether and subsequent extraction of polyphenols with aqueous organic solvent.

Modern instrumentation for PLE usually allow for single or multiple step extraction without need for operator intervention (Buldini et al., 2002). However, for good extraction efficiency, pressurised solvent temperature is normally kept between 80 and 200 °C, which is not compatible with thermolabile cocoa polyphenols.

In SFE the supercritical fluid being most likely carbon dioxide can be kept at somewhat lower temperatures (40 to 60 °C) suggesting the best compatibility with thermolabile polyphenols. However, the use of a more polar organic modifier such as methanol together with CO_2 was mandatory for the extraction of polar polyphenols including benzoic and cinnamic acids as well as catechin while recoveries still only ranged

between 30 and 70% (Tura and Robards, 2002). Recoveries of even more polar cocoa polyphenols, such as quercetin glycosides and particularly procyanidins, are likely to be even lower.

1.4.2 Techniques for isolation and purification of phenolic compounds from natural products

Many of the naturally occurring polyphenols in cocoa and other food commodities are not commercially available for unambiguous identification and use as reference for quantification. Moreover, there might be unknown or only tentatively identified substances that have been observed by analytical procedures such as HPLC-MS (Wollgast et al., 2001). Consequently, those substances have to be isolated and prepared in sufficient purity and quantity for subsequent structure elucidation including various techniques of partial and total hydrolysis, ultraviolet absorbance spectra, mass spectrometry, nuclear magnetic resonance spectroscopy etc.

Polyphenols and in particular proanthocyanidins have been isolated from cocoa (Porter et al., 1991, Sanbongi et al., 1998; Adamson et al., 1999; Hatano et al., 2002) as well as other plant matrices including grape seeds (Ricardo da Silva et al., 1991), unripe almond fruits (de Pascual-Teresa et al., 1998), peanut skins (Lou et al., 1999), litchi pericarp (Le Roux et al., 1998), apple (Shoji et al., 2003), and tea (Lakenbrink et al., 1999) by combinations of various techniques. These include liquid-liquid extraction (methyl-acetate, ethyl-acetate, n-butanol), open column chromatography (CC) for clean-up and crude fractionation (Diaion HP 2MG and HP-20, Polyamide, Silica), size exclusion chromatography (Sephadex LH-20, Toyopearl/Fractogel TSK HW-40, MCI-Gel CHP 20-P), thin-layer chromatography (cellulose, silica), and final purification by semi-preparative normal- and reversed-phase high-performance liquid chromatography. The necessity of multiple chromatographic separations in combination with irreversible absorption of higher polymeric procyanidins to solid phase materials makes these isolation and purification procedures both time-consuming and expensive. Adamson et al. (1999) used for example a costly preparative scale (50 x 2 cm) high-performance liquid chromatography column for normal-phase final purification of procyanidin oligomers from cocoa. Moreover, very high consumption of hazardous methylene chloride (more than 5 litres per separation) presents a strong environmental burden. Recently, new method developments for size exclusion chromatography (SEC)

(Yanagida et al., 1999) and countercurrent chromatography (CCC) (Degenhardt et al., 2000; Shibusawa et al., 2000; Shibusawa et al., 2001) have demonstrated to be relatively simple, rapid, inexpensive as well as capable of isolating pure compounds on a preparative scale. In particular high-speed countercurrent chromatography (HSCCC) has proven a valuable alternative to preparative HPLC as it avoids irreversible adsorption (Shibusawa et al., 2000; Shibusawa et al., 2001) and artifact formation (Degenhardt et al., 2000) of analytes due to the use of inert Teflon tubing and the absence of a solid phase. Moreover, load capacity is usually superior to that of preparative HPLC (Degenhardt et al., 2000). Nevertheless, both SEC and HSCCC are limited to the isolation of procyanidins up to the trimer with insufficient resolution of higher oligomers. On the other hand, Rohr (1999) reported on very high instability of oligomeric procyanidins with more than three sub-units hampering the practicability of these compounds as quantitative reference.

1.4.3 Colorimetric methods for polyphenol analysis

Three of the most commonly applied colorimetric methods for the quantitative estimation of polyphenols in food or plants include the Folin-Ciocalteu, Prussian-Blue, and Vanillin-HCl assays. All methods produce quantitative estimates of total polyphenol (Folin-Ciocalteu and Prussian-Blue) and total flavan-3-ol (Vanillin-HCl) content, respectively, given as equivalents of one standard phenolic compound, mostly gallic acid or catechin. However, no information on single phenolic compounds can be obtained (Wollgast and Anklam, 2000a).

Briefly, both the Folin-Ciocalteu and the Prussian-Blue assays are based on redox reactions. For the latter a reduction of ferric to ferrous ions is followed by the formation of the deep blue hexacyanoferrate-(II)-chelate (Price and Butler, 1977, Budini et al., 1980). In the Folin-Ciocalteu assay complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids are reduced by phenolic (and other reducing) compounds to complex molybdenum-tungsten blue (Singleton and Rossi, 1965; Rohr, 1999). Thus, the colour yield in both assays depend on the redox potential of the reference standard used as well as the phenolic compounds and other interfering cocoa matrix compounds such as aromatic amines, carbohydrates or Maillard reaction products formed during chocolate manufacture processing. Consequently,

quantitative results have to be interpreted carefully and should be seen as total polyphenol estimate rather than total polyphenol content, particularily when comparing results from different matrices or from different assays in different laboratories (Rohr, 1999).

In contrast to the former two assays the Vanillin-HCl assay is specific for flavan-3-ols, dihydrochalcones and proanthocyanidins which have a single bond at the 2,3-position and possess free metahydroxy groups on the B-ring (Butler et al., 1982). The method is based on the condensation of protonated vanillin forming an electrophilic carbocation in acidic solutions with the nuclephilic sites of the phenolic compounds mentioned above (figure 1.8) (Shahidi and Naczk, 1995).

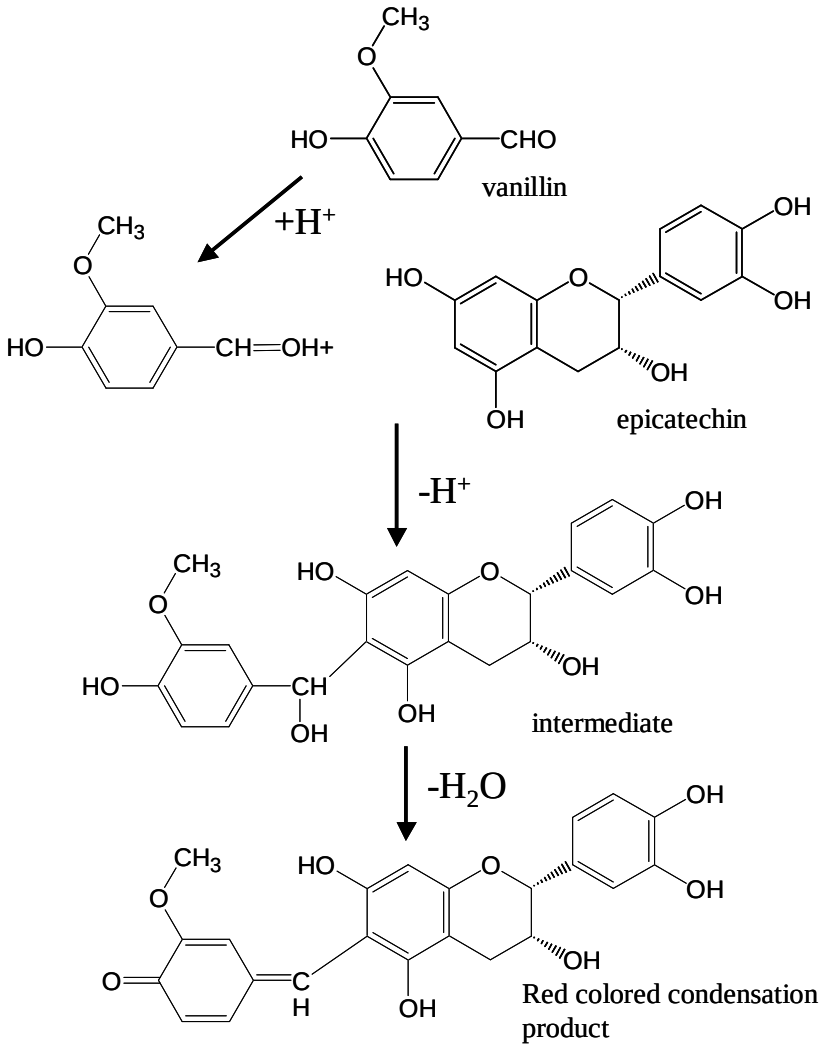


Figure 1.8 Condensation reaction of epicatechin with vanillin under acidic conditions (adapted from Shahidi and Naczk, 1995).

In substituted benzaldehydes like vanillin (4-hydroxy-3-methoxybenzaldehyde) the electrophility is reduced by the delocalisation of the positive charge and therefore reactions only occur with phenolic compounds which show a phloroglucinol- or resorcinol-type oxygenation pattern (Rohr, 1999). However, differences in molar absorption coefficients between catechin and its epimers have been observed, chromophore production is more complex in oligomeric and polymeric proanthocyanidins and is neither stoichiometric on a weight nor on a molar basis and the reaction kinetic depends on the chemical structure of the polyphenol (Butler et al., 1982; Rohr, 1999). Thus, as with the former two colorimetric assays, interpretation of quantitative results for cocoa flavan-3-ols and procyanidins should be done carefully. Nevertheless, despite of the discussed limitations of theses assays, for the purpose of a quick screening of polyphenol content in various cocoa matrices, for the set-up of sample extraction procedure and as simple tool for quick estimation of polyphenol content of raw material for cocoa processing and process control. these simple to conduct assays still represent valuable quantitative tools when interpreted appropriately.

1.4.4 Non-extractable procyanidins through hydrolysis with butanol-hydrochloric acid (proanthocyanidin assay)

It is well known that, despite all efforts, proanthocyanidins cannot be extracted exhaustively from plant material (Rohr, 1999). After proanthocyanidin extraction from tropical forages with 70% acetone the reported fraction of insoluble proanthocyanidins varied from 6 to 20% (Schofield et al., 2001). The acid-butanol assay (synonyms: butanol-hydrochloric acid-assay, proanthocyanidin assay) has been proposed as a method for estimating the amount of matrix-bound proanthocyanidins (Porter et al., 1986). The proanthocyanidin assay uses an acid-catalysed oxidative depolymerisation of condensed tannins (proanthocyanidins) to yield red anthocyanidins (figure 1.9). As the resulting anthocyanidins, mostly being either cyanidin or delphinidin, depend on the catechin units of the condensed tannin, this assay has been used for identification of the polyflavan structure (Schofield et al., 2001).

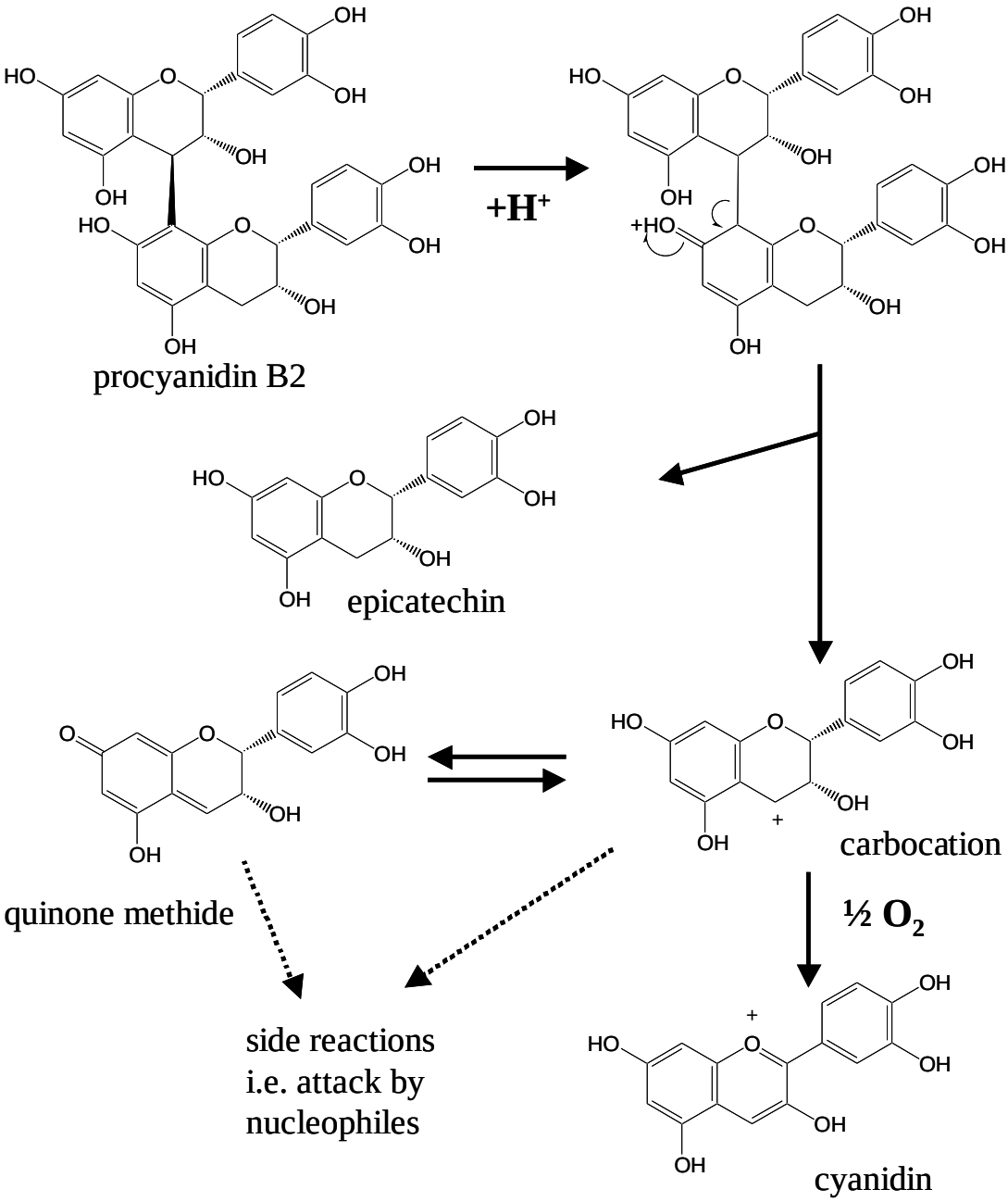


Figure 1.9 Reaction sequence of procyanidin B2 in the proanthocyanidin assay.

According to Schofield et al. (2001) the assay's greatest strength lies in the qualitative confirmation of a polymeric interflavan structure. Indeed, it has efficiently been applied as one element in structure elucidation of polymeric proanthocyanidins in grape seeds (Ricardo da Silva et al., 1991), cider apple skin and pulp (Guyot et al., 1997), litchi pericarp (Le Roux et al., 1998) as well as unripe almond fruits (de Pascual-Teresa et al.,

1998). Nevertheless, the method has also been used in slightly modified forms for the quantitative estimation of non-extractable protein- and fibre-bound condensed tannins in carob pods (Saura-Calixto, 1988), forage plants, protein concentrate meals and cereal grains (Terrill et al., 1992) as well as grape pomace (Bravo and Saura-Calixto, 1998). However, Makkar et al. (1999) suggested that not all bound condensed tannins react quantitatively in the acid-butanol assay and may thus be underestimated. Also Rohr (1999) mentioned the presence of side reactions resulting in a mixture of not well-defined oxidation products with an UV-absorbance maximum at around 450 nm leading to underestimation of bound proanthocyanidins. Moreover, the amount of side reactions appear to depend on the type of plant matrix and the type of condensed tannin as well as the critical control of reaction conditions in the assay (Rohr, 1999).

1.4.5 Normal-phase-high-performance liquid chromatography

Rigaud et al. (1993) developed an HPLC method using a normal-phase (NP) silica column and a gradient of dichloromethane into methanol with constant 4% of formic acid-water (1:1) mixture as the eluent to separate procyanidins on a molecular mass basis without derivatisation. This method was repeatedly applied to the analysis as well as for semi-preparative and preparative HPLC for isolation, purification, and subsequent structure analysis of procyanidin extracts from cocoa (Romanczyk et al., 1997, Kealey et al., 1998; Adamson et.al., 1999; Hammerstone et al., 1999; Hammerstone et al., 2000; Natsume et al., 2000) and other food commodities (Cheynier et al., 1998; Lazarus et al. 1999, Kennedy and Waterhouse, 2000; Yanagida et al., 2000a; Yanagida et al., 2000b; Guyot et al., 2001; Lazarus et al., 2001). In addition, preparative normal-phase HPLC has been successfully applied for isolation and purification of oligomeric and polymeric procyanidins according to their molecular weight and has proved particularly beneficial in combinations with other chromatographic techniques such as size exclusion chromatography (SEC) or semi-preparative reversed-phase HPLC (Guyot et al., 1997; Le Roux et al., 1998; Adamson et al., 1999; Cheynier et al., 1998).

Nevertheless, in normal-phase HPLC the epimeric pairs catechin and epicatechin or procyanidin dimers B2 and B5 as reported by Porter et al. (1991) cannot be resolved in contrast to reversed-phase HPLC (Hammerstone et al., 1999; Natsume et al., 2000). Moreover, separation or identification of other more or less minor phenolic compounds

such as quercetin and quercetin glycosides or clovamides in cocoa have not been reported when normal-phase HPLC was applied to cocoa (Romanczyk et al., 1997, Kealey et al., 1998; Adamson et.al., 1999; Hammerstone et al., 1999; Hammerstone et al., 2000; Natsume et al., 2000). In addition, an important drawback of this method is the use of dichloromethane as main solvent compound, a highly toxic chemical. In fact, chlorinated and fluorinated carbons are banned by the Montreal Protocol on substances that deplete the ozone layer (Montreal Protocol, 2000).

1.4.6 Liquid chromatography with mass spectrometric detection

Comprehensive information on electron impact (EI) mass spectrometry fragmentation patterns is available for the major classes of aglycone flavonoids. Only when the individual flavonoids are subjected to EI-MS analysis can their fragmentation be assigned and distinguished (Lin et al., 1993b). EI-MS is commonly coupled to gas chromatography (GC), however, polyphenols are usually non-volatile thermolabile compounds that have to be transferred to their trimethylsilyl or permethyl derivates prior to GC-MS. LC-MS with moving belt has been used to study flavonoids under EI conditions but has been hampered by difficulties with high background contribution and diminution of the molecular ion, especially with highly polar non-volatile compounds (Games and Martinez, 1989).

Fast-atom bombardment mass spectrometry (FAB-MS) (Porter et al., 1991; Ricardo da Silva et al., 1991) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (Ohnishi-Kameyama et al., 1997; Yanagida et al., 2000) have both been successfully applied to the identification of polyphenols. Whereas polyphenols or polyphenol-rich fractions had to be isolated and prepared for off-line FAB-MS or MALDI-TOF-MS analysis first, HPLC coupled on-line to thermospray mass spectrometry (Kiehne et al., 1997) and tandem mass spectrometry (MS-MS) (Lin et al., 1993b), respectively, has been used for the direct analysis of polyphenols from tea extracts.

However, only with the recent developments of interfacing and ionisation technologies for liquid chromatography, particularly the ionisation in atmospheric pressure (API) including atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI), respectively, as well as advances in computer technologies, mass spectrometry

(MS) coupled to HPLC has become a powerful tool in online detection and identification (Willoughby et al., 1998; Wollgast et al., 2001). In an API-MS system, the ion source region, located outside the mass spectrometer at ambient pressure, is separated from the high-vacuum mass analyser by a small ion sampling orfice and the LC effluent is sprayed in the vicinity of this orfice (Aramendia et al., 1996). ESI as a soft ionisation technique producing only pseudomolecular ions with almost no fragmentation has become the technique of choice for the analysis of polyphenols by HPLC-MS (e.g., Justesen et al., 1998; Andlauer et al., 1999; Friedrich et al., 2000), although APCI has also been applied for polyphenol analysis in tea (Zeeb et al., 2000).

The modified normal-phase HPLC method described in section 1.4.4 has been applied to the identification of procyanidins in chocolate and fresh cocoa beans, respectively, coupling the HPLC system to a quadrupole mass analyser with an ESI interface (Hammerstone et al., 1999). Catechin monomers and procyanidin oligomers have been identified due to the mass-to-charge ratio (m/z) of their single or multiple-charged pseudomolecular ions in the negative ion mode. However, neither the HPLC separation nor the MS allowed for the determination of stereoisomers, such as catechin and epicatechin both having the same retention time and mass-to-charge ratio of the pseudomolecular ion. In addition, the identification of compounds only due to their corresponding pseudomolecular ions can be difficult in the presence of interfering matrix compounds showing the same mass-to-charge ratios of their respective pseudomolecular ions. As a consequence the sample preparation still required a solid phase extraction (SPE) step to remove interfering sugars. Moreover, the analysis time of 70 minutes per sample is rather long and the method requires the use of toxic chlorinated solvents that are considered to be an ecological hazard (see discussion in section 1.4.4).

Very recently, Gu et al. (2003) applied both normal-phase- and reversed-phase HPLC-MS to the screening for proanthocyanidins in different kinds of foods. Thirty-nine foods including fruits, cereals, beans, nuts, beverages, spices, and one vegetable out of eighty-eight selected food items were found to contain proanthocyanidins. Oligomeric and polymeric procyanidins both of the B-type and A-type were identified by tandem-mass spectrometry (MS-MS) and thiolytic degradation for confirmation demonstrating the

potential of the former technique for fast and sensitive identification of phytochemicals in foodstuff and plant material (Gu et al., 2003).

1.4.7 Liquid chromatography-mass spectrometry for quantitative analysis

Colorimetric methods are commonly used for quantitative estimations of total polyphenol content and proanthocyanidins in cocoa (Waterhouse et al., 1996;Vinson et al., 1999) and other food commodities (Schofield et al., 2001). However, for the more accurate analysis of single compounds more sophisticated analytical techniques have to be employed, such as HPLC or CE coupled to various detection systems including ultraviolet, fluorescence, electrochemical and mass spectrometric detection systems.

Nevertheless, there have been discussions on the applicability of LC-MS for quantitative use (Willoughby et al., 1998). In fact, Justesen et al. (1998) and Andlauer et al. (1999) preferred HPLC with photo-diode array detection and Adamson et al. (1999) HPLC coupled to fluorescence detection, respectively, for determination of polyphenol contents in food. Both groups used mass spectrometry solely for compound identification purpose. Compared to mass spectrometric detection methods using ultraviolet or fluorescence detection have been reported to be more sensitive and to cover wider ranges in detector linearity often combined with higher precision (Huck et al., 2002). Moreover, co-eluting matrix compounds can lead to hardly predictable ion-suppression or ion-enhancement effects in the ion source of the mass analyser and the ionisation process is also sensitive to changes in mobile phase conditions, all of which can result in poor accuracy (Zoellner et al., 1999; Choi et al., 2001).

Nonetheless, modern LC-MS instrumentation with atmospheric pressure ionisation (API) techniques, mostly atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI), in combinations with recent technical developments in LC-MS related interfacing, ionisation and mass analysis offer improved robustness and ease of use for wide application both in research and routine quantitative analysis (Niessen, 2003). Due to its high degree of selectivity LC-MS allows fast development of relatively short analytical methods combined with reduced requirements on sample preparation allowing high throughput methodologies also in more complex matrices (Zeng et al., 2003). Moreover, tandem-mass spectrometry (MS-MS) providing the

highest degree of certainty in analyte identification may be employed in accordance with recent European guidelines (European Commission, 2002) to obtain data with relevant unambiguity slowly replacing more laborious and time-consuming GC-MS methods (Zoellner et al., 2003).

1.4.8 Capillary electrophoresis as alternative to high performance liquid chromatography

The vast majority of methods for the analysis of polyphenols in food described in literature is based on reversed-phase high-performance liquid chromatorgraphy due to the high resolution, high efficiency, high reproducibility, and relatively short analysis time without the need for derivatisation and no restriction on sample volatility. HPLC is also easily coupled to a variety of detectors (Lee and Widmer, 1996; Markham and Bloor, 1998; Merken and Beecher, 2000; Wollgast and Anklam, 2000a).

Capillary electrophoresis (CE) is a refinement of traditional electrophoresis in which separating power has increased to an extent up to 120,000 theoretical plates and thus exceeding that of HPLC by up to 65 times (Lee and Widmer, 1996; Markham and Bloor, 1998). This efficient separation capacity is related to the use of high separation voltages and efficient dissipation of Joule heat in a narrow fused-silica capillary, typically having inner diameters (I.D.) of 25-100 µm (Rodriguez-Delgado et al., 2000). The capillary tube is filled with a buffer and its ends are in buffer-filled reservoirs containing electrodes. The sample is applied by various means to one end of the tube and a tension voltage applied between the electrodes. Either positively or negatively charged species can be selected by changing electrode polarity. Neutral components travel together without separation through the capillary with the electro-osmotic or electro-endosmotic flow (EOF) of the buffer, a bulk flow induced by the potential difference between electrodes leading to a surface charge of the capillary (Lee and Widmer, 1996). Charged sample molecules are separated as they migrate through the capillary (against the flow) determined essentially by the charge-to-mass ratio of the molecule (Morin et al. 1997).

Thus, flavonoids not carrying a charge must be ionised by use of a suitable buffer. Borate buffers with a pH of 8-11 and a concentration of 25-200 mM are commonly used. Apart from the suitable pH range of these buffers, sodium borate can form

complexes with ortho-dihydroxyl groups on the flavonoid nucleus and with vicinal cis-dihydroxyl groups on sugar residues (Larger et al., 1998). Thus, the separation is determined by the mass-to-charge ratio of the deprotonated polyphenols or through differences in borate-phenol association (Vaher and Koel, 2003). In alkaline conditions the resolution generally increases with the increasing concentration of complex-forming buffer (Morin et al., 1997).

Separation of compounds that are not readily ionised or are hydrophobic may be effectively handled using micellar electrokinetic (capillary) chromatography (MEKC or MECC), a technique first introduced by Terabe and Co-workers (Terabe et al., 1984; Terabe et al., 1985). In addition, MEKC is often used to improve the separation of ionic compounds as well (Morin et al., 1997). In MEKC a surfactant, often sodium-dodecyl sulphate (SDS), is introduced to produce charged micelles in which the flavonoid migrates. In the majority of separations the very large EOF causes both the flavonoid and the micelles to be driven toward the cathode. The extent of this effect is determined by molecular size with smaller molecules being faster and thus, the micelles migrate slower than the flavonoid and both slower than the water molecules (i.e., the EOF).

In addition, flavonoid anions, which are produced by the alkaline pH of the buffer, as well as negatively charged micelles, are also attracted to the anode, with the strength of this attraction being determined by the degree of ionisation. Moreover, flavonoids migrate into the hydrophobic micelles according to their own hydrophobicity and thus, move to a certain amount together with the micelles at slower mobility rates. The separation is achieved due to differential partitioning of the analyte into the electrophoretically driven micelles of SDS added to the background electrolyte (Breadmore et al., 1999). When using borate as background electrolyte this partitioning is influenced by the presence of complexes between flavonoids and borate anions (Pietta et al., 1994). The increased size should facilitate its partitioning into the micelle, although this will be opposed to some degree by electrostatic repulsion between the negatively charged micelles and complexes (Larger et al., 1998).

Since in most cases all compounds move towards the cathode with the EOF, in MEKC the separation of the analytes takes place in a so-called micellar window, i.e. between the electrophoretic mobility of the EOF (water) and the micelles. This window and with

it the theoretical plates for separation can be increased by reducing the EOF through decreased pH, dynamic or permanent coating of the capillary surface or the presence of organic modifiers, such as acetone, methanol, acetonitrile etc. (Issaq, 1997; Issaq 1999; Melchior 2001)

Thus, the balance of these effects determines the net rate at which flavonoids migrate along the capillary column to the cathode. Consequently, MEKC method development and optimisation lies in finding that combination of mainly pH, background electrolyte concentration, surfactant concentration, and organic modifier type and concentration for achieving sufficient separation with acceptable analysis time. Other parameters include applied voltage, capillary inner diameter, and temperature, although these parameters have little influence on separation efficiency (Issaq, 1997).

MEKC as a well-suited technique complementary to HPLC offers the advantage of relatively short analysis times in combination with good resolution of compounds. In addition and unlike HPLC, only tiny amounts of organic solvent are consumed thereby reducing costs and environmental burden. Direct injection of complex matrices, such as food extracts or biological fluids, is possible with CE in general without the risk of column obstruction (Torres-Lapasio et al., 2000).

The use of CE or MEKC for preparative isolation and purification of polyphenols is possible (e.g., Ng et al., 1992). However due to the very small amount of sample introduced to the capillary (usually 1 to 50nl) this is very tedious and usually semi-preparative HPLC is preferred.

The sensitivity of CE methods is lower by a factor of 5 to 20 compared to HPLC methods for the same reason of injection of small amount of sample (Mellenthin and Galensa, 1999). This can be partly compensated by several techniques of on-capillary sample enrichment or the use of extended light capillaries in the UV-detection part of the capillary (Lee and Ong, 2000; Chen et al., 2003; Starkey et al., 2002).

Besides UV-DAD, which covers the vast majority of CE detection systems, laser-induced fluorescence detection (LIF) as well as more recently mass spectrometry and rarely even nuclear magnetic resonance have been coupled to CE (Spikmans et al. 2000). Although the hyphenation of CE with mass spectrometry (CE-MS) is desirable

owing to the high selectivity for structure elucidation, and technically this is also possible nowadays, the range of applications is still rather limited. This might reflect the short renaissance period (Spikmans et al., 2000). However, with mass spectrometric detection the use of volatile buffer systems is preferred in order to avoid signal suppression caused by the ionisation of non-volatile buffers such as phosphate or borate (Aramendia et al., 1995, Lafont et al., 1999). This makes it difficult to transfer readily available CE methods to CE-MS.

This is particularly true also for MEKC with CE-MS systems due to the presence of micelles in the buffer system, although only partial filling of capillaries with SDS containing buffer for the separation (Nelson et al., 1996; Muijselaar et al., 1998; Tanaka et al., 2000b), techniques applying reverse migrating micelles (Molina et al., 2001), or recent developments of low-flow CE-MS interfaces (Chen et al., 2003) have circumvented this problem. Other techniques of CE, such as non-aqueous capillary electrophoresis (NAEC) (Vaher and Koel, 2003) or capillary electrochromatography (CEC) the latter of which achieves sharp chromatographic separations using capillaries packed with stationary phases known from HPLC (Spikmans et al., 2000) represent better potential for the hyphenation of CE and MS. Recent optimisation of CEC-MS systems, such as the introduction of sheathless coupling of CE with MS through nano-electrospray interfaces as well as development of very rapid CEC-MS methods using gradient elution have greatly increased speed and sensitivity of analyses (Spikmans et al., 2000). However, the scarce availability of automated systems and the handling of fragile CEC capillaries still require further technical development.

1.5 Cocoa polyphenols – potential health implications

1.5.1 Epidemiological evidence

Results from epidemiological studies on flavonoids and the risk of the development of cancer remain inconclusive. Knekt et al. (1997) observed an inverse association among approximately 10,000 Finnish men and women between the intake of flavonoids and incidence of all sites of cancers combined. The sex- and age-adjusted relative risk of all sites of cancers combined between the highest and lowest quartiles of flavonoid intake was 0.80, mainly due to the low relative risk for lung cancer (0.54). However, Hertog (1996) pointed out in a recent review of epidemiology of flavonoids that flavonoid intake was not related to the incidence of cancer in the two prospective studies conducted by then. It has to be mentioned that in all studies flavonoid intake was calculated from the determination of the content of only five flavonols and flavones in foods. The average intake in the Netherlands was calculated to be approximately 23 mg/d with quercetin being the most predominant at 16 mg/d. The estimated flavonoid intake was highest in Japan at 64 mg/d and lowest in Finland at 6 mg/d. However, tea and wine (as well as cocoa) are rich sources of flavanols, proanthocyanidins and anthocyanins, all being strong antioxidants *in vitro*, rather than of flavonols and flavones.

Results of epidemiological studies for single flavonoid-rich beverages such as green and black tea, and wine could not confirm cancer-protective effects consistently. Hertog (1996) concluded that the foods often associated with low cancer rates in epidemiological studies, such as green-yellow vegetables and cruciferous vegetables, are not important sources of flavonols and flavones and that those flavonoids only play a minor role in the explanation of the cancer protective effect of vegetables and fruits. Moreover, the more consistently reported inverse relationship between consumption of onions as particular rich in quercetin and the risk of cancer development could also be explained by other potential anti-carcinogens present in onions, for example diallysulphides (Hertog, 1996).

As with cancers, an increasing body of epidemiological evidence links high intakes of antioxidants with reduced risk of cardiovascular disease (CVD). The evidence is

strongest for vitamin E, limited but promising for β-carotene, and inconsistent for vitamin C. However, protective effects of vitamin E may be evident only at high doses – much more than can be obtained from a normal diet (Langseth, 1995).

So far, the association between the intake of flavonoids and the risk of CVD, namely coronary heart disease (CHD) and stroke, has been investigated only in one prospective cohort study, the Zupthen Elderly Study (Hertog et al., 1993; Keli et al., 1996). The CHD mortality was approximately 65% lower in the highest tertile of flavonoid intake compared to the lowest tertile of flavonoid intake. The inverse relationship between flavonoid intake and first myocardial infarction was less pronounced as well as the intakes of tea, onions, and apples in relation to CHD mortality (Hertog et al., 1993). Dietary flavonoids were inversely associated with stroke incidence after adjustment for potential confounders, including antioxidant vitamins (Keli et al., 1996). In other studies, tea consumption showed no relation to the prevalence of CHD. However, the mean tea consumption was very low in those studies. In contrary, wine consumption has been found more consistently to be related to a lower risk of CHD (Hertog, 1996).

In another study (Lee and Paffenberger Jr., 1998), consumption of sugar candy and chocolate was associated with greater longevity of approximately one year. It could not be differentiated between consumption of sugar candy and chocolate, but the authors suggested the presence of phenolic antioxidants in chocolate as plausible explanation for the observed effect. However, the correlation is not very strong and greater consumption of candy and chocolate was not associated with progressively lower mortality.

It is evident that the science of epidemiology has inherent limits. Although epidemiology is very effective in identifying strong links between an environmental factor and disease (e.g., the link between smoking and lung cancer), it is less effective in discerning weaker associations (Taubes, 1995; Langseth, 1996). The link between fruit and vegetable intake and several cancers can be considered to be a strong one in magnitudes like smoking and lung cancer or alcohol and oral/oesophageal cancers (Block et al., 1992). However, many associations between diet and disease are relatively subtle. It may be impossible to determine from epidemiology alone, whether such relatively weak associations are real or whether they reflect some type of subtle bias or

measurement error (Langseth, 1996). This might be the case for associations between intake of a single nutrient or non-nutrient and disease as well as consumption of a single food commodity and disease. Bellisle et al. (1998) suggest that human intervention studies in particular bioavailability studies and dose-response studies in combination with the development and application of biomarkers might be a more successful research strategy.

1.5.2 Bioavailability

Cocoa polyphenols including most of the ones discussed in section 1.3 have been demonstrated to have strong antioxidant activity using *in vitro* systems with individual compounds or enriched cocoa fractions (Sanbongi et al., 1998; Adamson et al., 1999; Arteel et al., 2000; Richelle et al., 2001; Counet and Collin, 2003). However, in order to comprehend whether cocoa polyphenols exert their antioxidant activity in the target tissue protecting biological macromolecules from oxidative damage and thereby reducing the risk for the development of degenerative diseases, such as cancer and cardiovascular diseases, the fate of cocoa polyphenols in the human body has to be better understood.

The absorption and metabolism of food phenolics are determined primarily by their chemical structure, which depends on factors such as the degree of glycosylation/acylation, their basic structure (i.e., benzene or flavone derivatives), conjugation with other phenolics, molecular size, degree of polymerisation, and solubility (Bravo, 1998). Catechins and procyanidins can only be found as aglykones in plants and plant-derived food products and thus, molecular size and solubility might be the determining properties for absorption.

After absorption of flavonoids, the subsequent metabolism and excretion of flavonoids is rather well known from animal studies, but only few data in humans are available. Catechin, however, has been object of many studies in different mammalian species (Heilmam and Merfort, 1998b). Hydroxyl groups of the intact molecule are conjugated with glucuronic acid or sulphate in the small intestine and in the liver, respectively. In addition, methylation may occur. The conjugates have been found in urine but excretion in bile of glucuronides and sulphates seems to be important as well. Micro-organisms in the colon hydrolyse conjugates, which is supposed to enable absorption of the liberated

aglykones. Thus, conjugates can be reabsorbed and enter an enterohepatic cycle. However, these micro-organisms also substantially degrade the flavonoid moiety by cleavage of the heterocyclic ring leading to different phenolic acids (Rechner et al., 2002; Spencer, 2003). In the case of catechin, different γ-phenyl-valerolactones have been found, a class of intermediate metabolites that could not be detected as metabolites of other flavonoids (Heilmann and Merfort, 1998b). The phenolic acids are absorbed and excreted with urine. A wide range of mammalian species has shown considerable species variation in this secondary metabolism (Griffiths, 1982; Hollman, 1997; Heilmann and Merfort, 1998b).

Recently, Scalbert and Williamson (2000) have proposed a simplified general scheme for polyphenol metabolism in the small intestine and liver (figure 1.10) according to which:

- Polyphenols bound to glucose, galactose or xylose are hydrolysed to free polyphenol aglykon and sugar by cytosolic β-glucosidase (CBG) or lactase phlorizin hydrolase (LPH) prior to absorption by enterocytes – polyphenols bound to other sugar moieties proceed mostly unabsorbed to the colon where microbial degradation may occur.

- Absorbed polyphenols are then methylated by catechol-O-methyltransferase (COMT) at both catecholic hydroxy-groups in the B-ring prior to specific demethylation in the 4'-position to lead 3'-O-methyl-polyphenols.

- Both methylated and free polyphenols are subsequently conjugated by uridine diphosphate-glucoronosyl transferase (UDPGT) and phenol sulfotransferase (SULT) to lead polyphenol glucuronides, sulphates as well as mixed sulfoglucoronides.

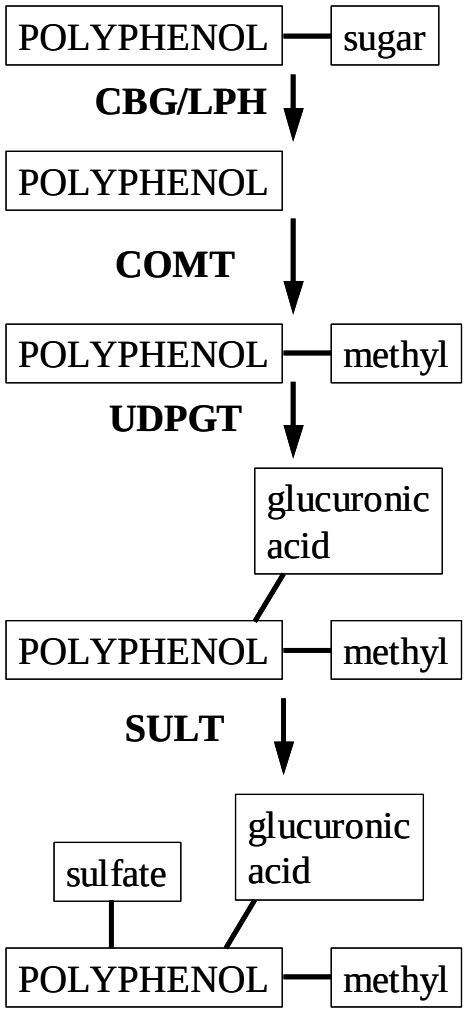


Figure 1.10 Schematic outline of polyphenol metabolism – **CBG** = cytosolic β-glucosidase, **LPH** = lactase phlorizin hydrolase, **COMT** = catechol-O-methyltransferase, **UDPGT** = uridine diphosphat-glucoronosyl transferase, **SULT** = sulfotransferase (adopted from Scalbert and Williamson, 2000).

Although evidence of absorption and metabolism of polyphenols in the gut exists, less is known about the efficiency of such uptake and the permanence of phenolic compounds or their conjugates and derivatives in the body. Blood concentrations of total catechins of 0.17 µmol/l after ingestion of black tea and up to 0.55 µmol/l after green tea were reported recently (Van het Hof et al., 1998). These data agree with another estimation that the absorption of tea catechins correspond to about 0.2-0.9% of the ingested dose (Lee et al., 1995). Mainly conjugates of the administered catechins were present in plasma. After a single oral application of 0.5, 1 and 2 g catechin in

human volunteers, the peak plasma concentration was reached after 1-2 hours (Balant et al., 1979). Independently of the doses, 0.5% of the catechin was found in the free unbound form in plasma and the same amount of free catechin was excreted in the urine. However, 25% of the administered doses were found as different metabolites, indicated by an intact A ring. The elimination half-life was estimated to be 1-1.5 hours (Balant et al., 1979). This is in agreement with the observation in rats after being fed 10 mg of ^{14}C labelled catechin of which 34% have been excreted with bile and 20% with urine (Shaw and Griffiths, 1980). Data on the permanence of polyphenols in the body are of great importance, because some of the physiologic effects of food polyphenols depend on their circulating levels (e.g., their antioxidant capacity). Van het Hof et al. (1998) reported that maximum blood levels of tea catechins occurred 2 hours after tea ingestion and elimination half-life varied between 4.8 and 6.9 hours for green and black tea catechins. In contrast, quercetin concentrations after ingestion of onions reached maximum plasma levels after 3.3 hours and the elimination half-life was 16.8 hours (Hollman, 1997).

Richelle et al. (1999) studied plasma kinetics of epicatechin in man after consumption of 40 g and 80 g of black chocolate. Epicatechin increased markedly after chocolate consumption, reaching a maximum between 2 and 3 hours. The maximal concentration and area under the curve of plasma kinetics correlated very well with the dose of chocolate. It has been concluded that epicatechin is absorbed from chocolate and is rapidly eliminated from plasma. Attainable plasma levels were 0.7 µmol/l (free epicatechin and epicatechin conjugates) from 80 g of black chocolate containing 164 mg of epicatechin. Similar plasma concentrations of epicatechin were reported by Rein et al. (2000b) as well as Wang et al. (2000) after the consumption of 80 g of procyanidin-rich chocolate. These increments correlated well with increases in plasma antioxidant capacities as measured by chemiluminescence and reduction in plasma lipid oxidation products (thiobarbituric acid reactive substances, TBARS). Bioavailability of epicatechin from chocolate and cocoa powder has been also demonstrated by others both in humans (Baba et al., 2000a) and rats (Baba et al., 2001). In both cases epicatechin was extensively conjugated to methylated and non-methylated sulphates, glucuronides, and sulfoglucuronides, respectively. In the human study, intake of approximately 220 mg epicatechin with chocolate and cocoa powder resulted in notably

higher peak plasma levels than the reported concentrations by Richelle et al. (1999), Rein et al. (2000b), and Wang et al. (2000) at 2 hours after ingestion of 4.7 to 4.9 µmol/l of methylated and non-methylated epicatechin metabolites, respectively. Urinary excretion of total epicatechin metabolites within 24 hours after chocolate or cocoa intake was 29.8 ± 5.3% and 25.3 ± 8.1% of total epicatechin intake. More than 80% of these epicatechin metabolites were excreted within the first 8 hours confirming a short elimination half-life (Baba et al., 2000a). Data on the absorption of other polyphenols in cocoa was absent until the discovery of procyanidin dimer B2 in human plasma after the consumption of a flavanol-rich cocoa beverage by Holt et al. (2002a) as well as Steinberg et al. (2002). Nevertheless, absorption efficiency of the dimer appeared to be much lower than that for epicatechin monomer as can be deduced from the 70-fold lower peak plasma concentrations after ingestion of both approximately 300 mg of epicatechin and procyanidin dimer, respectively.

Attention should be given to the fact that in most cases, and mainly because of the difficulty in their analysis and characterisation, the study of digestive fate and physiological effects of insoluble polyphenols – highly polymerised or bound tannins – is usually neglected (Bravo, 1998; Heilmann and Merfort, 1998b). Spencer et al. (2000; 2001c) concluded from studies of isolated trimeric to hexameric procyanidins in stimulated gastric juice (pH 2.0) that procyanidins decompose to give lower oligomers and monomeric catechin and epicatechin during gastric transition and subsequent absorption of monomers in the small intestine. Similarly, Zhu et al. (2002) observed decomposition of procyanidin dimers B2 and B5 in stimulated gastric juice but not of epicatechin and catechin monomers. Interestingly, both procyanidins and catechin monomers degraded quickly in stimulated intestinal juice (p 8.5) (Zhu et al., 2002) but the stability was significantly increased through the addition of ascorbic acid suggesting oxidative degradation under alkaline conditions (Zhu et al., 2003). Nevertheless, these observations result from *in vitro* studies and Rios et al. (2002) showed that *in vivo* procyanidins were stable during 50 to 60 minutes gastric transit time in 6 human volunteers after consumption of 500 ml of a cocoa beverage. Rios et al. (2002) also measured gastric pH, which increased from a baseline of 1.9 to 5.4 just after cocoa beverage ingestion. This might explain the contrasting results from the *in vivo* and *in*

vitro observations as it is well known that procyanidins are stable at slightly acid conditions at around pH 5 (Zhu et al., 2003).

Facino et al. (1998) demonstrated an increased antioxidant activity of plasma and decreased ischemia/reperfusion damage in rats after being fed a procyanidin-enriched diet for 3 weeks. This indicates that procyanidins are absorbed from the diet, at least in experimental rats. In a relatively new approach, studies using ^{14}C-labelled oligomers and cultured human colon cells (Caco-2) indicate that the monomer catechin is taken up passively through tight junctions. There is more limited uptake of dimers and trimers by the same route, but the higher oligomers and polymers appear to enter by transcytosis as it is known to happen for carageens, β-lactoglobulin and α-lactalbumin (Clifford, 1999).

Relying on the few data mostly obtained from *in vitro* studies, Heilmann and Merfort (1998b) suggest the microbial depolymerisation of procyanidins to give the catechin subunits as first step in the colon. Subsequently catechins are either absorbed or further metabolised by the colonic microflora as desribed above. However, the metabolic fate *in vivo* of procyanidins and whether procyanidins can be absorbed as intact molecules has yet to be determined (Heilmann and Merfort, 1998b).

Bravo et al. (1994) have suggested a classification of polyphenols for nutritional purposes in ‘extractable’ polyphenols (EPP) and ‘non-extractable’ polyphenols (NEPP). EPP are low- and intermediate-molecular-mass phenolics including some hydrolysable tannins and proanthocyanidins that can be extracted using different solvents, such as water, ethanol, methanol and in some cases aqueous acetone. NEPP are high-molecular-weight compounds or phenols bound to dietary fibre or protein that remain insoluble in the usual solvents. Results from *in vitro* tests with digestive enzymes and from animal studies suggest the non-availability of some polyphenolic compounds, mainly NEPP. NEPP from carob pod concentrate, which is rich in highly polymerised condensed tannins, have been extensively (98%) recovered in the faeces of rats, confirming the resistance of these compounds to intestinal digestion and/or absorption as well as degradation by colonic microflora. Conversely, EPP were excreted only in minor amounts suggesting that extensive digestion and/or absorption as well as microbial degradation of these phenolic compounds occur (Bravo, 1998). A similar observation

has also been reported by Clifford (1999), namely that anaerobic gut flora micro-organisms extensively degraded monomer, dimer and some higher oligomers.

Heilmann and Merfort (1998 a and b) as well as Scalbert et al. (2002) have reviewed the literature on microbial degradation of polyphenols and have proposed a large number of ring fission products with intact A-Ring. These include groups of compounds with C_6-C_1 (benzoic and hippuric acids), C_6-C_2 (phenylacetic acids), C_6-C_3 (phenylproprionic and cinnamic acids) as well as C_6-C_5 carbon bodies (phenyl-γ-valerolactons) having 3 or 4 hydroxy-, 3,4-dihydroxy-, or 4-hydroxy-3-methoxy-substitutions. As for intact polyphenols described above these ring fission products can be found in their free form or as glucuronide and sulphate conjugates.

Olthof et al. (2003) found hippuric acid as the main metabolite after ingestion of chlorogenic acid and black tea solids, respectively, but not after ingestion of quercetin rutinoside. Other colonic metabolites included benzoic (C_6-C_1) and phenylproprionic (C_6-C_3) acids after chlorogenic acid and black tea solids intake as well as phenylacetic acids (C_6-C_2) after quercetin rutinoside ingestion. In contrast to the relatively high amounts of colonic metabolites, only comparably small amounts of intact chlorogenic acid or quercetin both in free or conjugated form were detected in urine (Olthof et al., 2003). Also Rechner et al. (2002) have found that the majority of the *in vivo* forms derive from cleavage products of the action of colonic bacterial enzymes and subsequent metabolism in the liver. After a polyphenol-rich meal those metabolites included the glucuronides of 3-hydroxyphenylacetic, homovanillic, vanillic and isoferulic acids as well as un-conjugated 3-(3-methoxy-4-hydroxyphenyl)-propionic, 3-(3-hydroxyphenyl)-propionic, and 3-hydroxyhippuric acids. In contrast, intact conjugated polyphenols themselves, such as the glucuronides of quercetin, naringenin and ferulic, p-coumaric, and sinapic acid were detected at much lower levels. Gonthier et al. (2003) detected 14 colonic metabolites in the 24-hour urine of a human subject consuming a polyphenol-rich diet containing various fruits, vegetables, orange juice, and coffee. The by far most abundant metabolites were 3- and 4-hydroxyhippuric acid, vanillic acid, and 3-hydroxyphenylacetic acid. The consumption of chocolate containing approximately 147 mg catechin monomers and 439 mg procyanidin oligomers resulted within 48 hours in significant increases in 6 urinary phenolic acids, including m-hydroxyphenylpropionic acid, ferulic acid, 3,4-dihydroxyphenylacetic acid, m-

hydroxyphenylacetic acid, vanillic acid, and m-hydroxybenzoic acid in healthy human subjects (Rios et al., 2003). Interestingly, no increase in urinary hippuric acid was observed. However, this could have been a result of 3-fold higher levels and wide variations in the baseline concentrations during the two-day polyphenol-free diet. Possibly benzoic acid as a food conservative or other food ingredients, which should be controlled for, have caused these effects. Nevertheless, both Rechner et al. (2002) and Rios et al. (2003) suggest that consideration should be given to the cleavage products as having a putative role as physiologically relevant bioactive components *in vivo*. In fact, Spencer (2003) has recently reviewed the metabolism of tea polyphenols and many studies have suggested that the extent of absorption of dietary polyphenols in the small intestine is relatively small (10–20%). The implications of this low absorption in the small intestine is that the majority of ingested polyphenols, including those absorbed and conjugated in the enterocytes and/or the liver before transport back out into the lumen either directly or via the bile, will reach the large intestine where they encounter the colonic microflora. Spencer (2003) suggests the formation of hippuric acid and hydroxyhippuric acids as being a possible central metabolic pathway for dietary flavonoids in which the colon microflora and the liver are active metabolic sites.

Due to the fact that some polyphenols form complexes with proteins it has been suggested that addition of milk to black tea reduces the bioavailability of tea polyphenols. This was corroborated by the observation that ingestion of green or black tea significantly increased the total plasma antioxidant capacity, but addition of milk to tea abolished the effect (Serafini et al., 1996). However, in this study the concentration of polyphenols in plasma was not determined. More recently, it was found that addition of milk to green or black tea had no effect on the concentrations of catechins or quercetin in blood when compared with plain tea (Hollman et al., 1997; Van het Hof et al., 1998). This difference in the observations has been attributed to the fact that in the study of Serafini et al. (1996) much higher amounts of added milk had been used (Van het Hof et al., 1998). However, the inhibitory effect of milk on plasma antioxidant activity could be also due to a reduction of antioxidants other than catechins or quercetin, such as the condensed tannins (theaflavins and thearubigens) (Hollman et al., 1997). Very recently, Serafini et al. (2003) showed that consumption of plain, dark chocolate resulted in an increase in both the total antioxidant capacity and the

epicatechin content of blood plasma, but that these effects were markedly reduced when the chocolate was consumed with milk or if milk was incorporated as in milk chocolate. As for black tea they suggest that milk reduces the absorption of antioxidants from chocolate and may therefore negate the potential health benefits that can be derived from eating dark chocolate. However, these conclusions derive from the measurement of the total antioxidant capacity of plasma by only one rather unspecific parameter, the ferric-reducing antioxidant potential (FRAP) assay (Serafini et al., 2003). In contrast to the observations made by Serafini et al. (2003), neither protein nor lipid-rich meals effected flavanol absorption when consumed together with flavanol-rich cocoa and carbohydrate-rich meals even enhanced the flavanol absorption from cocoa (Schramm et al., 2003).

Probably even more important to the bioavailability of polyphenols, in particular to complex phenolics and tannins (CPT), are some protein fractions in the saliva. These include the so-called salivary proline-rich proteins (PRPs) and salivary histatins. Both fractions have a much higher affinity to CPT than for example enzymes or bovine serum albumin and gelatine (Bacon and Rhodes, 1998). In model solutions, it has been shown that complexes of CPT and salivary PRPs and histatins, respectively, remain stabile in conditions similar to those in the stomach. The complexes of CPT and salivary histatins remained stable also under model conditions similar to that in the small intestine (Naurato et al., 1999). In contrast, the complex of CPT and salivary PRPs showed decreased stability under the same conditions, in particular in the presence of bile salts (Lu and Bennick, 1998). Interestingly, in some mammals (e.g., in rats) it has been demonstrated that a diet rich in CPT caused an increased production of salivary PRPs by the parotid glands, whereas the production of histatins seems to be independent of CPT consumption (Jansman et al., 1994; Lu and Bennick, 1998). Whether this is valid also in humans has to be established.

In conclusion, it appears most likely that if cocoa polyphenols are biologically active it would be their numerous metabolites rather than the original compounds ingested with the diet responsible for such bioactivity. In contrast to the matter of interest, only little is known about the actual composition of polyphenol metabolites in the human body after consumption of polyphenol-rich food mainly due to analytical difficulties. The significance of results obtained in laboratory animals into humans is mostly unclear,

because animal studies have shown considerable species variation in metabolism of flavonoids. High plasma levels of flavonoids are not found although according to Hollman (1997) the concentrations are potentially high enough to give biological effects, at least for monomeric flavonoids. The question of absorption of proanthocyanidins and the effects on bioavailability that could have proteins in foods and of the saliva, respectively, as well as other food constituents must be addressed to the future.

1.5.3 Potential health effects of chocolate, cocoa, and cocoa polyphenols – evidence from *in vitro*, *ex vivo*, animal and human intervention studies

Several authors have recently reviewed the potential effects on human health of plant polyphenols in general (Chung et al., 1998; Harborne and Williams, 2000; Birt et al., 2001; Nijveldt et al., 2001; Heim et al., 2002) or from various polyphenol-rich food sources including wine (German and Walzem, 2000) as well as green and black tea (Wiseman et al., 2001; Lambert and Yang, 2003; Rietveld and Wiseman, 2003; Vita, 2003). Nevertheless, in recent years much research has also been carried out directly with chocolate, cocoa, polyphenol-rich cocoa fractions or even single phenolic compounds isolated from cocoa. The potential health effects include general antioxidant effects protecting against reactive oxygen species (ROS) that are involved in the aetiology of many degenerative diseases, such as cancer, cardiovascular diseases (CVD), cataract, diabetes and rheumatoid disease, many of which may be age-related (Wollgast and Anklam, 2000b). Moreover, cocoa polyphenols have been studied with respect to non-radical scavenging effects related to cancer, CVD, and the immune system. The following gives an overview on papers reporting on potential health effects of cocoa polyphenols in the before mentioned fields of disease thereby reviewing the current existing evidence.

1.5.3.1 Antioxidant effects

Many studies have involved the determination of the *in vitro* antioxidant capacities of cocoa extracts, single polyphenol-rich fractions of cocoa or isolated polyphenols from cocoa (e.g., Sanbongi et al., 1997 and 1998; Vinson 1998; Vinson et al., 1998; Serafini et al., 2003). Waterhouse et al., (1996) were the first to demonstrate that cocoa

flavonoids are potent inhibitors of LDL oxidation *in vitro*. Pure catechin inhibited LDL oxidation even more effectively than cocoa phenols did (87% versus 75%, respectively). Hirano et al. (2000) demonstrated a dose-dependent prolongation of *in vitro* LDL oxidisability of cacao liquor polyphenols, whereas Richelle et al (2000 and 2001) proved the prolongation of the lag-time of LDL oxidation of cocoa beverages. Lee et al. (2003) revealed a higher *in vitro* antioxidant capacity of cocoa compared to red wine, green tea and black tea, respectively, as determined by the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. Bearden et al. (2000) showed both decreased LDL oxidisability and increase in the antioxidant capacity *in vitro* as determined by the Oxygen Radical Absorbance Capacity (ORAC) assay by adding extracts of cocoa powder as well as monomeric catechins and oligomeric procyanidin fractions isolated from cocoa to the test systems. Furthermore, purified epicatechin based oligomeric procyanidins obtained from cocoa have been shown to protect LDL and liposomes from oxidation in several *in vitro* studies and have also been shown to protect against peroxynitrite-dependent oxidation reactions (Arteel and Sies, 1999; Arteel et al., 2000; Lotito et al., 2000; Pearson et al., 2001; Osakabe et al., 2002; Counet and Collin, 2003). This suggests that procyanidins can protect against reactive nitrogen species as well as reactive oxygen species. Steinberg et al. (2002) have recently demonstrated that such protective effect on LDL-oxidation by epicatechin and procyanidins can occur at physiologically relevant concentrations of 0.1 to 0.5 µM. A sparing of α-tocopherol was also observed and proposed as possible mechanism of LDL protection against oxidation. Among other polyphenols isolated from cocoa liquor by Sanbongi et al. (1998) and Osakabe et al. (2000), clovamide most strongly inhibited linoleic acid autoxidation, followed by epicatechin, catechin, quercetin, quercetin-3-glucoside, quercetin-3-arabinoside, and dideoxyclovamide. The first four of these compounds were essentially equally effective inhibitors of erythrocyte ghost membrane oxidation induced with tert-butylhydroxide, and quercetin-3-glucoside was only slightly less potent. The two remaining substances were less inhibitory in this system (Sanbongi et al., 1998). Interestingly, Cren-Olivé et al. (2003) demonstrated an equally strong protective effect on *in vitro* LDL-oxidation of O-methylated metabolites of catechin compared to non-metabolised catechin. More such studies investigating on actual *in vivo* metabolites of cocoa polyphenols rather than

on the unchanged compounds found in chocolate and cocoa would enhance the knowledge on potential *in vivo* antioxidant effects of cocoa polyphenols.

Evidence for *in vivo* antioxidant effects was gained from the observation of increased plasma antioxidant capacity in rats and rabbits after feeding of cocoa liquor and cocoa powder, respectively, as measured by enhanced *ex vivo* LDL oxidisability as well as reduced thiobarbituric acid reactive substances (TBARS), lipid peroxides and consumption of α-tocopherol (Baba et al., 2000b; Osakabe et al., 2000). Yamagishi et al. (2001b) observed reduced amounts of lipid peroxides in liver, kidney, heart and brain as measured by TBARS in rats fed a vitamin E-deficient diet supplemented with cocoa liquor polyphenols compared to the control group fed only the vitamin E-deficient diet. However, in this study levels of α-tocopherol decreased in the same manner in both supplemented and control groups. In another four-week study, daily polyphenol-rich cocoa extracts added to the diet reduced oxidative liver injury provoked by contemporary providing 10 to 14 g/kg ethanol per day in rats. Additionally, inflammation and necrosis in the liver were reduced as well (McKim et al., 2002). Very recently, Orozco et al. (2003) described a dose-depended reduction in oxidative DNA damage in rat testes after being fed diets containing 0.5 to 2% cocoa rich in flavanols for two weeks.

The reduction of *ex vivo* LDL oxidation after intake of chocolate or cocoa powder was also demonstrated in human volunteers. In two studies the consumption of 35 mg of delipidated cocoa induced a slight but significant increase in the lag time of LDL oxidation two hours but not four hours later (Hirano et al., 2000; Kondo et al., 1996). In healthy volunteers, the consumption of increasing amounts of procyanidin-rich chocolate resulted in an approximately linear rise in plasma epicatechin concentrations both in peak plasma levels after two hours and in the area under the curve. This was accompanied by a dose-responsive increase in the antioxidant capacity of the plasma studied by chemiluminescence and a dose-responsive reduction in plasma lipid oxidation as determined by TBARS (Rein et al., 2000b; Wang et al., 2000). It was suggested that the changes in plasma antioxidant capacity and lipid peroxidation potential occurred secondary to the rise in plasma epicatechin levels, although this conclusion cannot be established unequivocally from these results (Borchers et al., 2000). Serafini et al. (2003) reported on an increase of plasma antioxidant capacity as

measured by the ferric reducing antioxidant potential (FRAP) assay in healthy volunteers after the consumption of 100 g of dark chocolate but not after dark chocolate consumed with milk or milk chocolate, respectively. As these findings correlate with plasma epicatechin area under the curve levels, the authors conclude a reduced bioavailability of cocoa polyphenols due to the presence of milk proteins as well as a loss of beneficial effects of chocolate. However, levels of epicatechin and/or other antioxidants derived from chocolate or present in plasma, such as uric acid, albumin or ascorbic acid have not been reported at baseline and after two and four hours when FRAP measurements were made. This would have improved the interpretation of the actual reasons of changes in FRAP values, whereas the reduced area under the curve determined with only two time points at two and four hours could well be a sign of delayed or prolonged absorption of epicatechin from chocolate in the presence of milk rather than an actual reduced bioavailability. Moreover, the authors' conclusion of potential health benefits of dark chocolate and not of dark chocolate consumed with milk or milk chocolate based solely on the measurement made on blood plasma with a single measure of ferric reducing ability (FRAP assay) of plasma at two hours after chocolate consumption does not appear to be sufficiently substantiated.

Increased resistance of LDL to *ex vivo* oxidation was not only shown under acute conditions (mostly two hours after cocoa intake) but also after supplementation of 36 g cocoa powder per day for two weeks (Osakabe et al., 2001). These results have been confirmed by Wan et al. (2001) over a period of four weeks with volunteers consuming a diet supplemented with a mixture of cocoa powder and dark chocolate. However, the observed increases in LDL oxidation lag time was only 8% and plasma total antioxidant capacity as measured by the ORAC assay was increased by only 4%. Although statistically significant, it must be questioned whether such modest increases are biologically relevant. This is valid also for the results obtained with a six-week supplementation of a similar mixture of cocoa powder and dark chocolate leading to an equally modest increase in LDL oxidation lag time of 10% to baseline and 5% to washout phases. Moreover, urinary F_2-isoprostanes, a marker for lipid peroxidation, was not affected during the experimental period, which also may suggest a possible negligible biological relevance (Mathur et al., 2002).

1.5.3.2 Effects related to cancer

Oxidative damage is likely to play a major role at various steps of carcinogenesis. Therefore, the antioxidant activities of polyphenols including those described above for cocoa polyphenols might contribute to the cancer-preventive effects that epidemiological studies have found to be associated with high consumption of fruits and vegetables (Wollgast and Anklam, 2000b).

Romanczyk et al. (1997) examined anticarcinogenic properties of cocoa extracts, using several human cancer cell lines. Interestingly, the effects were seen only with oligomeric procyanidins and of these in particular with oligomers of five to twelve sub-units with the most effective being the pentamer. It is suggested that the mechanisms by which procyanidins exert anti-carcinogenic activity include inhibition of DNA strand breaks, DNA-protein cross-links and free radical oxidation of nucleotides due to their antioxidative activity as well as inhibition of enzyme activities of cyclo-oxygenase 2 (COX-2) and DNA-topoisomerase II. Moreover, procyanidins modulate nitric oxide production by macrophages, possessing an inducible nitric oxide synthase (iNOS), and thereby affecting ribonuclease reductase, the enzyme that converts ribonucleotides to deoxyribonucleotides necessary for DNA synthesis. Inhibition of DNA synthesis may be an important way in which macrophages and other tissues possessing iNOS can inhibit the growth of rapidly dividing tumour cells or infectious bacteria (Romanczyk et al., 1997).

Because there is no evidence of absorption of intact procyanidins higher than the dimer it has been argued that the proposed anticarcinogenic activities of procyanidins might be limited to the gastrointestinal tract (Wollgast and Anklam, 2000b). In fact, Verstraeten et al. (2003) suggest that attention should be focused on the effects of monomers and dimers for postabsorptive tissues, while studies on the physiological effects of larger oligomers may be best focused on the oligomers' ability to act in the oral cavity and the upper gastrointestinal tract. The authors observed a protection of liposomes against lipid oxidation by catechins and procyanidins isolated from cocoa with higher oligomers being more protective even when corrected for monomeric units. In addition, cocoa polyphenols caused a decrease in liposome membrane surface potential and protected

membranes from detergent-induced disruption by interacting and binding to phospholipid head groups of the membrane bilayer (Verstraeten et al., 2003).

Yamagishi and colleagues (2000, 2001a, 2002, and 2003) have studied the antimutagenic and anticarcinogenic effects of cocoa liquor polyphenols (CLP) in various *in vitro* as well as animal models. In the Ames test, CLP showed antimutagenic effects in bacteria treated with heterocyclic amines (Yamagishi et al., 2000) as well as in bacteria treated with 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP) (Yamagishi et al., 2002). In the host mediated assay in mice, a method used to estimate the potential carcinogenicity of chemicals *ex vivo*, oral administration of CLP reduced the number of colonies of revertant bacteria recovered from the liver provoked by activated heterocyclic amines (Yamagishi et al., 2000). Subsequently, the inhibitory effect of CLP on DNA strand cleavage induced by mitomycin C *in vitro* as well as the anticlastogenic effect of CLP against formation of micronuclei in bone marrow cells and peripheral blood cells in mice treated with mitomycin C was demonstrated (Yamagishi et al., 2001a). Furthermore, CLP decreased incidences of prenoplastic eosinophilic foci in the exocrine pancreas in a dose-dependent manner in mice treated with PhIP eight times over four weeks indicating an inhibitory effect of CLP on pancreatic carcinogenesis in the initiation stage (Yamagishi et al., 2002). Although a similar tendency existed for mammary carcinogenesis as well in the same study these results were not statistically significant. Finally, Yamagishi et al. (2003) demonstrated an increased survival rate combined with significant reduction in incidence and multiplicity of lung carcinomas as well as a tendency in the decrease in thyroid adenomas after 36 weeks in rats supplied with CLP. Because CLP was given only one week after a four week treatment with various known carcinogens, these results suggest a chemopreventive effect of cocoa polyphenols against tumorigenesis even after the initiation stage (Yamagishi et al., 2003). Since these anticarcinogenic effects were observed in this rat multi-organ carcinogenesis model in the lung and thyroid but not in the small intestine and colon, prior absorption of cocoa polyphenols must be assumed and that the active compounds were unlikely to be intact procyanidin oligomers which to the current knowledge are not present postabsorptive. Nevertheless, cocoa powder and cocoa procyanidin-enriched fractions inhibited growth and polyamine biosynthesis of the human colonic cancer cell line Caco-2 in another *in vitro* study (Carnésecchi et

al., 2002) suggesting the potential site of action of cocoa procyanidins in the gastrointestinal tract. These effects were explained by a contemporary observed significant inhibition of ornithine decarboxylase and S-adenosylmethionine decarboxylase activities, two key enzymes of polyamine biosynthesis that are known to be enhanced in cancer.

Other proposed mechanisms by which polyphenols may prevent, delay or alleviate the progression of cancer that, however, have not yet been studied particularly with chocolate, cocoa or polyphenols isolated from cocoa include: complexation (chelates) of divalent cations, inhibition of the activity of enzymes including telomerase, xanthine oxidase, lipoxygenase, protein kinase C and protein tyrosine kinases, inhibition of MAP kinase as well as growth factor signalling, induction of hepatic electrophile-processing (Phase II) enzymes, modulation of the activity of enzymes such as cytochrome P-450 isozyme, induction of apoptosis, and inhibition of angiogenesis (reviewed in Birt et al., 2001; Nijveldt et al., 2001; Lambert and Yang, 2003).

1.5.3.3 Effects on cardiovascular health

In addition to the antioxidant effects, in particular those on LDL oxidisability and lipid peroxidation observed and discussed in section 1.5.3.1, cocoa flavonoids may influence cardiovascular health through other mechanisms. Current concepts of atherogenesis include involvement of the immune system and chronic inflammation as crucial steps in the initiation and progression of the atherosclerotic process. Activation of the vascular endothelium with upregulation of adhesion molecules is claimed to play a pivotal role in such vascular inflammation by allowing leucocyte and monocyte adhesion to the vascular endothelium during the earliest phases of atherogenesis (Ferri and Grassi, 2003). Increased platelet reactivity and aggregation in the presence of endothelial dysfunction can lead to the development of arterial thrombosis and the progression of atherosclerosis (Steinberg et al., 2003).

Rein et al. (2000a) recently published a study of the inhibition of platelet activation *ex vivo* by cocoa polyphenols. Several indexes of platelet activation were diminished between two and six hours after a cocoa beverage was consumed. ADP-stimulated P-selectin expression, an important biomarker of thrombogenicity, and epinephrine-induced fibrinogen binding conformation of glycoprotein IIb-IIIa, a target of modern

antiplatelet therapy, as well as platelet microparticle formation were all lessened. The same authors reported elsewhere (Rein et al., 2000c) that cocoa consumption suppresses both unstimulated and stimulated platelet activation in whole blood measured by platelet PAC-1 binding and ADP-induced P-selectin expression over six hours.

A study by Schramm et al. (2001) provides evidence that some of the effects of chocolate on platelet activity may be secondary to changes in eicosanoid metabolism. Eicosanoids are bioactive metabolites of arachidonic acid that mediate inflammatory processes. A beneficial change in the ratio of two eicosanoids (a decrease in leukotriene and an increase in prostacyclin) was observed after consumption of a flavonoid-rich dark chocolate compared with a flavonoid-poor dark chocolate (providing 147 mg and 3.3 mg procyanidins/ bar, respectively) in healthy volunteers. Prostacyclin has been shown to inhibit platelet aggregation and is also a potent vasodilator, whereas leukotriene stimulates platelet aggregation, is a vasoconstrictor, and is proinflammatory. The ratio of these two eicosanoids provides a measure of proinflammatory versus antiinflammatory balance and thus suggests that chocolate procyanidins may affect the inflammatory response via eicosanoid modulation. Holt et al. (2002b) demonstrated that smaller, in the population more likely to be regularly consumed amounts (25 g) of chocolate, contributing 220 mg of catechins and procyanidins, could also inhibit platelet function after two hours as described by Rein et al. (2000a) for four times the amounts of cocoa polyphenols. Reductions in both ADP/collagen-stimulated and epinephrine/collagen-stimulated platelet-related primary hemostasis correlated well with increases in plasma epicatechin levels. In addition, antithrombotic eicosanoid response measured by an increased prostacyclin to leukotriene ratio as described by Schramm et al. (2001) was observed at 2 hours post-consumption of chocolate (Holt et al., 2002b).

It has been reported that cocoa procyanidins and other flavanoids are capable of directly inhibiting mammalian 15-lipoxygenase-1 and human platelet 12-lipoxygenase (Schewe et al., 2001) as well as recombinant 5-lipoxygenase (Schewe et al., 2002) at low micromolar concentrations. This suggests a mechanism by which they may modulate eicosanoid metabolism as well as reduce lipid peroxidation in particular in LDL particles and thereby contribute to cardiovascular protection (Sadik et al., 2003).

In another study, researchers looked at the effects that a specially formulated cocoa beverage, aspirin or both had on platelet function (Pearson et al., 2002). On separate days in a crossover design, sixteen healthy adults consumed aspirin, a 300 ml flavanol-rich cocoa beverage (897 mg flavanols as epicatechin and procyanidins) or aspirin plus flavanol-rich cocoa beverage. Platelet activation was measured by surface expression of P-selectin and PAC-1 binding to the glycoprotein IIb/IIIa-act receptor. On three different days measurements were taken at baseline, two hours and six hours after ingestion of each of the three treatment modalities. Results showed decreases in platelet reactivity at two hours after ingestion of the cocoa beverage, at six hours after ingestion of the aspirin and at both two hours and six hours after ingestion of the aspirin plus beverage. The results suggest that there is an additive effect with aspirin plus cocoa and that the increased inhibition of platelet function is due to the flavanol content of the cocoa (Pearson et al., 2002).

However, in a human mid-term study over two weeks, twenty-four-hour urinary excretion of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ as well as the ratio of the two compounds did not change significantly between a diet supplemented with cocoa powder and dark chocolate together providing approximately 466 mg procyanidins per day and a control diet, respectively (Wan et al., 2001). Whereas a single plasma assessment as conducted by Schramm et al. (2001) and Holt et al. (2002b) is a measurement at one point in time of eicosanoid production in response to cocoa consumption after two hours representing a momentary indicator of inflammatory balance, Wan et al. (2001) determined the concentrations of the stable metabolites of platelet aggregating vasoconstrictor thromboxane A_2 (thromboxane B_2) as well as of its counterpart, the antiaggregatory vasodilator prostaglandin I_2 (6-keto-prostaglandin $F_{1\alpha}$) in twenty-four-hour urine samples, which are thus indicators of overall eicosanoid synthesis in the whole body over 24 hours. With this approach an antiaggregatory or antithrombotic effect for cocoa polyphenols over a longer period cannot be confirmed. Nevertheless, the researchers suggested favourable although probably only modest heart health benefits for the participants supplemented with cocoa and dark chocolate deduced from a slight decrease (8%) in LDL oxidation and small increases in total antioxidant capacity (4%) in the blood and HDL cholesterol (4%) without adversely affecting prostaglandins (Wan et al., 2001).

A study by Murphy et al. (2003) examined the longer-term effect of a lower dose of cocoa flavanols and procyanidins using a double-blind, randomised, placebo-controlled study with 32 healthy subjects. Subjects on the active diet consumed 234 mg of cocoa flavanols and procyanidins per day for four weeks, while subjects on the placebo tablet consumed an identically appearing tablet made from cocoa powder with a low level of flavanols and procyanidins (≤ 6 mg) for four weeks. Dietary restrictions were imposed to control additional dietary sources of flavanols. Platelet function was determined after aggregation and P-selectin expression was stimulated *ex vivo* using ADP and collagen. After four weeks plasma levels of epicatechin were significantly increased in the active group and platelet aggregation and activation was significantly lower in the active group ($P<0.05$) compared with the placebo group. No significant differences were observed between the two groups in terms of antioxidant or blood lipid status (Murphy et al., 2003).

Although other intracellular pathways may play a role, interactions of NF-κB, firstly with oxidants in the cytoplasm and then with inflammatory genes in the nucleus, represent the most important mechanisms underlying endothelial adhesion molecule upregulation. Concordant with this, endothelial activation *in vivo* and *in vitro* is promoted by oxidants and prevented by natural antioxidants. Interestingly, superoxide anion may transform nitric oxide into the oxidant peroxynitrite and thereby further promote atherogenesis, whereas intact nitric oxide markedly inhibits NF-κB activation and has potent antiatherogenic properties. (Ferri and Grassi, 2003). Thus, although allowing a normal endothelium-dependent vasodilatory capability, a normal nitric oxide bioavailability also inhibits endothelial adhesion molecule upregulation. As a consequence, an increased nitric oxide availability, either due to nitric oxide synthase stimulation or to antioxidants avoiding nitric oxide transformation into peroxynitrite, or both, is expected to simultaneously protect the vascular endothelium against abnormal vasoconstriction and accelerated atherogenesis (Ferri and Grassi, 2003).

Indeed, Romanczyk et al. (1997) suggest that although the polyphenolic compounds inhibit the oxidation of LDL, the more comprehensive effect is their multidimensional effects on atherosclerosis via nitric oxide. Beneficial effects of nitric oxide modulation include regulation of blood pressure, lowering nitric oxide affected hypercholesterolemia, inhibition of platelet aggregation and monocyte adhesion, all of

which are involved in the progression of atherosclerosis. Interestingly, enhancement of nitric oxide levels *in vitro* via the induction of endothelial nitric oxide synthase (eNOS) was seen only with oligomeric procyanidins and of these in particular oligomers of 2 to 10 sub-units with the most effective being the dimer, trimer, tetramer, and pentamer (Romanczyk et al., 1997).

Interestingly, a renal haemodynamic state consistent with nitric oxide system activation, low prevalence of atherosclerotic diseases and no rise in blood pressure with age was recently described in Kuna Indians (Hollenberg et al., 1997 and 1999), a small population of Amerinds living in the San Blas island chain off the Coast of Panama who drink large amounts of cocoa rich in a subclass of flavonoids known as flavanols (Chevaux et al., 2001). Obviously, Kuna Indians could be normotensive and demonstrate increased renal vasodilation due to peculiarities not linked to high flavanol ingestion. Nevertheless, by studying American volunteers, Fisher et al. (2003) furnished a plausible mechanism for the haemodynamic characteristics of Kuna Amerinds demonstrating for the first time in a human study that flavanol-rich cocoa is able to induce vasodilation via nitric oxide activation. The study prospectively assessed the effects of flavanol-rich cocoa (821 mg of catechins and procyanidins/day), using both time and beverage controls. Participants were blinded to intervention and also the endpoint was objective and blinded. Pulsatile artery dilation in the fingertip of 27 healthy subjects using a plethysmographic device designed to reflect only pulsatile arterial volume changes was investigated. After four days of cocoa ingestion there was a clearly defined increase in pulse wave amplitude. The effects of cocoa were observed after 12 h from the last cocoa dose and, on day 5, an additional dose of cocoa led to a further increase in pulse wave amplitude after 90 min. In this respect the data obtained by Fisher et al. (2003) support the role of cocoa-derived polyphenols in inducing arterial vasodilation.

Karim et al. (2000) reported that cocoa extracts dose-dependently caused vasorelaxation in aortic rings obtained from New Zealand White rabbits. In the same study the authors also demonstrated that procyanidins were mostly responsible for aortic ring relaxation and significantly increased nitric oxide synthase activity. The study by Fisher et al. (2003) substantially expands upon this topic, demonstrating that intravenous administration of an arginine-analog blocking nitric oxide synthesis, nitro-L-arginine

methyl ester, significantly reduced the post-ischemic vasorelaxation observed after cocoa ingestion. In addition, the authors compared the endothelial effects of flavanol-rich and flavanol-poor cocoa extracts. Flavanol-rich cocoa induced a more marked endothelium-dependent vasorelaxation measured one minute postischemia compared to a flavanol-poor control beverage. The modest vascular effects exerted by flavano-poor cocoa indicate that flavanols are the primary polyphenols responsible for cocoa-induced vasodilation. From these findings the authors concluded that in healthy humans flavanol-rich cocoa induced vasodilation via activation of the nitric oxide system, providing a plausible mechanism for the protection that flavanol-rich foods induce against coronary events (Fisher et al., 2003).

Taubert et al. (2003) recently reported significant decrements of both systolic and diastolic blood pressure in elderly patients affected by isolated systolic hypertension after eating 100 g of dark chocolate over a period of 14 days. Interestingly, when elderly patients were switched to 100 g of white chocolate per day, blood pressure returned to control. White chocolate bars were substantially polyphenol-free, whereas each daily dose of dark chocolate contained approximately 500 mg of polyphenols supporting the idea that flavanols were responsible for the observed hypotensive effects of cocoa. In this regard it must be noted that Fisher et al. (2003) reported no systemic blood pressure changes in their study population after flavanol-rich cocoa ingestion probably due to the shorter study period.

Evidence in favour of the effects of flavanols on nitric oxide production was provided by Heiss et al. (2003) who showed that the daily ingestion of 100 ml of a cocoa drink rich in flavan-3-ols, but not ingestion of 100 ml of a cocoa drink low in flavan-3-ols, acutely increased brachial artery flow-mediated dilation in six outpatients with at least one cardiovascular risk factor. Because the outpatients studied by Heiss et al. (2003) had a significant degree of endothelial dysfunction at baseline, it might be suggested that flavanols are able not only to further improve a normal endothelial function, but also to ameliorate endothelium-dependent vasorelaxation in subjects with endothelial dysfunction.

1.5.3.4 Effects on immune response and inflammatory diseases

Sanbongi et al. (1997) first investigated the *in vitro* effect of cocoa liquor polyphenols on a variety of human immune cells and their functions. In agreement with the findings that flavonoids are generally immunosuppressive cocoa polyphenols reduced the production of hydrogen peroxide and superoxide anion by phorbol myristate acetate (PMA)-stimulated granulocytes (polymorphonuclear cells [PMN]) and menadione-stimulated lymphocytes. They also suppressed lymphocyte proliferation and interleukin(IL)-2 mRNA and protein production in response to phytohemagglutinin (PHA). IL-2 is involved in the control of T-cell expansion and activation and the regulation of its production is thus critical for initiating an immune response (Mao et al., 2000c). In addition, polyclonal immunoglobulin synthesis by B-cells stimulated with pokeweed mitogen was repressed as well. When the effects of epicatechin on production of reactive oxygen species by granulocytes and on lymphocyte proliferation were compared with those of total cocoa polyphenols, similar levels of inhibition were observed (Sanbongi et al., 1997). The finding that cocoa polyphenols inhibited PHA-induced IL-2 mRNA production in human peripheral blood mononuclear cells (PBMC) was confirmed and extended by the observation that they did not influence IL-2 transcription in unstimulated PBMC (Mao et al., 1999 and 2000c). This inhibition was caused by the oligomers with at least five subunits. In contrast, the monomer fraction had no affect on PHA-stimulated IL-2 mRNA levels. All procyanidin fractions of cocoa stimulated IL-1β mRNA production and protein secretion in human PBMC in either the presence or absence of PHA, although the extent of this stimulation varied widely among subjects (Mao et al., 2000a and c). IL-1β is a multifunctional cytokine that acts on nearly every cell type and is central to the early onset of inflammation in humans (Mao et al., 2000c). The greatest stimulation was observed with fractions containing oligomers consisting of at least five subunits. In another study the same authors (Mao et al., 2000b) investigated whether cocoa, in its isolated procyanidin fractions (monomer through decamer), would modulate synthesis of IL-4. IL-4 is a cytokine that also affects a variety of target cells in multiple ways, however, it has anti-inflammatory properties (Mao et al., 2000c). Both resting and PHA-stimulated PBMC were investigated at the protein secretion level. In resting PBMC the smaller-sized cocoa fractions (tetramer or less) were unable to induce an IL-4 response whereas the larger oligomeric procyanidins (pentamer or greater) stimulated secretion of IL-4. Cells coincubated with

PHA showed a huge increase in secretory IL-4. Interestingly, only the monomeric fraction was able to enhance PHA-induced secretion by 48%. In contrast, the other procyanidin oligomers suppressed IL-4 production, in particular the hexameric, heptameric, and octameric fractions (Mao et al., 2000b). Very recently, Mao et al. (2003) examined whether individual fractions of catechins and procyanidins isolated from cocoa modulate secretion of cytokine transforming factor (TGF)-β_1 from resting human PBMC taken from 13 healthy subjects. Cells from individuals with low baseline levels of TGF-β_1 were stimulated by cocoa polyphenol fractions with the low-molecular fractions being more active than the high-molecular fractions. Interestingly, secretion of TGF-β_1 in cells from individuals with high baseline levels of TGF-β_1 was inhibited by cocoa polyphenols although in this case the high-molecular fractions were more potent (Mao et al., 2003).

Although the mechanism of action of polyphenols inhibiting cytokine transcription is not clear from the studies by Mao et al. (1999, 2000a, b, c, and 2003) the authors suggest a reduction in intracellular reactive oxygen species, which are known to activate nuclear transcription factor NF-κB and which in turn mediates transcription and secretion of many cytokines (Borchers et al., 2000; Mao et al., 2000c). It has been reported that quercetin can suppress the activation of NF-κB induced by either tumor necrosis factor (TNF)-a or hydrogen peroxide (Borchers et al., 2000).

With respect to anti-inflammatory and immune-modulating activity Romanczyk et al. (1997) suggested inhibition of phospholipase A2, cyclo-oxygenase and lipoxygenase thereby decreasing levels of the inflammatory prostaglandin E_2 (PGE_2) as underlying mechanisms. In fact, many flavonoids are powerful antioxidants and have reported to inhibit cyclooxygenases as well as lipoxygenases, the central enzymes in the pathways to eicosanoid production (Sadik et al., 2003). Eicosanoids are mediators of inflammation that not only are implicated in atherogenesis and cancer but also play an important role in rheumatoid and arthritic diseases as well as in allergies and asthma (Borchers et al., 2000).

Cocoa polyphenols have reported elsewhere (Ono et al., 2002) to inhibit the production of nitric oxide in lipopolysaccharide (LPS) and interferon-γ activated macrophages. Uncontrolled production of nitric oxide in macrophages, which are the primary targets

of bacterial LPS, e.g., may be particularly involved in deterioration of hemodynamics in septicemia patients. The suppression of nitric oxide production was suggested to be due to an inhibition of inducible nitric oxide synthase (iNOS) in macrophages (Ono et al., 2002).

Findings with cocoa procyanidins are consistent with results from other cell culture studies of polyphenolics such as quercetin and trans-reservatrol, indicating the ability of selected flavonoids to modulate cytokines involved in acute inflammatory responses (Steinberg et al., 2003). That cocoa flavonoids may have anti-inflammatory properties *in vivo* is suggested by the work of Osakabe et al. (1998), reporting that cocoa polyphenols can reduce the severity of ethanol-induced gastric lesions by reducing myeloperoxidase- and xanthine oxidase-mediated oxidative stress as well as reduction of leukocyte function. However, urinary markers of inflammation including the whole-blood cytokines IL-1β, IL-6 and tumor necrosis factor-α, high sensitive C-reactive protein and P–selectin all remained unchanged in human volunteers supplemented with daily dark chocolate and cocoa powder over six-weeks compared to baseline and washout phases (Mathur et al., 2002).

The possible clinical implications of these data are that cocoa, if proven as a potential immune modulator and anti-inflammatory agent *in vivo*, may have therapeutic advantages in human disease that involve activation of the immune system, such as eczema and arthritis or chronic inflammation including rheumatoid diseases (Mao et al., 2000c). However, it must be noted that the immune-modulating effects of oligomeric cocoa procyanidins have been shown only *in vitro* and because of the current scientific evidence that procyanidins higher than the dimer are not present postabsorptive in its intact form the significance of these finding for the situation *in vivo* has yet to be determined.

1.5.4 Nutrients and nutritional value of chocolate

In drug development and subsequent application for its legal approval, the producer must demonstrate both the efficacy and the safety of the product. In contrast, for food producers having the intention to claim a health effect on foodstuff, food safety is already covered by general rules of existing legislation and it is not necessary to prove again that the foodstuff itself is safe. However, the development of foods and food

components with health claims must take into account basic nutritional principles and nutritional safety considerations (Richardson et al., 2003). Foods with health claims have the potential to influence dietary habits as well as to encourage high levels of consumption of such foods in addition to the normal diet or in substitution of other elements of the diet. Hence, it is essential that their composition should contribute positively to a nutritionally adequate diet and that increased population-wide consumption should generally be in line with the recommendations for a healthy diet as described for example in the WHO/FAO expert consultation on diet, nutrition and the prevention of chronic disease (WHO and FAO, 2003).

Consequently, the extent of use of a product is important and health claims that could encourage high levels of consumption must not be made for any substance where there is evidence that high intakes of the food, which contains nutrients or constituents in amounts that increase the risk of disease or an adverse health-related condition, could be harmful, or be unlikely to contribute to a healthy diet (Codex Alimentarius, 2003; Richardson et al., 2003). In the European Commission proposal for a EU regulation on nutrition and health claims made on foods (European Commission, 2003) it is suggested that alcoholic beverages containing more than 1.2% alcohol (v/v) in general should not be allowed to be labelled with health claims due to the known detrimental effects of chronic high alcohol consumption on alcohol-related morbidity and of all-cause mortality. Similarly, the amount of total fat, saturates, trans fatty acids, sugars, sodium or salt, at variable levels are criteria currently under discussion for determining whether a product can bear claims as scientific research identifies an association between the high consumption of these nutrients and some chronic diseases, such as cardiovascular disease, diabetes, several types of cancer, obesity, osteoporosis and dental disease (European Commission, 2003).

With respect to chocolate it is the high fat content, the high proportion of saturates as well as the high amount of free sugars (added by the manufacturer) that have been discussed with respect to cardiovascular health and dental decay, respectively (Curzon, 1999; Lupien, 1999; Kris-Etherton and Etherton, 1999). These chocolate nutrients and their relation to disease as well as the high energy density of this product in view of the worldwide epidemic of obesity (Hill et al., 2003; Nestle, 2003) are thus discussed in the

following sections in view of the nutritional appropriateness of chocolate for bearing health claims.

Nevertheless, the following considerations are only valid for dark and milk chocolates commonly available on today's market but not for specially designed formulations, such as chocolates free of added sugars or reduced in fat as well as any forms of tablets or foods containing cocoa polyphenol extracts. Of course, the reflections for such products as well as for readily available cocoa powders low or almost free in fat and/or free sugars and consequently low in energy would lead to a different result, however, this is beyond the scope here.

1.5.4.1 Lipids (cocoa butter) in chocolate and cardiovascular health

Cocoa butter accounts for 50% to 57% of the dry weight of cocoa beans and is responsible for the melting properties of chocolate in which it makes up approximately 30% of the total weight. The predominant fatty acids in cocoa butter are saturated (stearic; 18:0, 35% and palmitic; 16:0, 25%) and monounsaturated (oleic; 18:1, 35%), with the remaining fat being primarily polyunsaturated linoleic (3%) (Bracco, 1994). Palmitic and stearic acid are chemically defined as saturated fatty acids (SFA).

Consumption of foods rich in saturated fatty acids and cholesterol, each independently elevating blood cholesterol and LDL concentrations, has long been recognised as an important precursor for the development of coronary heart disease (Connor, 1999). However, whereas the saturated fatty acids lauric (12:0), myristic (14:0), and palmitic (16:0) acids have been shown to raise plasma cholesterol concentrations, stearic acid (18:0) has failed to do so and thus, is considered benign or neutral in this regard (Aro et al., 1997; Katan et al., 1994; Yu et al., 1995).

Evidence for several mechanisms explaining the lack of adverse effect of stearic acid on blood cholesterol and LDL concentrations has been provided. Reduced absorption of stearic acid from the gut has been reported, probably due to the *sn*-1 and *sn*-3 positions of stearic acid in natural triglycerides (Bracco, 1994; Kritschevsky, 1994). After absorption in the gut stearic acid is transported in chylomicrons and remnant particles to the liver where excess amounts are converted to oleic acid (18:1) by a hepatic desaturase (Grundy, 1994; Lin et al., 1993a). Whereas lauric, myristic and palmitic

acids down-regulate the hepatic receptor for LDL and increase the LDL-cholesterol production rate, the 9-cis 18:1 fatty acid (oleic acid) restores receptor activity and lowers the production rate. Stearic as well as 9-trans 18:1 fatty acids are biologically inactive in this context (Woollett and Dietschy, 1994). Finally, Emken (1994) reported on preferential incorporation of stearic acids into phospholipids rather than into cholesteryl-esters and if incorporated into LDL-cholesterol particles those might be more resistant to oxidation (Fuller and Jialal, 1994).

That cocoa butter could have a neutral effect on blood cholesterol in humans was first reported by Grande and colleagues in 1970 (Grande et al., 1970). More evidence for this has been derived from human studies involving semi-synthetic diets including cocoa butter (Denke, 1994a; Kris-Etherton and Mustad, 1994), but also other rich sources of stearic acid such as beef tallow (Denke, 1994b), and sheanut oil (Dougherty et al., 1994). Kris-Etherton et al. (1993) studied subjects who consumed 280 g of chocolate per day incorporated into foods supplying 80% of the approximately 37% of total calories contributed by fat in a controlled diet. Despite the fact that the chocolate-enriched diet was high in saturated fat (approximately 20% of calories), subjects experienced a neutral cholesterolemic response compared with their usual diet that did not include chocolate and which contained about 14% of calories from saturated fatty acids. A subsequent study demonstrated that the daily substitution of a 46 g milk chocolate bar, in place of a high carbohydrate snack in a National Cholesterol Education Program/American Heart Association Step One Diet, did not adversely affect LDL-cholesterol levels (Kris-Etherton et al., 1994). The authors thus suggest that strategies to reduce dietary fat should emphasise the reduction of the cholesterolemic SFA that are regularly consumed in relatively larger quantities rather than stearic acid.

However, in the Nurses' Health Study of 80082 women, stearic acid was associated to an increased risk of coronary heart disease (CHD) to a greater extent than lauric, myristic and palmitic acids (Hu et al., 1999). Connor (1999) proposes for this positive relation mechanisms in the prehepatic phase, prior to the conversion of stearic into oleic acid, including depression of the protective HDL, activation of factor VII, increased lipoprotein(a) concentrations, and impairment of fibrinolysis. Furthermore, dietary stearic acid has been associated with the progression of coronary lesions (Watts et al., 1996) and suggested explanations include increased thrombogenicity via postprandial

lipemia (chylomicrons) or increased platelet activity as well as the activation of coagolation. These reflections led Connor (1999) to the conclusion that chocolate with its high stearic acid content must be put in the category of foods that increase the risk of CHD.

However, the effects of stearic acid on thrombosis and hemostasis are less clear than the neutral effects on cholesterol and LDL, respectively, and seem to vary among *in vitro* and *in vivo* studies (Steinberg et al., 2003). Hoak (1994) states that stearic acid causes hypercoagulability of the blood by activation of factor XII and by aggregation of blood platelets. However, these observations may be mainly based on *in vitro* studies (Connor, 1999), whereas *in vivo* some preliminary studies show favourable effects of stearic acid with regard to factor VII coagulant activity, platelet activity or bleeding time in comparison with fats high in palmitic acid or high in myristic and lauric acids (Schoene et al., 1994; Tholstrup et al., 1994), whereas others confirm the procoagulant effect of stearic acid on factor VII (Mitropolous, 1994). It must be notified that the *in vivo* beneficial effect of stearic acid was shown against fats rich in the other SFAs whereas the *in vivo* adverse effect was shown in comparison to fats high in monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), respectively.

Other *in vivo* studies have studied the effect of stearic acid on proaggregatory (mainly thromboxane A_2) and antiaggregating eicosanoids (prostaglandin I_2, prostacyclin) by measuring the urinary excretion of their stable metabolites as described in chapter 1.5.3.3. When stearic acid rich diets were compared to diets rich in the other SFAs there appeared to be no (Blair et al., 1994) or beneficial effects of stearic acid (Mustad et al., 1993).

Nevertheless, like for factor VII coagulant activity, platelet activity or bleeding time it appears also in the case of eicosanoid biosynthesis that the evaluation of the thrombogenic and hemostatic effects of stearic acid in clinical studies has been mainly determined by the fatty acid pattern of the control diet. In this context it must not be forgotten that diets rich in butter (rich in lauric and myristic acids) or the typical American diet rich in SFAs, which have both been used as controls in the studies cited above, are associated with enhanced thrombosis tendency and adverse cardiovascular effects (Kris-Etherton and Etherton, 1999). Thus, it seems that the long-term effects of

stearic acid consumption on thrombogenic factors are not well elucidated yet and require further research (Kris-Etherton and Etherton, 1999; Steinberg et al., 2003).

In conclusion, there is still debate on the role in cardiovascular health of stearic acid as main constitute of cocoa butter and whether this makes chocolate a unique foodstuff rich in saturated fats making a recommendation for reduced consumption outdated or "pathogenic" with respect to cardiovascular health as concluded by Connor (1999). In fact, Kris-Etherton et al. (2000b) replied to this editorial comment by Connor (1999) mentioning that chocolate specifically was not shown to affect risk of CHD in the study by Hu et al. (1999). This was not unexpected given the very small contribution of chocolate to total SFA intake and the negligible to non-existent effect of chocolate on the PUFA to SFA ratio of the diet. Thus, Kris-Etherton et al. (2000b) considered the comment made by Connor (1999) that chocolate is pathogenic not justified on the basis of either chocolate's stearic acid content or its contribution to the PUFA to SFA ratio. However, Hu et al. (2000) pointed out that their study (Hu et al., 1999) **drew no conclusions** regarding chocolate consumption and risk of coronary heart disease (CHD), but stearic acid as well as the sum of all long-chain SFAs and CHD showed a positive, although modest association. This being the case Connor (2000) emphasises that it is important that the total saturated fat content of the diet be reduced and that the saturated fat content of the diet is derived from individual foods, of which chocolate is one containing 59% of total fatty acids as saturates. In Connor's calculations the consumption of even one chocolate bar per day would use up one-third to one-half of the day's saturated fat allowance (Connor, 2000).

It seems obvious that the inclusion of a moderate amount of chocolate into the diet is not predicted to have adverse effects on the lipid and lipoprotein profile of individuals, as long as the total fat and caloric intake is held constant. Consumption of large amounts of chocolate, however, which provides excess fat and calories to the diet beyond estimated maintenance needs, could contribute to obesity and negatively impact CVD incidence (Steinberg et al., 2003).

1.5.4.2 Free sugars in chocolate

Dental diseases include dental caries, developmental defects of enamel, dental erosion and periodontal disease. Dental diseases are a costly burden to health care services,

accounting for between 5% and 10% of total health care expenditures and exceeding the cost of treating cardiovascular disease, cancer and osteoporosis in industrialised countries. Although not life-threatening, dental diseases have a detrimental effect on the quality of life in childhood through to old age, having an impact on self-esteem, eating ability, nutrition and health. In modern society a significant role of teeth is to enhance appearance. Facial appearance is very important in determining an individual's integration into society and teeth also play an essential role in speech and communication. Oral diseases are associated with considerable pain, anxiety and impaired social functioning. Dental decay may result in tooth loss, which reduces the ability to eat a nutritious diet, the enjoyment of food, the confidence to socialise and the quality of life (WHO and FAO, 2003).

There is a wealth of evidence from many different types of investigation, including human studies, animal experiments and experimental studies *in vivo* and *in vitro* to show the role of dietary sugars in the aetiology of dental caries, although not in the other dental diseases (Rugg-Gunn, 1993; WHO and FAO, 2003). Collectively, data from these studies provide an overall picture of the cariogenic potential of carbohydrates. Sugars are undoubtedly the most important dietary factor in the development of dental caries (WHO and FAO, 2003). For clarification, the term ''sugars'' refers to all monosaccharides and disaccharides while the term ''sugar'' refers only to sucrose. The term ''free sugars'' refers to all monosaccharides and disaccharides added to foods by the manufacturer.

Research has consistently shown that when annual sugar consumption exceeds 15 kg per person per year (or 40 g per person per day) dental caries increase with increasing sugar intake. When sugar consumption is below 10 kg per person per year (around 27 g per person per day), levels of dental caries are very low. Exposure to fluoride (i.e. where the proportion of fluoride in drinking water is 0.7-1.0 ppm, or where over 90% of toothpastes available contain fluoride) increases the safe level of sugars consumption (WHO and FAO, 2003).

With respect to chocolate data from human studies are very limited. The many human observational studies on diet and dental caries rarely mention chocolate and, if they do, it is included with confectionary which makes it difficult to draw conclusion for

chocolate (Curzon, 1999). Among human intervention studies, the Vipeholm Study (Gustafsson et al., 1954), which plays a major role in any consideration of diet and dental caries (Curzon, 1999), remains the only intervention study involving chocolate. Over a study period of several years groups of adult subjects were given dietary supplements either between-meals snacks or added to meals. All candy-eating (confectionery) groups including the chocolate group developed more cavities than the no-candy groups. However, the chocolate group had lower caries increments than the groups using caramels or toffees between the meals. Nevertheless, the caries activity of chocolate appeared to be higher than for sucrose (Gustafsson et al., 1954).

Additional data for the cariogenic potential of chocolate derive from intra-oral plaque studies. These involve the measurement of pH in plaque on or in between the teeth. The ability of a food to encourage or create an acid plaque is related to the food's ability to initiate or encourage the development of dental caries. In such studies chocolate usually falls in the middle of scores and is therefore considered only of moderate cariogenicity. Moreover, it appears that added ingredients, such as nuts or skim milk, decrease the acidogenic potential of chocolate (Curzon, 1999). Nevertheless, the acidogenicity of a foodstuff cannot be taken as a direct measurement of its cariogenic potential. Plaque pH studies take no account of protective factors in foods, salivary flow and the effects of other components of the diet (WHO and FAO, 2003).

Quite some animal models have been used for investigating the cariogenic potential of chocolate. Cariogenic Potential Indices (CPI) ranged from 0.7 to 0.8 with sucrose being the reference at 1.0. Interestingly, several of theses studies reported on a reduced cariogenicity of chocolate when calcium caseinate or milk products were added (Curzon, 1999). Grenby and Mistry (1995) found a 30% decrease in caries scores in rats for milk chocolate compared to dark chocolate. Obviously, caution needs to be applied when extrapolating the results of animal studies to humans because of differences in tooth morphology, plaque bacterial ecology, salivary flow and composition, and the form in which the diet is provided (usually powdered form in animal experiments). Nonetheless, animal studies have enabled the effect on caries of defined types, frequencies and amounts of carbohydrates to be studied. (WHO and FAO, 2003).

Finally, the speed with which a food or beverage clears from the mouth has been associated with its potential cariogenicity, slow-clearing foods being thought to be more cariogenic. The little information available suggests that chocolate products, particularly those containing added ingredients such as nuts and milk products cleared from the mouth relatively quickly (Curzon, 1999). In addition, Curzon (1999) suggested that the observed moderate cariogenicity of chocolate might be related to the cocoa fraction, which contains phytochemicals inhibitory to oral bacteria.

1.5.4.3 High energy-density of chocolate

Obesity is a global public health concern that claims an increasing number of lives worldwide (Hill et al., 2003). The number of overweight people is higher than ever. Between 1999 and 2000 in the United States almost 65% or two thirds of the adult population was overweight, i.e. having a body mass index (BMI) greater than 25 kg/m^2 and 31% are obese (BMI greater than 30 kg/m^2) compared to 56% and 23%, respectively, between 1988 and 1994. Probably even more dramatically, the prevalence in obese children in the U.S. has increased from 11% to 15% (1988-1994) to 36% in 1999 and 2000. Although the actual numbers might be highest for the U.S., most countries experience more dramatic relative increases in obesity and there is no sign that this tendency will halt or reverse (Hill et al., 2003).

Obesity is strongly related to at least 30 medical conditions including heart disease, diabetes, hypertension, some cancers and sleep apnea, and is estimated in the United States to contribute to over 300,000 deaths and $103 billion each year in health care costs (McGinnis and Foege, 1993; Wolf and Colditz, 1998). Particularly disturbing in this context is the 10-fold increase in incidences of type-2 diabetes among children between 1982 and 1994 (Pinhas-Hamiel et al., 1996). Consequently, the World Health Organisation (WHO) has declared overweight as one of top ten risk conditions in the world and one of the top five in developed nations (WHO, 2002).

Although obesity is a complex and multifactorial issue spanning diet, physical activity, genetics, and behaviour there is growing agreement among experts that the environment, rather than biology, is driving the epidemic (Hill and Peters, 1998; Hill et al., 2003). Biology clearly contributes to individual differences in weight and height, but the rapid weight gain that has occurred over the past three decades is a result of the

environment. When energy intake exceeds energy expenditure over time an increase in weight results. Possible factors in the environment that promote overconsumption of energy include the easy availability of a wide variety of good-tasting, inexpensive, energy-dense foods and the serving of these foods in large portions (Hill et al., 2003).

There is convincing evidence that a high intake of energy-dense foods promotes weight gain (WHO and FAO, 2003). Energy-dense foods tend to be high in fat (e.g. butter, oils, fried foods), sugars or starch and low in non-starch polysaccharides and micronutrient density. Several trials have covertly manipulated the fat content and the energy density of diets, the results of which support the view that so-called ‘‘passive over-consumption’’ of total energy occurs when the energy density of the diet is high and that this is almost always the case in high-fat diets (WHO and FAO, 2003). In particular excess energy consumption (in relation to expenditure) has been observed in diets predominantly made up of fat-rich foods due to a low satiety quotient (Bolton-Smith and Hetherington, 1999). A meta-analysis of 16 trials of ad libitum high-fat versus low-fat diets of at least 2 months duration suggested that a reduction in fat content by 10% corresponds to about a 1 MJ reduction in energy intake and about 3 kg in body weight (Astrup et al., 2000). At a population level, 3 kg equates to about one BMI unit or about a 5% difference in obesity prevalence (WHO and FAO, 2003).

In line with Astrup et al. (2000) other studies in both lean and obese adult women show that energy density results in increased energy intake, however, this was independent from the content in fat and other macronutrients (Bell et al., 1998; Rolls et al., 1999). The authors suggest that energy density is more closely related to factors such as the water and fibre contents of foods. In contrast, Bray and Popkin (1998) found that high-fat diet increases the likelihood of developing obesity. This is in line with the findings from another meta-analysis by Yu-Poth et al. (1999) showing that fat-reduced diets cause a dose-dependent decrease in energy intake and body weight. Nevertheless, Kris-Etherton et al. (2000a) mentioned that a higher-fat weight-loss diet was not tested, so one can question whether weight loss might be less, the same or maybe even greater because of better adherence to the higher-fat (i.e., high MUFA), energy-reduced diet. Although the authors agree that an energy imbalance is the root cause, they see no compelling evidence that this is due to changes in fat intake. Another case in point relates to the increase in the incidence of overweight and obesity with concurrent little

or no change in absolute intake of fat and a decrease in percentage of energy from fat in the U.S. and other countries (Kris-Etherton et al. 2000a). However, Marckmann and Astrup (2000) warn against considering high-fat monoene diets to be the most healthy diets arguing that in many trials comparing diets with various fat contents, energy intake was fixed and constant (i.e., isoenergetic conditions were maintained) to keep body weight constant, which however, is an inappropriate design because spontaneous energy intake would normally differ if diets with different fat contents were eaten ad libitum. Besides, even the olive oil consuming Greeks have become more obese during the past decades due to their increasingly sedentary lifestyle (Marckmann and Astrup, 2000).

Like for dietary fat it is considered that restriction of free sugars likely contributes to reducing the risk of unhealthy weight gain, noting that free sugars contribute to the overall energy density of diets thereby promoting a positive energy balance. Moreover, it is recognised that higher intakes of free sugars threaten the nutrient quality of diets by providing significant energy without specific nutrients (WHO and FAO, 2003). Diets that are limited in free sugars have been shown to reduce total energy intake and induce weight loss (Mann et al., 1970; Smith et al., 1996). The CARMEN study (Carbohydrate Ratio Management in European National diets) was a multi-centre randomised trial that tested the effects of altering the ratio of fat to carbohydrate on body weight and blood lipids in overweight individuals, as well as the effects of the ratio of simple to complex carbohydrate per se. A greater weight reduction was observed with the high complex carbohydrate diet relative to the simple carbohydrate one, although the difference was not statistically significant (Saris et al., 2000). Nevertheless, an analysis of weight change and metabolic indices for those with metabolic syndrome revealed a clear benefit of replacing simple by complex carbohydrates (Poppitt et al., 2002).

Chocolate, with an energy content of approximately 480 kcal/ 100 g, of which fat calories account for ca 55% of total energy and those of sugars for ca 43%, has not been studied much with respect to its relation to obesity because of its high energy-density or fat content (Bolton-Smith and Hetherington, 1999). Because laboratory studies of fat preferences in obese consumers reveal a close relationship between taste preferences for fat and body fat (Drewnowsky et al., 1985; Mela and Sacchetti, 1991), Drewnowsky et al. (1992) asked a large clinical sample of obese men and women to list their ten favourite foods. Chocolate was rated in the top ten favourite foods by 32% of women

and 15% of men, respectively. Together with the other foods rated this study confirmed the preference for fat-rich foods including chocolate, although gender differences appear to be important as well (Drewnowski et al., 1992). The same group noted cravings for chocolate amongst obese binge eaters in another study (Drewnowski et al., 1991). However, it is not clear whether such taste preferences develop as a function of adiposity or as a function of habitual dietary patterns (Bolton-Smith and Hetherington, 1999). In fact, the palatability of chocolate is a powerful element in its consumption. Thus, it is suggested that a preference for chocolate may not be predicted on the basis of body weight or body fat, but rather on liking, intake and patterns of eating across weight categories (Bolton-Smith and Hetherington, 1999). Nevertheless, by providing a highly pleasurable, readily available and energy-dense snack, chocolate may perhaps contribute to weight gain or maintenance in obese by providing the typical hedonistic properties of high-fat and high-sugar foods which can override regulatory mechanisms and result in excess energy intake (Green and Blundell, 1996).

1.5.4.4 Conclusions

It should be emphasised that it was not the purpose in this section to evaluate, whether chocolate in general should be considered as “good” or “bad” foodstuff. It was rather tempted to view chocolate in the light of a future application for a health claim in advertisement or labelling that needs to be approved by regulatory bodies taking into account the impact that an enhanced population-wide consumption due to a more positive consumer perception may have. Moreover, together with the health claim, both in the Codex Alimentarius work on guidelines for the use of nutrition and health claims (Codex Alimentarius, 2003) and in a European Commission proposal for a EU regulation on nutrition and health claims made on foods (European Commission, 2003) a requirement for a statement of frequency and amount of consumption, for which the claimed effect can be expected, is proposed.

For example, in the experiment by Taubert et al. (2003) described in chapter 1.5.3.3, fourteen consecutive daily doses of 100 g of dark chocolate decreased systolic blood pressure by 5.1 mmHg and increased calorie intake by 480 kcal/day. If this was the targeted amount needed for obtaining the desired effect, in the long run (i.e. in real life),

this would result in a progressive increment of body weight, which in turn, is likely to counterbalance the positive effects of cocoa on vascular function.

In fact, even though fat, free sugars, and energy density and each relation to major diseases including CVD, dental disease, and obesity have been treated separately in the discussions above, it is obvious that they are inherently linked. Consequently, the joint WHA/FAO expert consultation on diet, nutrition and the prevention of chronic diseases formulated "population nutrient intake goals for preventing diet-related chronic diseases" that include a daily energy intake from saturated fats as well as from free sugars of each less than 10% (WHO and FAO, 2003). For a 2500 kilocalories diet this calculates to less than 250 kcal for each, or less than 28 g saturated fat and 63 g free sugars, respectively. The consumption of 100 g dark chocolate, containing around 18 g of saturated fat and normally about 45 g of free sugars (Würsch and Finot, 1999), would alone consume up 65% of "allowed" saturated fat and 71% of "allowed" free sugars.

There is strong evidence that high intakes of fruits and vegetables are associated with less coronary heart disease and cancer morbidity. Of course, the example of 100 g chocolate daily was only an example to illustrate the nutritional dimension of daily consumption of very high quantities. Actually, it has been repeatedly suggested that including flavonoid-rich dark chocolate in *"small or moderate amounts"* may allow for greater flexibility, adherence, and palatability of an individual's meal plan, yet still promote cardiovascular health (e.g., Steinberg et al., 2003). However, as discussed earlier, a possible future use of health claims with chocolate might lead to a significant promotion of chocolate consumption both on an individual and population level due to consumer perception of chocolate as a "good" foodstuff. Apart from the possible direct health implications of an excess consumption of chocolate as discussed above, indirect adverse health effects may result because high-fat, high-sugar energy-dense diets prevent high intakes of fruits and vegetables owing to the low energy ceiling of modern sedentary societies. (Marckmann and Astrup, 2000). Consequently, energy density is perhaps the most important determinant of how much chocolate can be included in a moderate-fat dietary pattern as is recommended by the American Heart Association. Based on the current pandemic explosion of obesity, which is particularly evident in children (Hill et al., 2003), we should be careful when recommending chocolate ingestion to the public, including the use of health claims.

1.6 Scientific substantiation of health claims – the biomarker approach

Stahl et al. (2002) consider any compound that enters the 'systemic circulation' bioactive, in that it has a metabolic impact, even if it is only the stimulation of the detoxification process or the use of energy for its excretion. It is on the other hand important to emphasise that a compound need not enter the systemic circulation to be bioactive as the activity can already be exerted in the gastrointestinal tract (Stahl et al., 2002). Thus, the objective should be to demonstrate the efficacy of functional foods or food constituents with regard to a desired function, such as disease prevention or enhancement of a desired function rather than a general bioactivity of food compounds.

Large-scaled human interventions trials with a hard end-point such as disease occurrence or cause-specific mortality are considered as the gold standard for testing the effectiveness of functional food components in humans. However, these trials are extremely time-consuming often having a long latency time of diseases such as cancer. In addition they are of enormous costs. Still, just a limited number of compounds and doses can be tested often only in high-risk population groups, which have to be of a high initial number of participants to evaluate a health effect. Human intervention studies with markers of intermediate endpoints reduce the costs and the time taken for evidence to accumulate to support a beneficial effect of a functional food. They can be used to establish the optimal intake of an agent and, thus, provide the scientific basis for the intervention strategy in major human intervention trials, which will in turn validate or disprove the biomarker concept. Finally, instead of waiting for many years for a disease to develop, one may be able to quantify susceptibility markers and intermediate endpoint markers and intervene before the disease fully develops (Diplock et al., 1998; Halliwell, 1999; Loft and Poulsen, 1999; Roberts, 2002).

As described in section 1.5.3.1 polyphenols including those from cocoa have gained much attention recently owing to their antioxidant capacity (free radical scavenging and metal chelating) and their possible beneficial implications in human health such as in the treatment and prevention of cancer, cardiovascular disease, and other pathologies. The link between oxidative stress and increased risk of chronic diseases has generated the hypothesis that dietary antioxidants contribute to health and well-being and reduce the risk for chronic diseases. A substantial number of so-called scavenging assays have

been developed and applied to study total antioxidant capacity of plasma, serum or other biological fluids including the trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), 1-diphenyl-2-picrylhydrazyl (DPPH), and ferric reducing ability (FRAP) assays (Crews et al., 2001). A general problem with these relatively simple assays is that they are poorly validated with respect to their pathological relevance in disease development and therefore their specificity as biomarkers is still under debate.

Instead of scavenging assays the measurements of biomarkers of oxidative protein and DNA damage as well as lipid peroxidation can be used to establish the role of antioxidants in this protection and the optimal intake of these antioxidants. This concept is based on the well-accepted presumptions that oxidative DNA damage is a significant contributor to age-related development of some cancers, that lipid peroxidation plays a key role in the development of cardiovascular disease and that elevated protein oxidation is involved in various pathologies, such as cataractogenesis, atherosclerosis, rheumatoid arthritis, diabetes, and neruodegenerative diseases including Alzheimer's disease (Halliwell, 1999; Griffiths, 2000).

Probably the most promising biomarkers for oxidative damage are various families of isoprostanes for lipid peroxidation and of multiple DNA base oxidation products, in particular 8-hydroxydeoxyguanosine (8-OHdG), in human fluids, such as urine and plasma (Diplock et al., 1998; Halliwell, 1999). Several markers of protein oxidative damage have been proposed as well including protein carbonyls, nitrotyrosine, dityrosine, L-DOPA and others (Griffiths, 2000). However, the complexity of protein structure, arising from the primary sequence and involvement of carbohydrate moieties in structure stabilisation, together with a lack of specific and sensitive methodologies, has hindered the identification of a biomarker that sufficiently fulfils the criteria defined in at least two consensus documents of leading scientists in the field (Diplock et al., 1999; Griffiths et al., 2002).

Antoine and Diplock (2000) have subdivided the criteria in biological or patho-physiological validity as well as analytical validity of markers. For the former a marker of oxidative damage must be demonstrated to be a major product of oxidative modification with a relatively immediate sensitive and specific outcome that is clearly

linked to the development of a disease in order to replace later and more remote outcomes in intervention studies. Moreover, those markers must be minimally invasive and ethical in experimental studies. Whereas there is general agreement on the patho-physiological relevance of both 8-OHdG (Cooke et al., 2000; Diplock, 2000; Halliwell, 2000a; Loft and Poulsen, 2000; Griffiths et al., 2002; Halliwell, 2002) and isoprostanes (Lawson et al., 1999; Liu et al., 1999; Morrow et al., 1999; Praticò, 1999; Halliwell, 2000b; Mezzetti et al., 2000; Cracowski et al., 2002a), there is still debate on their analytical validity. The marker must be a stable product not susceptible to artefactual induction or loss during storage as well as free of confounding factors from the dietary intake. The requirements further include the availability of a reliable analytical procedure that is sufficiently specific, sensitive, reproducible, and robust (Antoine and Diplock, 2000; Griffiths et al., 2002).

In the following the patho-physiological disease links as well as the available methodologies for the measurement of 8-OHdG and F_2-isoprostanes are reviewed and discussed as the set-up, validation and application of fast methodologies for the measurement of these two markers of oxidative stress in human urine has been selected as one of the objectives for the experimental part of this work.

1.6.1 8-OHdG as a marker of oxidative DNA damage

Damage to DNA generates a multiplicity of different modifications including sugar and base modifications, strand breaks and DNA-protein cross-links (reviewed in Dizdaroglu et al., 2002). Of the few base modifications, which have so far been used as markers for oxidative DNA damage, the hydroxylation product of guanosine, 8-hydroxydeoxyguanosine (8-OHdG) (figure 1.9), has been the most investigated lesion (Loft and Poulsen, 2000), after its formation was first reported in 1984 by Kasai (reviewed in Kasai, 1997). 8-OHdG is known to be mutagenic (Kasai, 1997) and it is therefore repaired by excision and unchanged and independently from dietary sources (Gackowski et al., 2001; Topp et al., 2002) excreted into the urine where it remains remarkably stable during storage (Diplock et al. 1998; Griffiths et al., 2002).

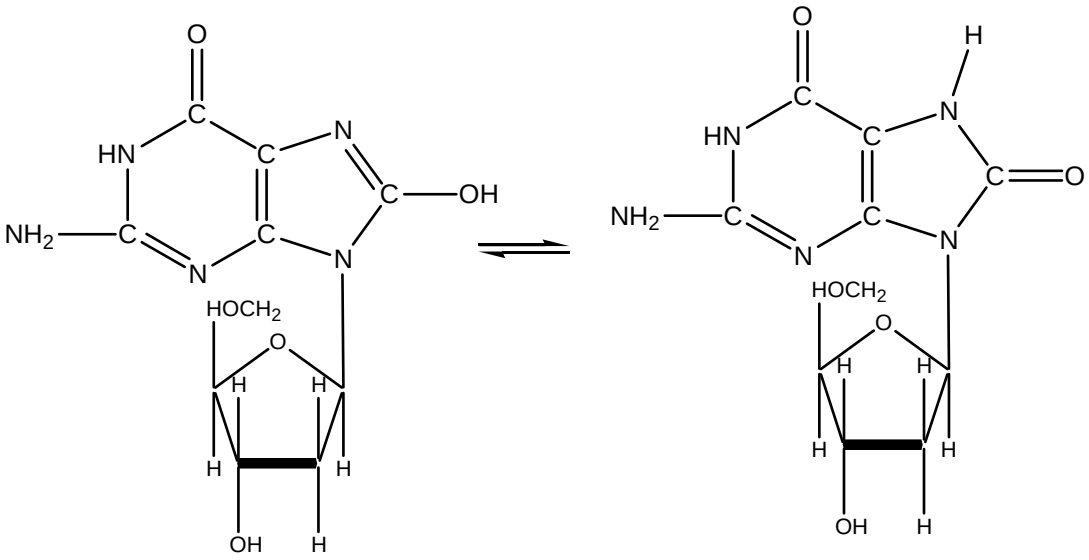


Figure 1.9 Chemical structures of 8-hydroxydeoxyguanosine (8-OdHG) (left) and its keto-isomer 8-oxo-deoxyguanosine (8-oxo-dG) (right).

The most widely used analytical methods for 8-OHdG determination in urine and DNA purified from cells are high performance liquid chromatography with electrochemical detection (HPLC-ECD) (Germandik et al., 1997; Helbock et al., 1998) and to a smaller extent gas chromatography with mass spectrometric detection (GC-MS) (Teixera et al., 1993; Rehman et al., 1998). However, estimated levels of oxidative DNA damage in human cells, calf thymus DNA and liver tissue and even of standard 8-OHdG showed wide variations between laboratories and between methods applied (Collins et al., 1997; Lunec, 1998). In order to resolve methodological problems and to reach agreement on the basal level of oxidative damage in human cells, the European Standards Committee for Oxidative DNA Damage (ESCODD) was set up in 1997 and a number of inter-laboratory studies with different study material involving mainly HPLC-ECD and GC-MS based methodologies have been conducted (ESCODD 2000, 2002a and 2002b, 2003; Riis and ESCODD, 2002). Protocols have been optimised in order to limit the problem of oxidised artefacts during sample preparation in particular for GC-MS analysis. In most cases, HPLC-ECD was superior to GC-MS with respect to reproducibility and sensitivity. However, this might be true only for isolated DNA from human cells and not for the analysis of urinary 8-OHdG, which was so far not subject of an inter-laboratory study. Indeed, analysis of urinary 8-OHdG by HPLC-ECD requires time-consuming clean-up procedures such as multiple solid phase extraction (SPE)

steps (Shigenaga et al. 1989; Germandik et al. 1997; Pilger et al., 2001; Pilger et al., 2002), column switching techniques (Loft and Poulsen, 1999), carbon column capture (Bogdanov et al., 1999), or immunoaffinity clean-up (Degan et al., 1991). Additionally, interfering compounds can easily co-elute and obscure the 8-OHdG peak and by using electrochemical detection there is no structural evidence for identification of the analyte (Helbock et al., 1998). Mass spectrometric detection providing structural evidence represents a means to clearly identify and quantify different compounds (Offord et al. 2000) and it is more versatile than the HPLC-ECD assay, since a wide array of lesions can be measured (Ravant et al., 1998; Dizdaroglu et al., 2002). Nevertheless, measurement of urinary 8-OHdG by GC-MS also requires extensive sample work-up including both solid-phase extraction and/or HPLC prepurification as well as a derivatisation step, which has limited the use of this technique (Holmberg et al., 1999; Cooke et al., 2002). In contrast, with the recent development of HPLC with tandem mass spectrometric detection comparatively little sample clean-up is required while offering both measurement and confirmatory identification of 8-OHdG (Ravant et al., 1998; Weimann et al., 2001).

1.6.2 F_2-isoprostanes as a marker of lipid peroxidation

Lipid peroxidation, which is the oxidation process of poly unsaturated fatty acids (PUFA), involves a cascade of events characterised by the disappearance of PUFAs, the appearance of radicals within the PUFAs and the formation of conjugated dienes, lipid peroxides, aldehydes or alkanes (reviewed in Abuja and Albertini, 2001). In recent years there has been an increasing consensus that F_2-isoprostanes are the best currently available markers of lipid peroxidation in the human body (Halliwell, 2000b; Roberts II and Morrow, 2000). In 1990 Morrow and Roberts first reported that stable prostaglandin-like compounds are produced *in vivo* in humans by a free radical-catalysed mechanism (Morrow et al., 1990). Unlike the classic prostaglandins (PG), which are formed through the action of cyclooxygenase (COX), these compounds, being analogous to $PGF_{2\alpha}$, and therefore termed F_2-isoprostanes are generated by free radical attack of arachidonic acid in phospholipids of cell membranes (Morrow et al., 1992). Depending upon which of the labile hydrogen atom is first abstracted by free radicals, up to 64 different F_2-isoprostanes, as either 5-, 12-, 8- or 15-series, can theoretically be formed. Esterified F_2-isoprostanes are cleaved by phospholipase(s) and

both metabolised and excreted into urine (reviewed by Praticò, 1999; Lawson et al., 1999; as well as Roberts II and Morrow, 2000). Detectable quantities of F_2-isoprostanes are present in virtually all tissues and human biological fluids including urine, plasma, bile, cerebrospinal fluid, seminal fluid, pericardial fluid, and exhaled breath condensate (Morrow and Roberts II, 1999; Cracowski et al., 2002a).

The free radical product that is used most as a marker for oxidative stress is 15-F_{2t}-IsoP[1], a major isoprostane that has received considerable attention because it is also biologically active in terms of exhibiting potent renal pulmonary and arterial vasoconstrictor activity in various animal models (Praticò, 1999; Tarcan et al., 2000). 15-F_{2t}-IsoP is believed to interact with a novel class of prostaglandin receptors exerting discrete effects on platelets and the endothelium (Minuz et al., 1998). The chemical structure of 15-F_{2t}-IsoP, to date the only commercially available isopostane, is depicted in figure 1.10.

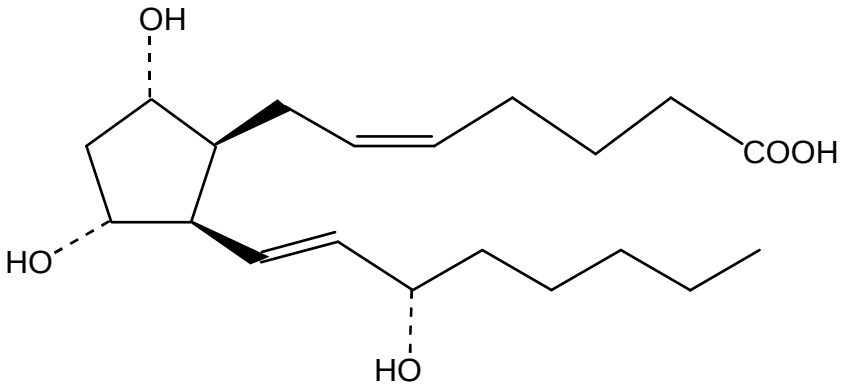


Figure 1.10 Chemical structure 15-F_{2t}-isoprostane (15-F_{2t}-IsoP).

There is a vast body of evidence from many clinical studies supporting the patho-physiological validity of 15-F_{2t}-IsoP as a marker of lipid peroxidation, which is involved in various forms of cardiovascular, systemic and inflammatory, hepatic and

[1] Nomenclature refers to the nomenclature system approved by the International Union of Pure and Applied Chemistry (IUPAC) (Taber et al., 1997). Other terms for 15-F_{2t}-IsoP found in the literature are the initial terms 8-*epi*-$PGF_{2\alpha}$ and 8-*iso*-$PGF_{2\alpha}$ or $iPF_{2\alpha}$-III according to the nomenclature system proposed by Rokach et al., 1997.

gastrointestinal, pulmonary, neurological, nephrological, and obstretric diseases (reviewed in Lawson et al., 1999; Liu et al., 1999; Morrow et al., 1999; Praticò, 1999; Mezzetti et al., 2000; and Cracowski et al., 2002a). Increased levels of 15-F_{2t}-IsoP have also been reported in humans in conditions not causally linked with lipid peroxidation but that predispose to accelerated development of cardiovascular diseases (CVD) partly mediated by lipid peroxidation such as diabetes (Murai et al., 2000), hypercholesterolemia (Nourooz-Zadeh et al., 2001), hyperhomocysteinemia (Liu et al., 1999), inflammatory diseases (Cracowski et al., 2002a), as well as chronic alcoholism (Meagher et al., 1999) and cigarette smoking (Obata et al., 2000). It is noteworthy that 15-F_{2t}-IsoP concentrations were approximately twice as high in umbilical cords from newborn babies of smoking mothers compared with those of non-smoking mothers (Obwegeser et al., 1999). Interestingly, the elevated urinary F_2-isoprostane levels were reduced following improved metabolic control over 4 weeks in type 2 diabetes, 30 days abstinence in patients with severe alcoholic liver disease and following treatment in patients with chronic obstructive pulmonary disease (Cracowski et al., 2002a). The reduction of F_2-isoprostane levels correlated with clinical improvement, further suggesting that oxidative stress might be an important factor in such diseases. Supplementation of vitamin E, vitamin C, beta-carotene and combinations of these antioxidant vitamins induced a reduction of urinary F_2-isoprostane levels in a dose-dependent manner in patient populations that are characterised by high rates of lipid peroxidation including patients with cystic fibrosis, type 2 diabetes, and chronic alcoholic liver disease (Cracowski et al., 2002a). In contrast, conflicting results are reported for the effect on urinary F_2-isoprostane excretion by antioxidant vitamin supplementation in healthy subjects. Patrignani et al. (2000) suggest that the basal rate of lipid peroxidation is a major determinant of the response to antioxidant supplementation. Nevertheless, Morrow et al., (1999) have shown a dose-dependent reduction of 25% and 37% of plasma lipid-esterified 15-F_{2t}-IsoP after two-week supplementation with 400 and 800 IU/day vitamin E in normal volunteers as well as a 21% decrease in plasma F_2-isoprostanes after one year of vitamin E administration in 100 healthy men. In further accordance with the antioxidant hypothesis, diets rich in fruits and vegetables diminish the excretion of urinary 15-F_{2t}-IsoP suggesting enhancement of antioxidant defence as an important factor of such diets (Thompson et al., 1999).

Among the biological fluids available most studies were performed on urine because of the non-invasiveness of the procedure and the lack of artefactual generation of 15-F_{2t}-IsoP during storage as observed for plasma or tissue 15-F_{2t}-IsoP (Cracowski et al., 2002a). Another disadvantage of measuring isprostanes in plasma is that it is not possible to measure them over a period of time, since plasma half time is only approximately 18 minutes and they are excreted rapidly into urine (Griffiths et al., 2002). Thus, alike urinary 8-OHdG reflecting overall oxidative DNA damage (Loft and Poulsen, 2000), urinary 15-F_{2t}-IsoP assessing its total endogenous production is expected to reflect "whole body" lipid peroxidation (Halliwell, 2000b; Roberts II and Morrow, 2000).

In recent studies it was shown that 15-F_{2t}-IsoP levels in urine and plasma are not confounded by the lipid content of the diet (Richelle et al., 1999; Gopaul et al., 2000), which contributes to the several favourable attributes of the measurement of 15-F_{2t}-IsoP in urine (Halliwell 2000b, Roberts II and Morrow, 2000). However, Richelle et al. (1999) showed that urinary 15-F_{2t}-IsoP excretion was elevated by 40 % following 24 h fasting due to induction of oxidative stress and/or the release of 15-F_{2t}-IsoP from adipose tissue. Also extreme endurance exercise is associated with an increased production of F_2-isoprostanes (Mastaloudis et al., 2001). As a consequence, urine samples should be taken at rest and not during long fasting periods to avoid these potential confounding factors.

The analytical methods for isoprostanes are mainly based on the sensitive and specific capillary GC-isotope dilution-negative ion electron capture chemical ionisation (NCI) MS technique (Tsikas, 1998; Lawson et al., 1999; Morrow et al., 1999; Mori et al., 1999; Walter et al., 2000; Caccetta et al., 2001; Hodgson et al., 2002). However, GC-MS analysis of urinary F_2-isoprostanes requires extensive sample clean-up consisting of one or two SPE steps and/or prepurification by HPLC or two-step thin-layer chromatography (TLC) (Ferretti and Flanagan, 1997; Praticò et al., 1998; Lawson et al., 1999; Morrow and Roberts II, 1999; Gopaul et al., 2000; Obata et al., 2000, Cracowski et al., 2002b). The introduction of GC-NCI-tandem mass spectrometry (MS-MS) has increased specificity in compound identification and allowed reducing sample preparation although still requiring one SPE and one TLC or HPLC clean-up (Tsikas et al., 1998; Richelle et al., 1999; Schwedhelm et al., 2000). A great simplification of the

sample preparation was achieved by replacing all SPE as well as TLC and HPLC clean-up steps by a single immunoaffinity extraction of 15-F_{2t}-IsoP prior to GC-NCI-MS (Bachi et al., 1996: Tsikas et al., 2003). However, the columns need to be reused because of the laborious generation of antibody and immunoaffinity columns, so sample carryover must be monitored, and the columns have a finite lifespan requiring a constant supply of antibody (Lawson et al., 1999). Finally, the conversion of the compounds of interest into volatile derivates is necessary, and this derivatization step, which is usually carried out at high temperatures, can contribute to artefactual formation of 15-F_{2t}-IsoP in plasma samples (Roberts II and Morrow, 2000; Halliwell, 2002). Generally, complicated preparation procedures before analysis often produce recovery problems (Loft and Poulsen, 1999), are time-consuming and thus clearly limit the analytical capacity.

Both radioimmunoassys (RIA) and enzyme immunoassays (EIA) have been developed for and applied to the analysis of isoprostanes, and more specifically for 15-F_{2t}-IsoP (Wang et al., 1995; Proudfoot et al., 1999; Basu et al., 2001; Helmersson and Basu 2001; Feillet-Coudray et al., 2002). However, Lawson et al., (1999) noted that cross-reactivity may be an insufficiently investigated problem of 15-F_{2t}-IsoP immunoassays due to the possible interference of 63 other possible isomers all sharing the same basic 1,3-syn-hydroxycyclopentane ring structure, which is believed to be the major prostaglandin antigenicity. In addition, urinary metabolites probably present in higher amounts than the parent isoprostanes might complicate the situation further. Finally, because the degree of antibody cross-reactivity can vary from batch to batch, quantitative comparisons of data from different antibodies should be made with caution (Lawrence et al., 1998). Indeed, a comparison of a commercially available EIA and GC-MS showed poor correlation with both significant fixed bias (where there is a consistent difference between the methods) and proportional bias (where one method gives values higher or lower than the other method by an amount proportional to the size of measurement) with the EIA giving lower 15-F_{2t}-IsoP levels at low concentrations and higher levels at high concentrations (Proudfoot et al., 1999).

More recently following the wider spreading of improved instrumentation, HPLC with tandem mass spectrometry has been applied for the analysis of urinary F_2-isoprostanes. Lawson et al., (1998) achieved separation of the four classes of F_2-isoprostanes, namely

the 5-, 8-, 12-, and 15-series (Taber et al., 1997) with good signal-to-noise ratio following a single SPE step without need for derivatisation by HPLC-isotope dilution-MS-MS. LC-MS is still 2 or 3 orders of magnitude less sensitive than GC-NCI-MS (Lawson et al., 1999). However, significantly reduced sample preparation and higher amounts of sample injected on column may compensate much of the lower sensitivity if not all with the introduction of more sensitive liquid chromatography mass analysers. Indeed, HPLC-MS-MS has been successfully applied to the analysis of 15-F_{2t}-IsoP and other F_2-isoprostanes both in urine and plasma as well as a urinary metabolite of 15-F_{2t}-IsoP with sufficient sensitivity and accuracy (Li et al., 1999; Murai et al., 2000; Ohashi and Yoshikawa, 2000; Liang et al., 2003). Liang et al. (2003) demonstrated the high throughput and thus the utility for large clinical studies of their method with a single SPE step for sample preparation together with short HPLC analysis time (less than 10 minutes) due to the high selectivity in the multiple reaction mode (MRM) during MS-MS analysis.

2 Materials and methods

2.1 Methods applied for the analysis of polyphenols in chocolate and chocolate raw products

2.1.1 Reagents and standards

Standards of (-)-epicatechin, (+)-catechin, (-)-epigallocatechin, (+)-gallocatechin, quercetin, quercetin-arabinoside, chlorogenic acid, procyanidin B2, caffeine, theobromine, and cyanidin chloride were purchased from Campoverde srl - Division Gigalabo (Milan, Italy). Acetonitrile, methanol, ethanol, butanol, isopropanol, dichloromethane, acetone, n-hexane, formic acid, phosphoric acid, and acetic acid (Sigma-Aldrich, Milan, Italy) were of HPLC grade. Folin-Ciocalteu's Phenol Reagent 2.0 N F-9250, sodium carbonate (anhydrous), vanillin, hydrochloric acid, ferric chloride, potassium ferricyanide, boric acid, sodium dodecylsulphate (SDS), sodium hydroxide, sodium tetraborate, sodium dihydrogenorthophosphate, MOPS (3-[N-morpholino]-propanesulfonic acid), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), TRIS (Tris(hydroxymethyl)-aminomethane) and CHES (2-[N-cyclohexamino]-ethanesulfonic acid), were all of analytical grade purchased from Sigma-Aldrich (Milan, Italy). Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Vimodrone Milan, Italy). Cocoa beans, cocoa nibs, cocoa liquor, dark chocolate, and milk chocolate were kindly provided by Kraft Foods Europe, Munich, Germany.

2.1.2 General procedure for the extraction of polyphenols from cocoa liquor and chocolate

Approximately one gram of milled cocoa beans, cocoa nibs, cocoa liquor or chocolate sample was defatted twice with 10ml n-hexane for 5 minutes in an ultrasonic bath at 30ºC and was subsequently centrifuged for 10 minutes at 3000 x g. Polyphenols were extracted from the air-dried sample with 10ml of a mixture of acetone, water and acetic acid (70+29.8+0.2, v/v/v) for 10 minutes at 30ºC in the ultrasonic bath. The sample was filtered through a folded filter (595 1/2, Schleicher & Schuell, Milan, Italy) and the organic solvent was then removed by rotary evaporation under partial vacuum at 40ºC. The remaining aqueous extract was quantitatively transferred to a 50 ml graduated flask

and filled up to volume with distilled water for Folin-Ciocalteu and Prussian-Blue assays and with methanol for the Vanillin-HCl assay, respectively. For all other chromatographic and capillary electrophoretic methods the aqueous extract was filtered through a 45mm PTFE (polytetraflourethylene) filter (Waters SpA, Milan, Italy) and was ready for instrumental analyses without further clean-up.

2.1.3 Colorimetric methods

The equipment used in the Folin-Ciocalteu, Vanillin-HCl and Prussian-Blue assays for measurement of ultraviolet (UV) and visible light (Vis) absorbance at various wavelengths was a variable length UV-Vis spectrophotometer (Type UV-1201; Shimadzu Scientific Instruments, Columbia, USA). Cuvets (10 × 10 × 45 mm) were purchased from Greiner Labortechnik (Milan, Italy). In case of the proanthocyanidin assay, an HP 1050 Series HPLC system equipped with a quaternary pump, an autosampler, and a variable wavelength UV-Vis detector (all Agilent Technologies, Palo Alto, CA, USA) was used. The HPLC-system was interfaced with a personal computer powered with Agilent ChemStation software.

2.1.3.1 Folin-Ciocalteu assay

The total phenolics were assayed colorimetrically as described by Shahidi and Naczk (1995) with few optimised modifications as follows: A 1.5 ml of ten-fold diluted Folin-Ciocalteu's Phenol Reagent, 1.5 ml of 7.5% sodium carbonate and 50 µl phenolic extract were mixed well in the cuvets. The absorbance was measured at 765 nm after 30 min at room temperature. A mixture of reagents and water was used as a blank. Catechin was used as calibration reference standard, and calibration solutions contained 100, 200, 300, 400, 500, and 600 mg/l catechin, diluted from the same catechin stock solution (1 g/l). The content of phenolics is expressed as catechin equivalents.

2.1.3.2 Vanillin-HCl assay

The sum of total flavan-3-ols and proanthocyanidins were assayed colorimetrically as described by Porter et al. (1978) with few optimised modifications as follows: To 200 µl of phenolic extract, 300 µl of methanol was added and mixed well in the cuvets. To this 2.5 ml freshly prepared 0.5% vanillin solution in methanol containing 4% concentrated HCl (sample) or 2.5 ml of 4% concentrated HCl in methanol (blank) was

added and mixed well. The absorbency of the sample or blank was measured at 500 nm after a 30 min standing at room temperature. Catechin was used as calibration reference standard, and calibration solutions contained 125, 250, 375, 500, 675, and 750 mg/l catechin, diluted from the same catechin stock solution (1 g/l). The content of flavan-3-ols plus proanthocyanidins is expressed as catechin equivalents.

2.1.3.3 Prussian-Blue assay

The total phenolics were assayed colorimetrically as described by Price and Butler (1997) with few optimised modifications as follows: To 100 µl of phenolic extract, 2.6 ml distilled water was added and mixed well in the cuvets. To this, 150 µl 0.1 M ferric chloride was added and this was followed immediately by timed addition of 150 µl 0.008 M potassium ferricyanide solution. The absorbency was then measured at 720 nm after a 10 min standing at room temperature. A mixture of reagents and distilled water was used as a blank. Catechin was used as calibration reference standard, and calibration solutions contained 5, 10, 12.5, 25, 37.5, and 50 mg/l catechin, diluted from the same catechin stock solution (1 g/l). The content of phenolics is expressed as catechin equivalents.

2.1.3.4 Non-extractable procyanidins through hydrolysis with a modified proanthocyanidin assay

The residue (approximately 0.5 g) from the extraction of lipids and extractable polyphenols following the procedures described in 2.1.2 and 2.3.2, respectively, was used for the determination of non-extractable polyphenols (NEPP).

The proanthocyanidin assay procedure described by Porter et al. (1986) and modified by Terrill et al. (1992) was adapted to be used for the analyses of NEPP in cocoa polyphenol extraction residues as follows: The residue was transferred into 50 ml centrifuge tubes and 20 ml of freshly prepared butanol/HCl reagent (95% butan-1-ol/HCl (36%) v/v) added. The tubes were placed in a boiling water bath for 75 min, cooled on ice, centrifuged for 10 minutes at 3000 x g and the supernatant transferred to a 100 ml graduated flask and filled up to volume with 20% aqueous methanol.

20 µl of the solution were injected to the HPLC system. Separations were performed on a 5-µ Hypersil ODS column (25 cm × 4.6 mm i.d.) with a pre-column (2 cm × 4.6 mm

i.d.) (Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of water/formic acid/methanol 49/2/49 (v/v/v). An isocratic elution was run at a flow rate of 1.0 ml/min and the column temperature was maintained at 30°C. The retention time of cyanidin was 4.9 min. Detection was achieved by setting the wavelength of the UV-Vis detector at 510 nm and peak area was used for quantification.

Cyanidin chloride was used as calibration reference standard, and calibration solutions contained 1, 5, 10, 15, 25, and 50 mg/l cyanidin chloride, diluted from the same stock solution (100 mg/l). The content of NEPP is expressed as cyanidin equivalents.

2.1.3.5 Parameters of method performance for assay comparison

Linearity was assessed by performing repeated analyses of calibration solutions followed by linear regression analysis as well as graphically by plotting analyte response factors (ratio of absorbency to analyte concentration) versus analyte concentration.

Precision of the assays was determined both for calibration standards and real samples by 10-12 repeated analyses of the same solution or sample, respectively, followed by calculating the relative standard deviation.

The “ease of use” was judged qualitatively from the authors’ experience performing the assays. In particular, characteristics considered included handling of the assay and chemical reagents, total analysis time, ready-for-use availability of reagents versus need for daily preparation of reagent solutions etc.

2.1.4 Normal phase and reversed phase high-performance liquid chromatography with ultraviolet and electrochemical detection

The equipment used for both normal phase and reversed phase high-performance liquid chromatography was an HP 1050 Series HPLC system equipped with a quaternary pump, an autosampler, as well as a variable wavelength UV-Vis detector and an HP 1049 electrochemical detector in series (all Agilent Technologies, Palo Alto, CA, USA). The HPLC-system was interfaced with a personal computer powered with Agilent ChemStation software.

2.1.4.1 Normal phase high-performance liquid chromatography

Normal phase high-performance liquid chromatographic (NP-HPLC) analyses were performed as described by Rigaud et al. (1993) using a 5 µm Supelcosil (Supelco, Milan, Italy) silica column (250 x 4 mm) at 37°C column temperature, an injection volume of 20 µl, and with a solvent system consisting of dichloromethane (A), methanol (B) and acetic acid-water (1:1 v/v) (C). Separations were effected by a series of linear gradients with a constant 4% C at flow rate of 1 ml/min as follows: elution starting with 14% B in A followed by a series of linear gradients from 14-28.4% B in A (0-30 min), 28.4-39.2% B in A (30-45 min), 39.2-86% B in A (45-50 min), isocratic 86% B in A (50-55 min), 86 to 14% B in A (55-65 min), and isocratic 14% B in A (65-70 min). The column was additionally re-equilibrated with 14% B in A for 5 minutes previous to each analysis. Ultraviolet (UV) detection was recorded at 280 nm wavelength and electrochemical detection was carried out in series at the optimised potential of +1.0 V.

2.1.4.2 Reversed phase high-performance liquid chromatography

Reversed phase high-performance liquid chromatographic (RP-HPLC) analyses were performed using a 5 µm Hypersil (Agilent Technologies, Palo Alto, CA, USA) ODS column (250 x 4.6 mm) at 25°C column temperature, an injection volume of 20 µl, and with a solvent system consisting of 1.0% acetic acid (A) and methanol/water/acetic acid (90:9:1, v/v/v) (B) under the following conditions: elution starting at 10% B in A, followed by a series of linear gradients from 10 to 50% B in A (0-40 min), 50 to80% B in A (40-45 min), isocratic 80% B in A (45-50 min), 80 to 10% B in A (50-52 min), and 10% B in A (52-55 min). The column was additionally re-equilibrated with 10% B in A for 5 minutes previous to each analysis. The flow rate was set to 1 ml/min. Ultraviolet (UV) detection was recorded at 280 nm wavelength and electrochemical detection was carried out in series at the optimised potential of +1.0 V. For compound identification, the column and experimental conditions were also transferred to the HPLC-system 1 described below (section 2.1.6.1) using fluorescence detection (HP 1046 fluorescence detector (FLD), Agilent Technologies, Palo Alto, CA, USA) used at 276 nm excitation wavelength and 316 nm emission wavelength as well as mass spectrometric detection (section 2.1.6.1).

2.1.5 Micellar electrokinetic capillary chromatography

A ^{3D}CE capillary electrophoresis system (Agilent Technologies, Palo Alto, CA, USA) equipped with an ultraviolet diode-array detector (DAD) and Agilent ^{3D}CE ChemStation software Version 6.03 was used to perform all separations. Uncoated fused-silica capillaries (75 mm I.D.) were purchased from Agilent Technologies (Palo Alto, CA, USA). Subsequently, they were cut to a length of 48.5 cm and a window was created for the detector lamp so that the effective capillary length to the detector was 40 cm.

New capillaries were conditioned by washing with 1 M sodium hydroxide (30 min), 0.1 M sodium hydroxide (10 min) and water (10 min) prior to first use. Before each sample injection the capillary was rinsed with 0.1 M sodium hydroxide (3 min), water (2 min) and buffer (2 min). The optimised buffer used consisted of 175 mM boric acid with 40 mM sodium dodecylsulphate (SDS) and 30 % methanol adjusted to pH 7.9 with sodium hydroxide. Standards or samples were injected hydrodynamically applying a pressure of 50 mbar for 3 seconds. Electrophoresis was carried out in the positive voltage mode at 15 kV, and the temperature of the capillary environment was maintained at 25°C. Detection for producing electropherograms was conducted at a wavelength of 280 nm for catechins and procyanidins, and at additional wavelengths of 200 nm and 254 nm, respectively, for other phenolic compounds. UV spectra were recorded over the whole electropherogram between 190 and 600 nm for compound characterisation. The buffer was replaced after four sample injections.

2.1.6 High-performance liquid chromatography with mass spectrometric detection applied for the identification of phenolic compounds in chocolate

Due to a change of the equipment in the laboratory some parts of the qualitative analysis were carried out with HPLC-MS system 1 and some with the other HPLC-system 2. Nevertheless, both mass analysers were equipped with an electrospray-ionisation (ESI) source, and both were also based on the ion trap technology. Thus, both systems gave substantially the same mass spectra as well as spectra resulting from collision induced dissociation (CID) experiments. As data from both instruments were used for separation and tentative compound identification, both experimental conditions are presented

separately in the following because of individual characteristics of the HPLC-parts as well as of each MS-system.

2.1.6.1 HPLC-MS system 1

Chromatographic separation was performed using a SpectraSystem (Finnigan Mat, San Jose, CA, USA) consisting of a SCM degasser, a P4000 (low flow) quaternary pump and an AS3000 autosampler. The HPLC system was coupled to an MS ion trap, LCQ-Deca (Finnigan Mat, San Jose, CA, USA) equipped with an ESI interface. The system was controlled with Xcalibur software version 1.2 (Finnigan Mat, San Jose, CA, USA).

Reversed phase high-performance liquid chromatographic (RP-HPLC) analyses were performed using a 5 µm Supelcosil LC-18 column (250 x 4.6 mm) (Supelco, Milan, Italy) at room temperature with a solvent system consisting of 0.2% acetic acid (A) and acetonitrile (B) under the following conditions: linear gradients from 6 to 25% B in A (0-18 min), 25 to 60% B in A (18-20 min), isocratic 60% B in A (20-25 min), 60 to 6% B in A (25-27 min), 6% B in A (27-30 min). The column was additionally re-equilibrated with 6% B in A for 5 minutes previous to each analysis. The flow rate was set to 1 ml/min and the injection volume to 20 µl. The outlet of the HPLC system was split to the waste – or an external fluorescence detector in one case as mentioned in section 2.1.4.2 – (ca. 3/4 of the flow) and to the ESI interface of the mass analyser (ca. 1/4 of the flow). After identifying the retention times of the relevant analytes, in subsequent analyses the first 5 minutes of the LC eluent containing matrix compounds that were not retained by the column was diverted to waste via the integrated three-way valve in the analyser, then the valve automatically switched over to the ESI source in order to reduce a quick decrease in sensitivity of MS analysis.

In addition to HPLC direct infusion experiments were carried out in order to obtain total ion mass spectra of chocolate extracts as well as MS-MS spectra of catechin, epicatechin, and procyanidin B2 standards at a flow rate of 10 ml/min using the LCQ syringe pump (Finnigan Mat, San Jose, CA, USA).

All mass spectrometric (MS) as well as single- and multiple-stage CID tandem-mass spectrometric (MS-MS and MS^n) analyses were carried out in the negative ion mode under the following optimised conditions: source voltage 3.9 kV, capillary voltage -31

V, capillary temperature 300ºC, sheath gas (N_2) flow 80 arb (arbitrary units), auxiliary gas (He) flow 10 arb. Full scan MS spectra (m/z 250 - m/z 2000) were first recorded during the chromatographic run and the pseudomolecular ions of each phenolic compound identified. MS-MS and MS^n spectra were recorded by isolating the pseudomolecular ion of interest in the ion trap followed by single- or multiple-stage CID. The collision energy required in this process was set to 30% of the total available collision energy. High-resolution scans (~10,000 resolution compared to ~2,000 in normal scan mode) were recorded in the range +/- m/z 5 near the electrospray (ESI) generated single- or double-charged pseudomolecular ions of procyanidins for direct resolution and observation of carbon isotope distribution.

2.1.6.2 HPLC-MS system 2

The RP-HPLC analyses were carried out on an Agilent 1100 Series HPLC equipped with a binary pumping system and an autosampler (all Agilent Technologies, Palo Alto, CA, USA) coupled to an ultraviolet diode-array detector (DAD) an the ion trap mass spectrometer (Agilent S1100 series LC/MSD Trap, Agilent Technologies, Palo Alto, CA, USA) in series interfaced with an electrospray ionisation (ESI) source. Instrument control, data acquisition, and data evaluation were achieved with Agilent ChemStation (Agilent Technologies, Palo Alto, CA, USA) as well as Bruker LC/MSD Trap analysis and DataAnalysis (Bruker Daltonics, Bremen, Germany) software packages on the interfaced personal computer.

The HPLC separations were performed on an Xterra RP 18 (Waters, Milford, MS, USA) column (150 x 2.1 mm) packed with 3.5 µm particles. The column temperature was set to 35°C and the solvent system consisted of 0.2% acetic acid (A) and acetonitrile (B) under the following conditions: linear gradients from 6 to 25% B in A (0-18 min), 25 to60% B in A (18-20 min), isocratic 60% B in A (20-25 min), 60 to 6% B in A (25-27 min), 6% B in A (27-30 min). The column was additionally re-equilibrated with 6% B in A for 5 minutes previous to each analysis. The flow rate was set to 0.2 ml/min and the injection volume to 10 µl. The outlet of the HPLC system was directly coupled to the ESI interface of the mass analyser. The first 5 minutes of the LC eluent containing matrix compounds that were not retained by the column was diverted to waste via the integrated three-way valve in the analyser, then the valve automatically

switched over to the ESI source in order to reduce a quick decrease in sensitivity of MS analysis.

The diode array detection (DAD) system was only occasionally connected for gathering additional information on phenolic compounds or background matrix. Chromatograms were created contemporaneously at wavelengths of 200, 254, 280, 360, and 450 nm for phenolic compounds and presumed oxidised polymeric substances and UV spectra were recorded over the whole chromatogram between 190 and 600 nm for compound characterisation.

MS, MS-MS, and MS^n analyses were carried out mostly in the negative ion mode under the following optimised conditions: source voltage 3.4 kV, capillary voltage –69.5 V, capillary temperature 280ºC, sheath gas (N_2) flow 10 l/min and auxiliary gas (He) pressure 30 psi. For more confidence in the identification of the molecular weight of the analyte, few MS and MS-MS analyses were carried out also in the positive ion mode under the following conditions: source voltage 3.9 kV, capillary voltage 67.4 kV, capillary temperature 280°C, sheath gas (N_2) flow 10 l/min and auxiliary gas (He) pressure 30 psi.

Full scan MS spectra (m/z 250 - m/z 2200) were first recorded during the chromatographic run and the pseudomolecular ions of each phenolic compound identified. MS-MS and MS^n spectra were recorded by isolating the pseudomolecular ion of interest in the ion trap followed by single- or multiple-stage CID.

2.2 Isolation, purification and characterisation of phenolic compounds from cocoa powder

The extraction of polyphenols from cocoa powder, subsequent isolation and purification of single phenolic compounds as well as the characterisation comprised a combination of various chromatographic and spectrometric techniques. After extraction of polyphenols and subsequent separation of phenolic compounds from matrix compounds and xanthine alkaloids by flash chromatography one branch of the further purification consisted of high-speed countercurrent chromatographic (HSCCC) and the other of size exclusion chromatographic (SEC) isolation of fractions enriched in single cocoa polyphenols prior to final purification and structure elucidation of the compounds.

Techniques applied complementarily for structure elucidation included nuclear magnetic resonance (NMR) spectroscopy, liquid phase hydrogen/deuterium exchange-mass spectrometry (H/D-MS), and liquid chromatography-mass spectrometry (LC-MS). The outline of the applied procedures is shown in figure 2.1.

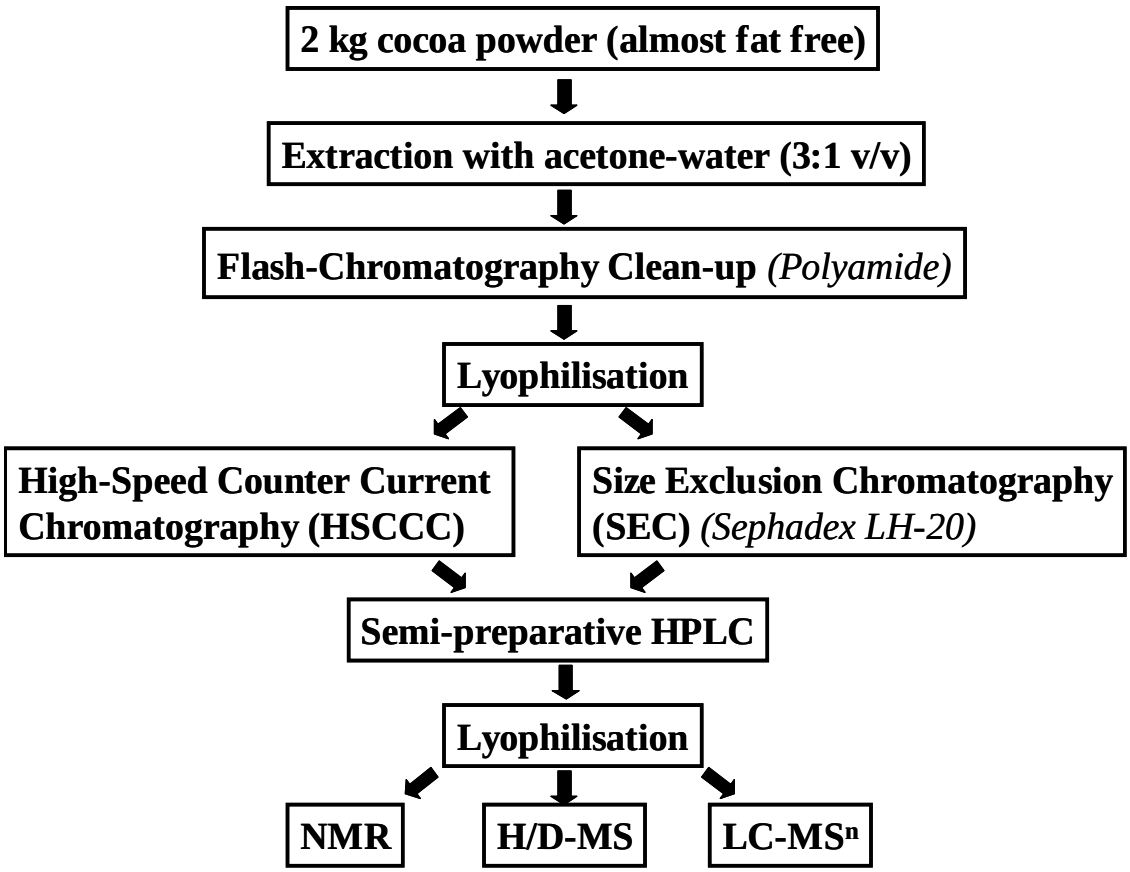


Figure 2.1 Outline of the isolation–purification–characterisation process – abbreviations are explained in the text.

2.2.1 Chemical reagents

Acetone (Sigma-Aldrich, Milan, Italy) used for extraction of cocoa powder and polyamide clean-up was of industrial grade. Polyamide-6 and Sephadex LH-20 were purchased from Fluka (Milan, Italy). Acetonitrile, methanol, and acetic acid (Sigma-Aldrich, Milan, Italy) for SEC and semi-preparative HPLC were of HPLC grade. Ethyl-acetate, ethanol, n-butanol used for HSCCC were of analytical grade and were purchased by the Technical University of Braunschweig. The NMR solvents acetone-d_6, methanol-d_4, DMSO-d_6 were purchased from Sigma-Aldrich (Milan, Italy). Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Vimodrone Milan,

Italy). Fat-free cocoa powder was kindly provided by Kraft Foods Europe, Munich, Germany.

2.2.2 Equipment

The equipment used for isolation and purification consisted of: a flash chromatography system (Aldrich, Milan, Italy) based on 400 ml glass columns with a possibility to apply a pressure up to 3 bar using pressurised air or nitrogen via a valve in order to control flow rates; a high-speed countercurrent chromatography (HSCCC) system model CCC-1000 (Pharma-Tech Research Cooperation, Baltimore, MD, USA) equipped with three preparative coils connected in series (total volume: 850 ml), a Biotronik HPLC pump BT 3020, a Knauer UV-vis detector, a Knauer L 250 E plotter, and a Pharmacia LKB Super Frac fraction collector was used at the Technical University of Braunschweig, Germany in collaboration with A. Degenhardt; an open glass column with an automated fraction collector (Waters, Milford, MA, USA) for size exclusion chromatography (SEC); a lyophiliser (FreeZone Plus 6 Freeze Dry system, LABCONCO, Kansas City, Missouri, USA); and the HP 1050 HPLC system described in 2.1.4. using only the UV-detector.

The equipment used for characterisation of semi-purified extracts and chromatographic fractions during the isolation and purification procedure as well as for structure elucidation of isolated compounds comprised: a Bruker AMX 500 MHz nuclear magnetic resonance (NMR) spectrometer (Bruker BioSpin, Karlsruhe, Germany); the HPLC-DAD-ESI-MS system described in section 2.1.6.2; and a Quattro micro triple quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) source with an integrated syringe pump and the system was powered with MassLynks 4.0 software (all Waters, Manchester, UK).

2.2.3 Procedure for extraction, isolation and purification

2 kg of cocoa powder were extracted in two steps, each twice with 2 litres of a mixture of acetone-water (75:25 v/v) by ultrasonication. Extracts were subsequently filtered using folded filters (Schleicher & Schuell, Milan, Italy), the organic solvent removed by rotary evaporation under partial vacuum at 40ºC and the aqueous extracts reunified and stored at 4°C for further use within a week.

Each 100-200 ml of aqueous extract were purified in several runs by flash chromatography (400 ml column, ca. 3 cm x 30 cm) using polyamide, which had previously been conditioned by covering the sorbent with methanol overnight. After applying the extract the column was washed with eight volumes of 500 ml distilled water for removal of undesired matrix compounds and xanthine alkaloids before elution of the phenolic fraction with 6 volumes of 500 ml acetone-water (75:25 v/v). The organic solvent was removed by rotary evaporation under partial vacuum at 40ºC and the aqueous extracts were lyophilised and stored at -25°C for further use.

In the procedure for further isolation of cocoa polyphenols using HSCCC, 700-900 mg of lyophilised polyamide extract were dissolved in either solvent system a), consisting of a polar two-phase solvent system of ethyl-acetate/ethanol/water (25:1:25 v/v/v) or in solvent system b), consisting of another polar two-phase system of ethyl-acetate/n-butanol/water (45:5:50 v/v/v). The solutions were injected into the system by loop injection and the separations were run at a revolution speed of 1000 rpm at flow rates of 2.5-3 ml/min. The upper phase was used as stationary phase, the lower phase as mobile phase in both cases. Five-millilitre fractions were collected automatically and the elution was monitored online by UV-spectrometry at 280 nm wavelength. According to the UV-chromatogram the small 5 ml fractions were reunified in a way to yield four homogeneous fractions and a fifth fraction (light phase), which remained in the Teflon coils of the HSCCC system. These fractions were lyophilised for structure elucidation or final purification, respectively.

In the other procedure using SEC, an open column (1.5 cm x 58 cm) was filled with Sephadex LH-20, previously conditioned in methanol for at least two hours. Approximately 400 mg of lyophilised polyamide extract were diluted in 20 ml methanol and applied on the column. Elution was carried out by gravity with pure methanol at a resulting flow rate of approximately 1 ml/min. The first 20 ml of eluate corresponding to the death volume of the column were discarded. Thirty seven-millilitre fractions were automatically collected and the distribution of the phenolic compounds monitored by off-line characterisation of the fractions by HPLC-MS. According to the identified characteristics the 7 ml fractions were reunified in a way to yield three homogeneous fractions, which were lyophilised for final purification.

Semi-preparative HPLC was performed on a Hypersil (Agilent Technologies, Palo Alto, CA, USA) ODS 5 µm (10 x 250 mm) column at a column temperature of 30°C with a solvent system consisting of distilled water (A) and acetonitrile (B). 50 µl of lyophilised extract resuspended in mobile phase at starting conditions were injected and various combinations of linear gradients and isocratic phases applied, depending on the compounds to be purified. The flow rate was set to 3 ml/min. Elution was monitored by UV-spectrometry and eluate was collected manually over several runs after a signal was registered. Eluates containing the same compound were reunified, lyophilised and stored at -25°C for structure elucidation.

2.2.4 Structure elucidation of isolated compounds

2.2.4.1 Nuclear magnetic resonance

All samples were prepared in 5 mm NMR tubes by diluting lyophilised chromatographic fractions in 500µl of deuterated solvent (aceton-d_6 for procyanidin B2; methanol-d_4 for procyanidin C1 and clovamide; dimethylsulfoxide (DMSO)-d_6 for dideoxyclovamide and monodeoxyclovamides).

^{1}H NMR and multidimensional spectra were acquired using a 5mm broadband inverse probe operating at the basic frequency of 500.14MHz (O1). The multidimensional experiments used were standard Bruker implementations of gradient selected versions of inverse (^{1}H-detected) Heteronuclear Single Quantum Coherence (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) experiments.

^{13}C NMR of dideoxyclovamide and procyanidins were acquired on 5mm ^{1}H/^{13}C dual probe.

2.2.4.2 Simple and collision-induced dissociation mass spectrometry

HPLC-MS and MS^n experiments were carried out on the HPLC-MS system 2 (section 2.1.6.2) for cocoa powder extracts during all phases of the isolation procedure as well as for purified polyphenols using the identical conditions as described in section 2.1.6.2 in order to obtain the information from mass spectrometry as well as from HPLC retention behaviour.

2.2.4.3 Hydrogen/deuterium exchange mass spectrometry

Liquid phase hydrogen/deuterium exchange mass spectrometry (H/D-MS) was carried out by dissolving the isolated compound in normal solvent (water-methanol 1:1 v/v) and separately in deuterated solvent (D_2O-methanol-d_4 1:1 v/v) at room temperature leading to immediate exchange of acidic protons of the isolated compound with protons (H^+) from the normal aqueous phase and deuterons (D^+) from the deuterated phase, respectively. Either normal or deuterated aqueous solutions were injected directly into the mass analyser using the integrated syringe pump at 10 µl/min flow rate.

MS and MS-MS analyses were carried out in both the negative ion mode using a capillary voltage of -2.8 kV and in the positive ion mode using a capillary voltage of 3.0 kV, respectively. In both cases the source and desolvation temperatures were set to 80°C and 150°C, respectively, and the desolvation and cone gas (both nitrogen) flows were optimised at 350 l/h and 20 l/h, respectively. Mass spectra of compounds were obtained by scanning ± 10 Da around the mass of the protonated or deprotonated ion of the compound in solution using only quadrupole 1 of the analyser. MS-MS spectra were obtained by isolating the most abundant precursor ion from the MS-scans in quadrupole 1, followed by CID in the collision cell (quadrupole 2) using argon as collision gas at approximately 3 x 10^{-3} mbar and a collision energy of 20-25 eV, and performing MS-scans with quadrupole 3.

2.3 Quantitative analysis of polyphenols in chocolate

Two HPLC-MS systems were used for quantitative analyses. System 1 was used only for quantification of catechin and epicatechin in baking chocolate (standard reference material SRM 2384, National Institute of Standards and Technology NIST, Geithersburg, Maryland, USA) in the frame of a proficiency testing exercise. For this, chromatographic separation was performed using an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of degasser, binary pump, and autosampler. The HPLC system was coupled to a Micromass Platform (Waters, Manchester, UK) equipped with an ESI interface. The outlet of the HPLC system was split (4:1) to the ESI interface of the mass analyser.

All other quantitative analyses in cocoa beans, cocoa nibs, cocoa liquor, dark chocolate, and milk chocolate were performed on system 2 which is the system described in section 2.1.6.2 above.

2.3.1 Chemicals and Reagents

Standards of (-)-epicatechin, (+)-catechin, (-)-epigallocatechin, (+)-gallocatechin, quercetin, quercetin-arabinoside, quercetin galactose, chlorogenic acid, and procyanidin B2 were purchased from Campoverde srl - Division Gigalabo (Milan, Italy). Standards of clovamide, monodeoxyclovamides, dideoxyclovamides, and procyanidin C1 were isolated and purity confirmed as described in section 2.2 and were used as calibration standards as well. Acetonitrile, acetone, n-hexane, and acetic acid (Sigma-Aldrich, Milan, Italy) were of HPLC grade. Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Vimodrone Milan, Italy). Cocoa beans, cocoa nibs, cocoa liquor, dark chocolate, and milk chocolate were kindly provided by Kraft Foods Europe, Munich, Germany.

2.3.2 Sample preparation

Approximately 100 grams of cocoa beans, that were first deshelled, or cocoa nibs were grinded under liquid nitrogen in an analytical laboratory mill. Of these, 30-35 portions of each 25-30 mg were taken with a micro-spatula and reunified to give approximately 1 g of sample. For cocoa liquor, dark chocolate, milk chocolate as well as for the baking chocolate reference material, which all were considered homogeneous from the homogenisation during the manufacturing processes only approximately 1 gram was cut in small pieces, transferred into a 50 ml Teflon beaker and ground under liquid nitrogen in the analytical laboratory mill.

To each of these samples of ca. 1 gram, 0.5 mg of taxifolin was added as internal standard. The mixture was defatted twice with 10ml n-hexane for 5 minutes in an ultrasonic bath at 30ºC and was subsequently centrifuged for 10 minutes at 3000 x g. Catechins and procyanidins were extracted from the air-dried sample with 10 ml of a mixture of acetone, water and acetic acid (70+29.8+0.2, v/v/v) for 10 minutes at 30ºC in the ultrasonic bath. Water was added to the sample to get a total volume of 50 ml. The diluted extract was filtered through a 45 µm PTFE (polytetraflourethylene) filter

(Waters SpA, Milan, Italy) and was ready for HPLC-MS separation without further clean up.

2.3.3 High-performance liquid chromatography with mass spectrometric detection

RP-HPLC analyses were performed using a 3.5 µm XTerra RP-18 column (150 x 2.1 mm) at 30°C with a solvent system consisting of 0.2% acetic acid (A) and acetonitrile (B) under the following conditions: linear gradients from 6 to 25% B in A (0-18 min), 25 to 60% B in A (18-20 min), isocratic 60% B in A (20-25 min), 60 to 6% B in A (25-27 min), 6% B in A (27-40 min). The flow rate was set to 0.2 ml/min and the injection volume to 10 µl. The first 5 minutes of the LC eluent containing matrix compounds that were not retained by the column was diverted to waste, then the valve automatically switched over to the ESI source in order to avoid a quick decrease in sensitivity of MS analysis.

2.3.3.1 MS-System 1

All MS analyses were carried out in the positive ion mode, single ion reporting (SIR) of m/z 291 and 305, respectively, under the following optimised conditions: cone voltage 30 V, capillary voltage 3.5 kV, source temperature 80ºC, drying gas (N_2) flow 300 l/h, nebuliser gas (N2) flow 15 l/h.

Calibration was carried out using authentic standards of catechin (2, 4, 6, 8, 10, 12 µg/ml) and epicatechin (5, 10, 15, 20, 25, 30 µg/ml) containing constant 10 µg/ml taxifolin as internal standard. Quantification was carried out using relative response of peak area of catechin or epicatechin to internal standard (taxifolin). Results are given in mg/g baking chocolate.

2.3.3.2 MS-System 2

MS and MS-MS analyses were carried out exclusively in the negative ion mode under the following optimised conditions: source voltage 3.4 kV, capillary voltage –69.5 V, capillary temperature 280ºC, sheath gas (N_2) flow 10 l/min and auxiliary gas (He) pressure 30 psi.

Full scan mass spectra (m/z 250 - 2200) were first recorded during the chromatographic run, and subsequently reconstructed mass chromatograms were obtained for each of the deprotonated ions of the phenolic compounds. The peak area was used for quantification.

Similarly, MS-MS spectra were obtained by isolating the respective precursor ion followed by collision-induced dissociation (CID) and MS-MS scan from the mass to charge ratio of the precursor ion to approximately 30% of that value. Mass chromatograms for characteristic daughter ions were reconstructed and the peak area was again used for quantification.

Calibration was carried out using a mix of authentic standards either commercially available or isolated from cocoa and characterised by NMR and MS. The stock solution contained: 1000 μg/ml of catechin and epicatechin, 500 μg/ml of procyanidin B2, 300 μg/ml of procyanidin C1, and 100 μg/ml of clovamide, monodeoxyclovamides, dideoxyclovamide, quercetin, quercetin arabinose and quercetin galactose. Step-wise dilution of the stock solution for calibration was done as follows: 1:10; 1:50, 1:100; 1:500, 1:1000; 1:5000 and 1:10000. All diluted calibration solutions contained constant 1 μg/ml taxifolin as internal standard. Quantification was carried out using relative response of peak area of polyphenol standard in the calibration solution to peak area of internal standard (taxifolin). Results are given in mg/kg chocolate or chocolate raw product.

2.3.4 Other techniques

The Folin-Ciocalteu assay was applied for estimation of total polyphenol content as described in 2.1.3.1 above and non-extractable polyphenols (NEPP) in the residue from polyphenol extraction (section 2.3.2) were determined by the modified proanthocyanidin assay described in section 2.1.3.4.

2.3.5 Method validation parameters

Linearity was assessed by performing repeated analyses of calibration solutions followed by linear regression analysis as well as graphically by plotting analyte

response factors (ratio of analyte response to analyte concentration) versus analyte concentration.

Trueness was studied by the standard addition method, i.e., by spiking a cocoa liquor sample of established polyphenol content (12 repeated analyses) with 25, 50, 75 and 100% of the polyphenol concentration of the non-spiked sample followed by triplicate analyses and calculation of recovery. Mean recovery was computed and tested against complete recovery (100%) by one-way student's t-test.

Precision of the method was determined by performing analysis of variance on the data set from the recovery experiments and calculating the relative standard deviation from the square root of the mean of squares of the error (MS_{error}).

2.4 Polyphenol content in chocolate and chocolate raw products – identification of important process parameters

2.4.1 Chocolate manufacturing process lines and sampling plan

For studying the influence of process parameters in chocolate and cocoa manufacturing on polyphenol content and profile as well as identifying key processes or process variables, samples from various production lines have been taken. The manufacturer provided the necessary information regarding temperature and time of the treatment in the monitored manufacturing lines that included a cocoa liquor production, a dark chocolate making procedure, a milk chocolate production line, and a combined cocoa nibs alkalising-roasting process. A schematic outline of these procedures including the time-temperature profile of the studied processes as well as the sampling plan and codes (indicated by an arrow to the right) are presented in figures 2.2 to 2.5.

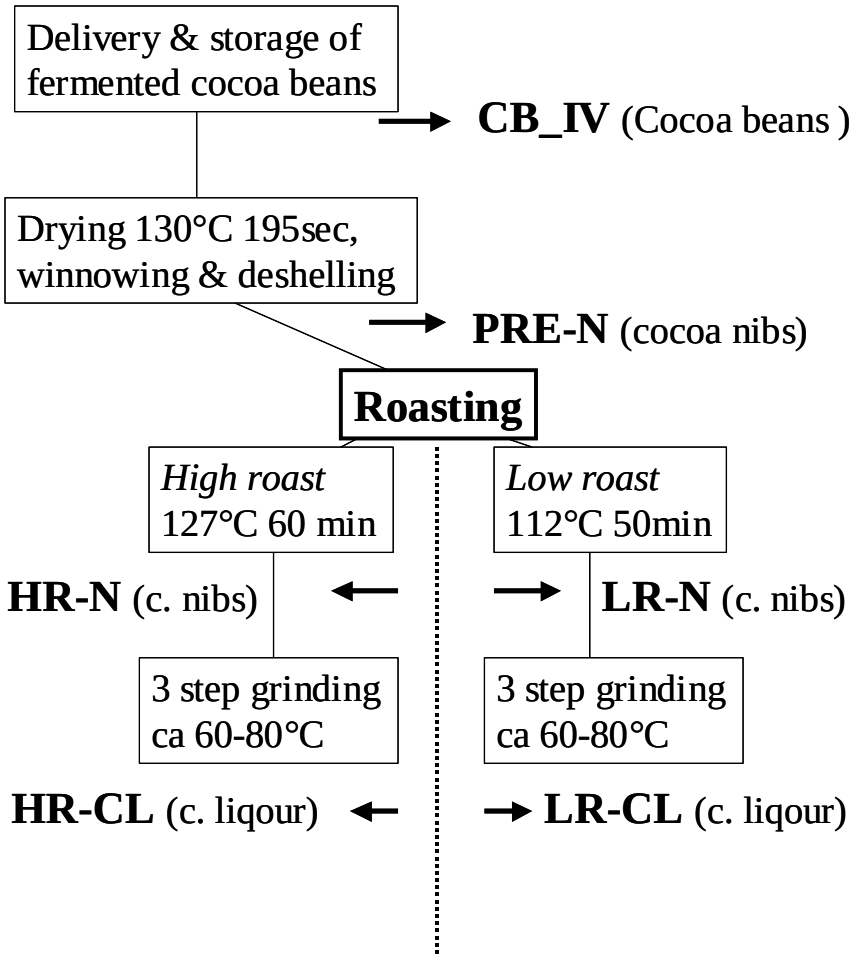


Figure 2.2 Outline of cocoa liquor manufacturing process and sampling plan – sample codes are given in bold capital letters.

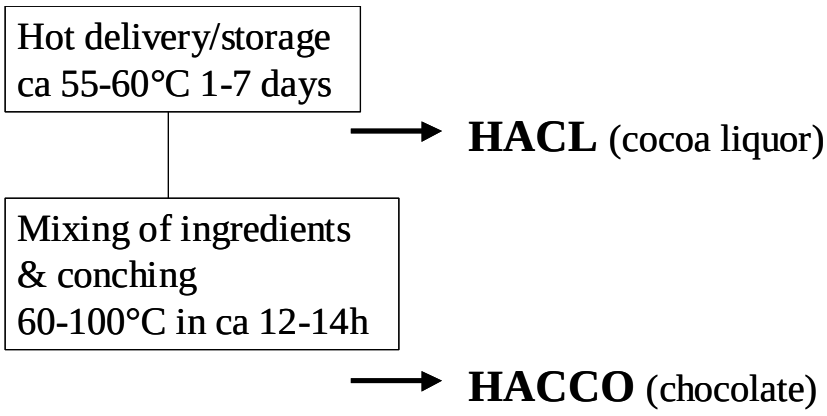


Figure 2.3 Outline of dark chocolate manufacturing process with cocoa liquor as start material and sampling plan – sample codes are given in bold capital letters.

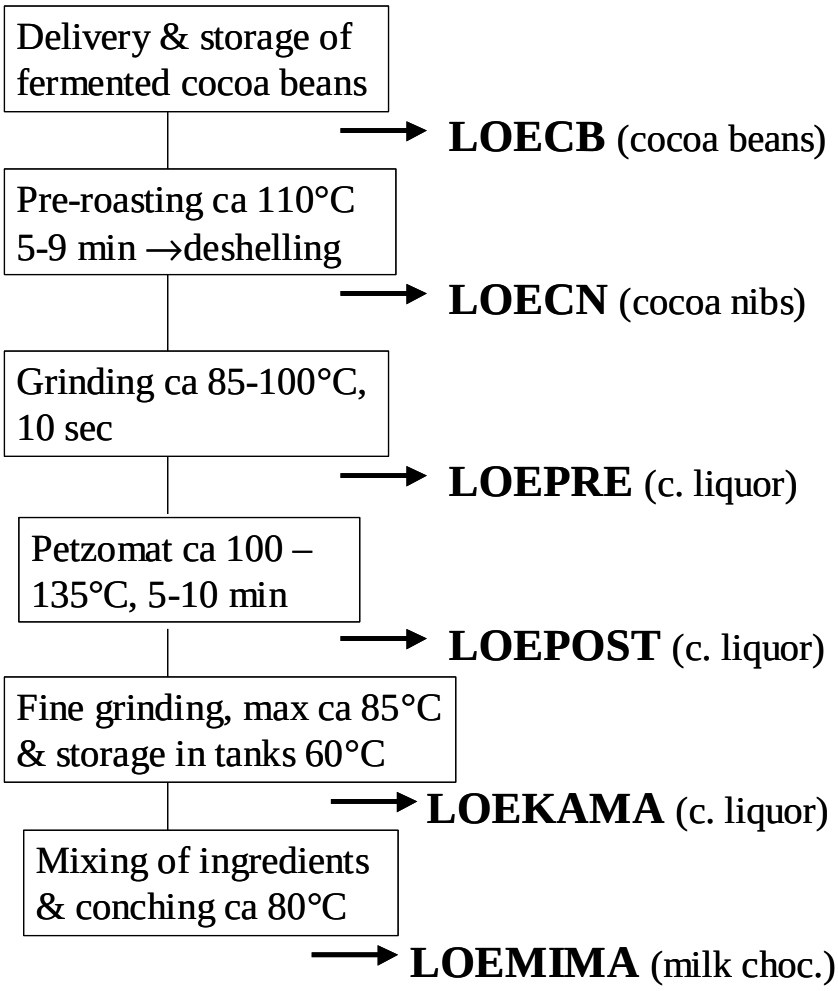


Figure 2.4 Outline of milk chocolate manufacturing process and sampling plan – sample codes are given in bold capital letters.

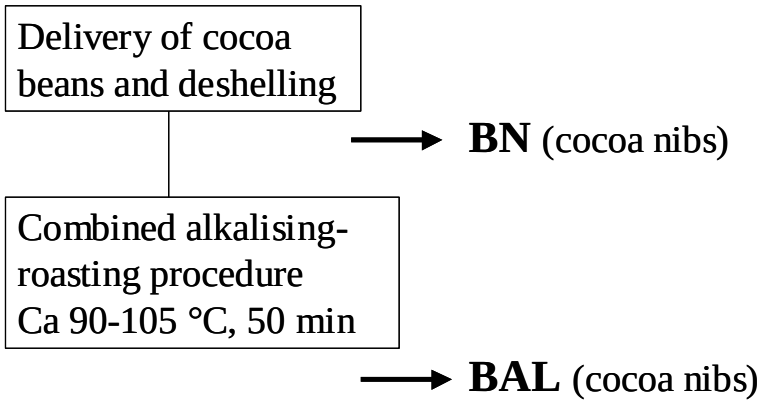


Figure 2.5 Outline of nibs combined alkalising-roasting procedure and sampling plan – sample codes are given in bold capital letters.

In addition to the samples from the chocolate and cocoa making processes samples of cocoa beans from various origins of samples were taken in one plant from 20 kg jute sacks, which were kept from various shipments for quality control, for studying natural and region-related variations in polyphenol contents and profiles. For studying variations in one product from the same manufacturer, but produced on different days, dark chocolates and milk chocolates from the same producer were purchased in different local markets and care was taken, not to take sample with the same production code. Moreover, samples from other manufacturers were included for estimating the magnitude of differences between products available on the local market.

2.5 Excursus 1: Bioavailability of polyphenols from chocolate in humans

2.5.1 Chemicals and Reagents

Standards of (-)-epicatechin, (+)-catechin, quercetin, chlorogenic acid, and procyanidin B2, were purchased from Campoverde srl - Division Gigalabo (Milan, Italy). Standards of caffeic acid, p-coumaric acid, 3-hydroxy-phenylacetic acid, 3-hydroxy-4-methoxybenzoic (vanillic) acid, 3-hydroxy-4-methoxyphenylacetic (homovanillic) acid, benzoic acid, p-hydroxybenzoic acid, theobromine, caffeine, and hippuric acid were from Sigma-Aldrich (Milan, Italy). Acetonitrile, ethyl acetate, ethanol, and acetic acid (Sigma-Aldrich, Milan, Italy) were of HPLC grade. Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Vimodrone Milan, Italy). Dark chocolate and milk chocolate were purchased in a local supermarket.

2.5.2 Subjects and study design

Two healthy volunteers (male, 32 years; female, 57 years) abstained from polyphenol-rich foods for two days, and after overnight fasting consumed 100 g chocolate or, on a separate occasion, 200 g milk chocolate both containing approximately 100 mg of epicatechin, 20 mg of catechin, 40 mg of procyanidin B2, 20 mg of higher procyanidin oligomers, 8 mg of hydroxycinnamic acid amides (clovamides), and 5 mg of quercetin compounds. Blood was taken before and 2 and 6 hours after consumption, urine was collected before, from 0 to 3, 3 to 6, 6 to 9, 9 to 12 and 12 to 24 hours after consumption.

2.5.3 Sample preparation

Plasma was obtained after centrifugation of venous blood samples stored in EDTA or sodium heparin containing tubes (Becton Dickinson, Italy) and stored at –80°C until further extraction. Urine samples were acidified to pH 4.0 with hydrochloric acid and frozen at –20°C until extraction.

Polyphenol metabolites were extracted from plasma samples with ethyl acetate. The organic layer was evaporated under a gentle nitrogen stream, re-dissolved in 5% ethanol at pH 4 and subjected to solid phase extraction (C18 cartridge). Urine samples were allowed to thaw, stirred and directly used for solid phase extraction as for plasma extracts.

Solid phase extraction (SPE) was performed by applying the re-suspended plasma sample or a volume of 5-10 ml of urine on a C18 cartridge (Waters™ Sep Pac®, Vac 3cc, 200 mg, Milford, MS, USA) previously conditioned with 5 ml methanol and 5 ml 0.2% acetic acid. After washing with 5 ml of 0.2% acetic acid and 3 ml of water the cartridge was dried by applying a vacuum using a 12 slot SPE-vacuum system (Supelco, Milan, Italy). Elution of phenolic metabolites was performed using 3 times 1 ml of methanol. The eluates were then reduced to dryness under a stream of nitrogen (Reacti-Vap III; Pierce Chemical Company, Rockford, IL, USA) and re-suspended in 250 µl of 5 % acetonitrile. All samples were filtered (0.45 mm) prior to HPLC analysis.

2.5.4 High-performance liquid chromatography with mass spectrometric detection

Chromatographic separation was performed using a SpectraSystem (Finnigan Mat, San Jose, CA, USA) consisting of a SCM degasser, a P4000 (low flow) quaternary pump and an AS3000 autosampler. The HPLC system was coupled to an MS ion trap, LCQ-Deca (Finnigan Mat, San Jose, CA, USA) equipped with an ESI interface. The system was controlled with Xcalibur software version 1.2 (Finnigan Mat, San Jose, CA, USA).

Reversed phase high-performance liquid chromatographic (RP-HPLC) analyses were performed on an Xterra RP 18 (Waters, Milford, MS, USA) column (150 x 2.1 mm) packed with 3.5 µm particles. The column temperature was set to 25°C and the solvent system consisted of 0.2% acetic acid (A) and acetonitrile (B) under the following

conditions: linear gradients from 6 to 25% B in A (0-18 min), 25 to 60% B in A (18-20 min), isocratic 60% B in A (20-25 min), 60 to 6% B in A (25-27 min), 6% B in A (27-30 min). The column was additionally re-equilibrated with 6% B in A for 5 minutes previous to each analysis. The flow rate was set to 0.25 ml/min and the injection volume to 5 µl. The outlet of the HPLC system was directly coupled to the ESI interface of the mass analyser. The first 5 minutes of the LC eluent containing matrix compounds that were not retained by the column was diverted to waste via the integrated three-way valve in the analyser, then the valve automatically switched over to the ESI source in order to reduce a quick decrease in sensitivity of MS analysis.

All mass spectrometric (MS) as well as single- and multiple-stage CID tandem-mass spectrometric (MS-MS and MS^n) analyses were carried out in the negative ion mode under the following optimised conditions: source voltage 3.9 kV, capillary voltage -31 V, capillary temperature 300ºC, sheath gas (N_2) flow 80 arb (arbitrary units), auxiliary gas (He) flow 10 arb. Full scan MS spectra (m/z 150 - m/z 700) were first recorded during the chromatographic run and the deprotonated ions of each phenolic metabolite identified. MS-MS and MS^n spectra were recorded by isolating in the ion trap the deprotonated ion of interest or by isolating ions corresponding to the deprotonated ion of a compound, which existence was presumed or investigated for, followed by single- or multiple-stage CID. The collision energy required in this process was varied between 25-50% of the total available collision energy.

2.6 Excursus 2: Measurement of urinary markers for oxidative DNA-damage and lipid peroxidation in humans

This study has been carried out in collaboration with and supervision of a diploma thesis (Lanfer, 2002). The experimental part of the collaboration being the task within this doctoral work included the development of a methodology for quantitative determination of the two markers in human urine based on high-performance liquid chromatography-tandem mass spectrometric (HPLC-MS-MS). The measurement of theses urinary markers by enzyme immunoassays, the method comparison based on method validation parameters as well as the preliminary human study for investigation of biological variation in marker levels was task of the diploma work and thus, the description of materials and methods for these studies are described in Lanfers' thesis

(Lanfer, 2002). Consequently, only the HPLC-MS-MS methodology is described in the following.

2.6.1 Chemicals and Reagents

Standards of 15-F_{2t}-Isoprostane (15-F_{2t}-IsoP) and [3,3,4,4-$^2H_4$]-labelled 15-F_{2t}-Isoprostane (15-F_{2t}-IsoP-d_4) were from Cayman Chemical (Ann Arbor, MI, USA) whereas 8-hydroxydeoxyguanosine (8-OHdG) standard was purchased from Sigma-Aldrich, (Milan, Italy). Acetonitrile, methanol, ethanol, ethyl-acetate, and heptane (Sigma-Aldrich, Milan, Italy) were of HPLC grade and potassium dihydrogen-phosphate (Sigma-Aldrich, Milan, Italy) of analytical grade. Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Vimodrone Milan, Italy).

2.6.2 Equipment

The HPLC measurements of 8-OHdG and 15-F_{2t}-IsoP were carried out on an Agilent 1100 Series HPLC equipped with a binary pumping system and an autosampler (all Agilent Technologies, Palo Alto, CA, USA). The high-performance liquid chromatographic separations were performed on an Xterra RP 18 column (150 x 2.1 mm) packed with 3.5 µm particles (Waters, Milford, MS, USA). The HPLC was directly coupled to an ion trap mass spectrometer (Agilent S1100 series LC/MSD Trap, Agilent Technologies, Palo Alto, CA, USA) interfaced with an electrospray ionisation (ESI) source. Instrument control, data acquisition, data evaluation and peak integration were achieved with Agilent ChemStation (Agilent Technologies, Palo Alto, CA, USA) as well as Bruker LC/MSD Trap analysis, DataAnalysis, and QuanAnalysis (Bruker Daltonics, Bremen, Germany) software packages on the interfaced personal computer.

2.6.3 Sample preparation for 8-hydroxydeoxyguanosine

For method development urine was collected from a healthy, non-smoking male volunteer (age 32 years; body weight 90 kg; BMI 23.0 kg/m^2, respectively) and used immediately for further sample preparation.

Solid phase extraction (SPE) was performed by applying a volume of 5 ml urine on a C18 cartridge (Waters™ Sep Pac®, Vac 3cc, 200 mg, Milford, MS, USA) previously conditioned with 5 ml methanol and 5 ml 25 mM potassium dihydrogen-phosphate (pH

5.5). After washing with 5 ml of 25 mM potassium dihydrogen-phosphate and 3 ml of water the cartridge was dried by applying a vacuum using a 12 slot SPE-vacuum system (Supelco, Milan, Italy). Before elution of 8-OHdG with 3 times 1 ml of methanol the cartridge was washed with 3 ml ethyl acetate:heptane (1/1 v/v) to remove further components of the urinary matrix. The methanolic eluates were reduced to dryness under a stream of nitrogen (Reacti-Vap III; Pierce Chemical Company, Rockford, IL, USA), resolved in 100 µl of 5 % acetonitrile.

2.6.4 High-performance liquid chromatography-tandem mass spectrometry for analysis of 8-hydroxydeoxyguanosine

10 µl of the reconstituted solution were injected to the HPLC system. A water/acetic acid/acetonitrile solution (97.8/0.2/2; v/v/v) was used as mobile phase. An isocratic elution was run at a flow rate of 0.2 ml/min and column temperature was maintained at 35°C. The retention time of 8-OHdG was 10.2 min. The experimental conditions of the MS were the following: Source voltage 3.9 kV, capillary voltage 81 kV, capillary temperature 265°C, sheath gas flow 12 l/min and auxiliary gas pressure 40 psi. ESI was used in the positive ion mode. Full scan mass spectra were acquired by scanning from m/z 100-350. MS/MS spectra were obtained by isolating $[M+H]^+$ m/z 284 as precursor ion followed by collision induced dissociation (CID) and scanning from m/z 100 to 300. The area under the peak in the reconstructed mass chromatogram of the most abundant product ion m/z 168 was used for quantification.

Standard solutions with 8-OHdG concentrations of 50, 100, 150, 200, 250, 300, 350, 400 ng/ml water were prepared and analysed as described above. The 8-OHdG standard curve was generated by plotting peak area versus concentration of the standards and the equation of the standard curve resulting from linear regression analysis was used to determine the 8-OHdG concentrations corresponding to the observed peak areas.

2.6.5 Sample preparation for 15-F_{2t}-isoprostane

For method development urine was collected from a healthy, non-smoking male volunteer (age 32 years; body weight 90 kg; BMI 23.0 kg/m^2, respectively) and used immediately for further sample preparation.

A 15-F_{2t}-IsoP immunoaffinity column (20 ml volume; Cayman Chemical, Ann Arbor, MI, USA) with 1 ml sorbent (mouse anti-15-F_{2t}-IsoP covalently bound to sepharose 4B) and a binding capacity of 10 ng 15-F_{2t}-IsoP was used for the immunoaffinity clean-up procedure, which was performed according to the description of the manufacturer. Before analysis the urine samples were centrifuged at 2000 × *g* for 3 min (Eppendorf centrifuge 5810 R) to avoid plugging the column. The storage solution of the column was allowed to pass through the packing material and a volume of 3 ml of the urine sample was applied to the column. After 20 ml of urine sample containing 2 ng 15-F_{2t}-IsoP-d_4 internal standard had passed through the sorbent the column was washed with 10 ml column buffer (Cayman Chemical, Ann Arbor, MI, USA), followed by 10 ml water and both of the washes were discarded. Elution was carried out with 5 ml elution solution (95% ethanol) into a glass vial. The eluate was evaporated to dryness under a stream of dry nitrogen (Reacti-Vap III/Reacti-Therm III heating module; Pierce Chemical Company, Rockford, IL, USA) and was reconstituted with 100 µl 5 % acetonitrile. The column was regenerated with 10 ml water, followed by 10 ml column buffer. It was then stored with column buffer in an upside position for further purification cycles. According to the recommendation of the supplier a column was not used after more than 5 regenerations.

2.6.6 High-performance liquid chromatography-isotope dilution-tandem mass spectrometry for analysis of 15-F_{2t}-isoprostane

80 µl of the reconstituted solution were injected to the HPLC system. The mobile phase consisted of water/acetic acid/acetonitrile 59.8/0.2/40 (v/v/v). An isocratic elution was run at a flow rate of 0.2 ml/min and column temperature was maintained at 35°C. The retention time of 15-F_{2t}-IsoP was 6.3 min. The experimental conditions of the MS were the following: Source voltage 3.9 kV, capillary voltage –106.5 kV, capillary temperature 300°C, sheath gas flow 12 l/min and auxiliary gas pressure 50 psi. ESI was used in the negative ion mode. Full scan mass spectra were acquired by scanning from m/z 100-400. MS-MS spectra were obtained by isolating $[M+H]^-$ m/z 353 of deprotonated 15-F_{2t}-IsoP and $[M+H]^-$ m/z 357 of deprotonated internal standard 15-F_{2t}-IsoP-d_4 as precursor ions followed by CID and scanning from 100 to 300 m/z. Quantification was carried out by calculating the ratio of the peak area of the sum of the most abundant product ions (m/z 193+253) for 15-F_{2t}-IsoP to the peak area of those

(m/z 197+257) for 15-F_{2t}-IsoP-d_4, respectively, in the reconstructed mass chromatograms.

Standard solutions with increasing concentration of 15-F_{2t}-IsoP (10, 20, 30, 40, 50 and 60 ng/ml) containing a constant amount (20 ng/ml) of 15-F_{2t}-IsoP-d_4 internal standard were prepared by aliquoting different volumes of a bulk standard solution. The standard curve was established with the response ratio (peak area of 15-F_{2t}-IsoP /peak area of 15-F_{2t}-IsoP-d_4) as dependent variable and standard concentrations as independent variable. Calculating the response ratios of the urine samples containing a known amount of internal standard 15-F_{2t}-IsoP-d_4 the standard curve obtained from linear regression analysis was used to determine 15-F_{2t}-IsoP concentrations in the urine samples.

2.6.7 Method validation parameters

Linearity was assessed by performing repeated analyses of calibration solutions followed by linear regression analysis as well as graphically by plotting analyte response factors (ratio of analyte response to analyte concentration) versus analyte concentration.

Trueness was studied by the standard addition method, i.e., by spiking a urine sample of established polyphenol content (12 repeated analyses) with 50, 100 and 150% of the polyphenol concentration of the non-spiked sample followed by triplicate analyses and calculation of recovery. Mean recovery was computed and tested against complete recovery (100%) by one-way student’s t-test.

Precision of the method was determined by 10 repeated analyses of the same urine sample and calculating relative standard deviation.

Sensitivity was determined according to Green (1996) by establishing limit of detection (LOD) and limit of quantitation (LOQ), respectively. The LOD was calculated as the analyte concentration that produces a signal three times over the noise level (S/N > 3). The LOQ was set at S/N > 10.

2.7 Statistical analyses

Results are generally expressed as means ± 95% confidence interval. However, for cocoa polyphenol and urinary marker concentrations determined by methods that have undergone validation, results are expressed as means ± expanded uncertainty with a coverage factor of 2 corresponding to a level of confidence of 95% following recommendations in official analytical guidelines (EURACHEM, 2000; Alder et al., 2001). This measure is determined in the validation process from random error (precision) and bias (trueness) and is immanent to the method used for quantification. Values were log-transformed before statistical analysis to compensate for unequal variances. Descriptive statistics (mean, standard deviation) and graphical presentations of results were performed using MS Excel 2000. Linear regression analyses and graphical presentation of standard curves were conducted with SigmaPlot Version 8.02 (SPSS Inc., Chicago. IL, USA). Statistical tests including student's t-test, calculation of Pearson's correlation coefficient, analysis of variance (ANOVA) and multiple comparisons of means (Tukey HSD) were carried out with Statistica software package version 5.5 (StatSoft Inc., Tulsa, OK, USA). Multivariate analyses (principal component analysis, PCA) were performed using the software package Unscrambler Version 7.6 (Camo ASA, Oslo, Norway). Statistical power analysis was done with SysStat Version 10 (SPSS Inc., Chicago. IL, USA).

Linear regression analyses were performed to establish equations for the relation of analyte response and analyte concentration as well as to test for linearity by proving significant difference from zero of the slope of the standard curve and no significant difference of the intercept. For wide concentration ranges over several orders of magnitude, linear regression was carried out using a weighting factor of 1/y in order to compensate for unequal variances.

One-way student's t-test was used to investigate for complete recovery in spiking experiments (determined recovery at various levels versus 100% fixed target value).

Analysis of variance (ANOVA) was used to test for any significant differences between samples. If the result of the F-test from the ANOVA was found to be significant ($p < 0.05$), Tukey's test was used *post hoc* to determine specific differences between mean values. Differences were considered significant at $p < 0.05$. ANOVA was also used for

proving representativeness of sampling by demonstrating non-significant differences in the variance of repeated sampling and analytical measurements.

Principal component analysis (PCA) was used for exploratory analysis allowing for data reduction, identification of patterns, and visualisation of clusters and outliers in the multivariate data set (results of 22 phenolic compounds in 112 samples).

The linear relationship between total polyphenols determined by the Folin-Ciocalteu assay and determined by the sum of polyphenols from HPLC-MS as well as between the latter and non-extractable polyphenols (NEPP) were determined calculating Pearson's' correlation coefficient r including test of significance at $p < 0.05$.

Statistical power analysis was performed for establishing the minimum number of samples for being able to show significant differences at given (biologically relevant) differences and determined biological variability.

3 Results and discussion

3.1 Evaluation of available methods for the analysis of polyphenols in chocolate and chocolate raw-products

This section describes the evaluation of available methods as part of this work followed by the selection of the most suitable ones for further use. This is mainly based on the comparison of performance characteristics of the practically applied methodologies in the laboratory but also on the observations made and conclusions drawn by other researchers taken from literature. Generally, analytical methods have first been set-up by adapting existing methodologies either in-house, or published in instrument suppliers' application notes, as conference contributions, or in peer-reviewed journals.

In order to optimise and compare the methodologies for the analysis of polyphenols in cocoa matrices a harmonised procedure of sample preparation has been used throughout this part of the study as described in chapter 2.1.2.

3.1.1 Colorimetric methods for polyphenol analysis

Three of the most commonly applied colorimetric methods have been compared for further evaluation: the Folin-Ciocalteu, Prussian-Blue, and Vanillin-HCl assays. All methods produce quantitative estimates of total polyphenol (Folin-Ciocalteu and Prussian-Blue) and total flavan-3-ol plus proanthocyanidin (Vanillin-HCl) content, respectively, given as equivalents of one standard phenolic compound, mostly gallic acid or catechin but no information about single phenolic compounds. Despite of various limitations of these assays as discussed in chapter 1.4.3, for the purpose of a quick screening of polyphenol content in various cocoa matrices, for the set-up of sample extraction procedure, and as simple tool for quick estimation of polyphenol content of raw material for cocoa processing and process control, these simple to conduct assays still represent valuable quantitative tools when interpreted appropriately.

Thus, for the purpose of this work, the three assays have been compared according to the assay precision both for the standard and for real samples, linearity of the assay over the tested concentration range as well as ease of use, the latter being judged

qualitatively according to the authors' experience when performing the assays. As cocoa contains mainly catechins and their oligomeric and polymeric procyanidin derivates, catechin has been used here as reference standard compound for calibration of all assays.

The precision was determined for calibration standards by calculating the mean relative standard deviations over the repeated analysis of calibration solutions that have been performed to establish standard curves. Whereas the Folin-Ciocalteu assay showed a high precision of 4.6% (expressed as coefficient of variation, CV%) and the Vanillin-HCl-assay a still acceptable precision of 7.3%, the precision determined for the Prussian-Blue assay, being 17.5%, is outstandingly high, especially when considering the absence of interfering matrix compounds in the calibration solutions. For real samples, both the Folin-Ciocalteu and Vanillin-HCl assays showed somewhat higher coefficient of variations of 9.9% and 9.8%, respectively, calculated from the variations in 10-12 repeated analyses of the same cocoa liquor sample. This lower precision in real samples is likely due to the presence of matrix compounds as well as due to the error contribution of the extraction procedure. The Prussian-Blue assay showed the lowest precision of the tested assays (14.6%) also for real samples. However, this precision is higher than the precision for the same assay determined with calibration solutions. It is not clear which could be the reasons for such a rather illogic but repeatable observation.

Assay linearity was assessed by performing repeated analyses of calibration solutions followed by linear regression analysis as well as graphically by plotting analyte response factors versus analyte concentration. Figures 3.1a to 3.3a show the standard curves of the assays including the resulting equation from linear regression analysis and the 95% confidence intervals for the regression line. Linearity was judged from the quality of the fitting of the linear regression (r-square value, R^2) as well as the statistical tests for significant ($p < 0.05$) difference from zero of the slope (should be different from zero) and the intercept (should NOT be different from zero) of the curve equation. In addition, linearity was judged visually by plotting response factors versus analyte concentration (figures 3.1b to 3.3b). Response factors should vary within borders of ±20% of the mean response factor and not showing any trend with catechin concentration.

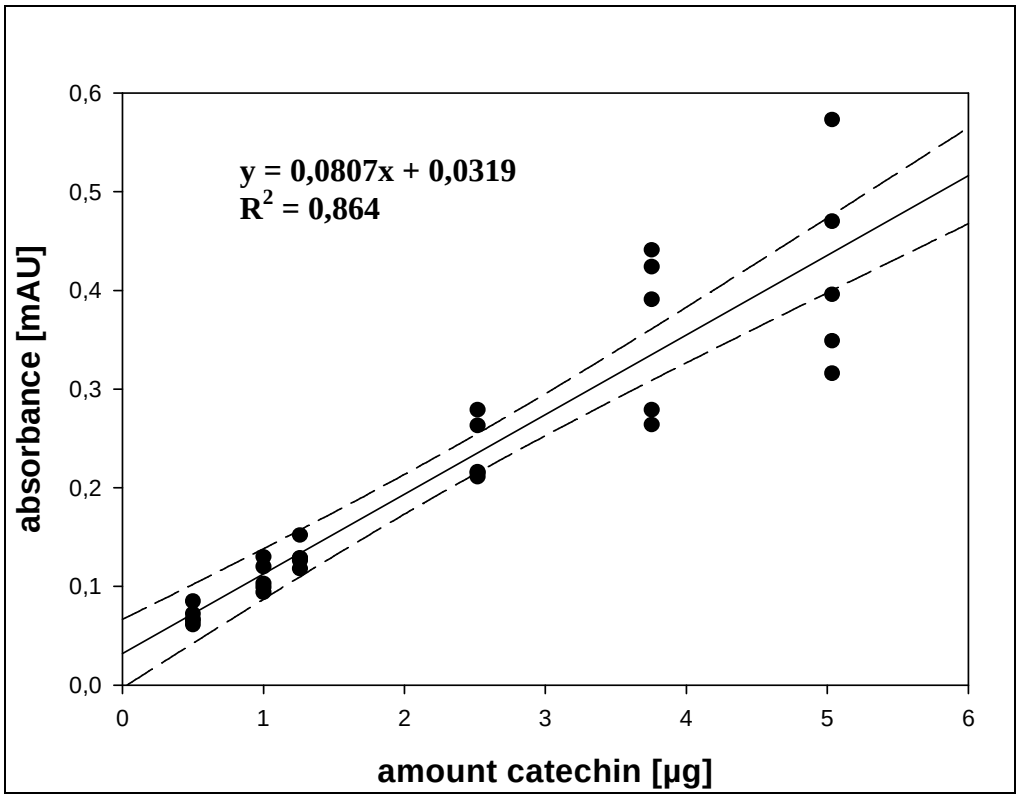


Figure 3.1a Standard curve for Prussian-Blue assay (linear regression) - dashed lines represent 95% confidence interval.

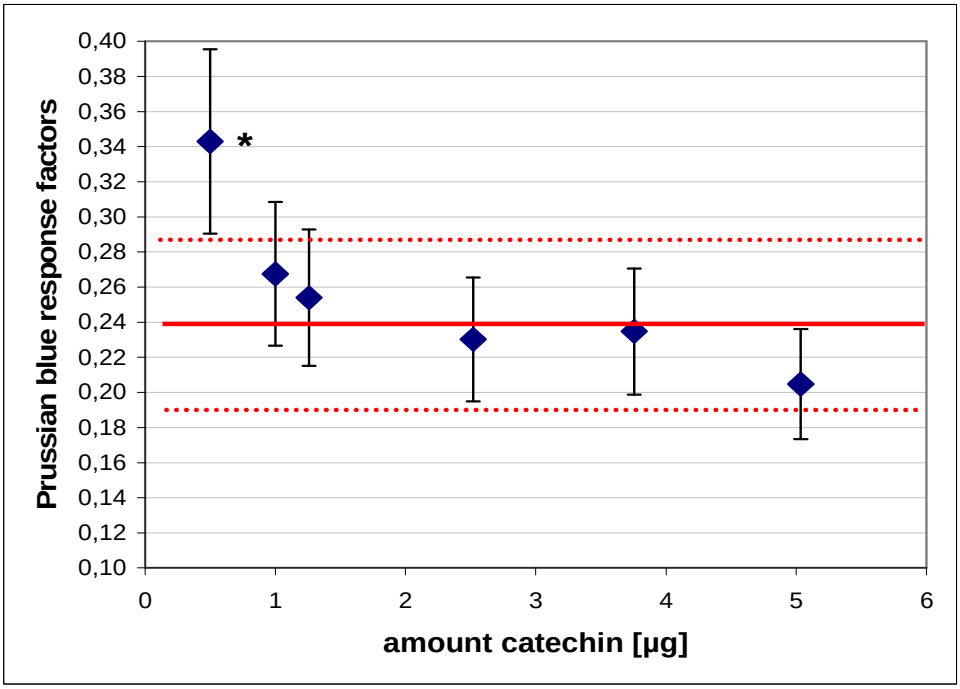


Figure 3.1b Linearity of Prussian-Blue method – horizontal lines represent average ± 20%, error bars represent 95% confidence intervals, * is an outlier.

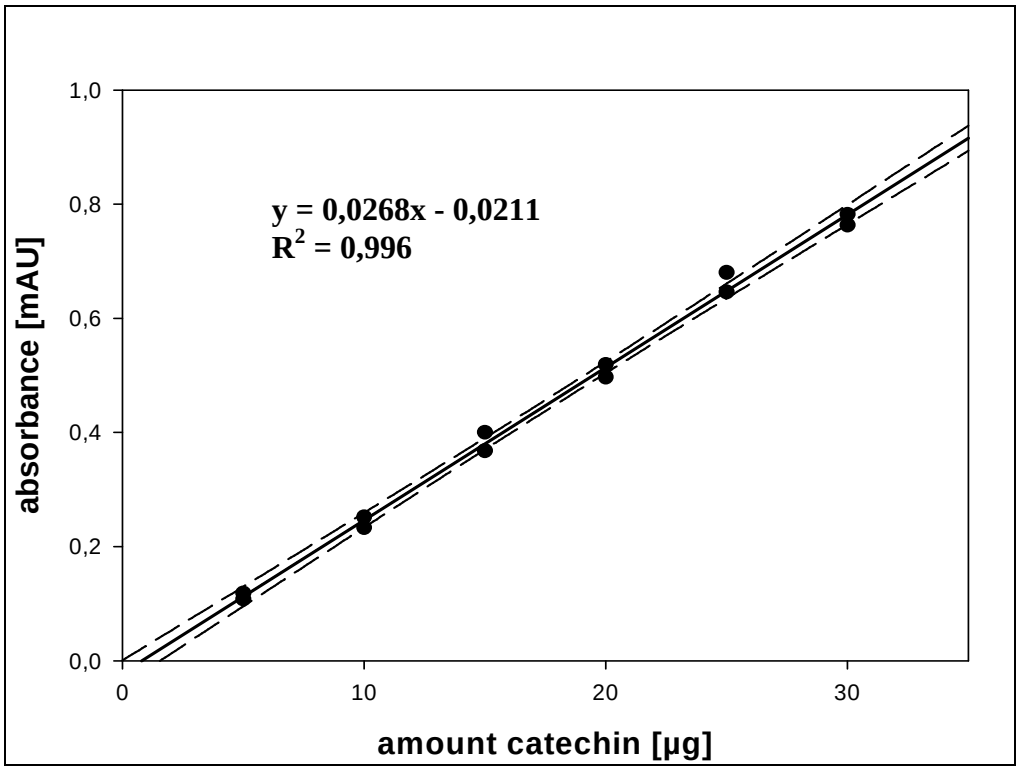


Figure 3.2a Standard curve for Folin-Ciocalteu assay (linear regression) - dashed lines represent 95% confidence interval**.**

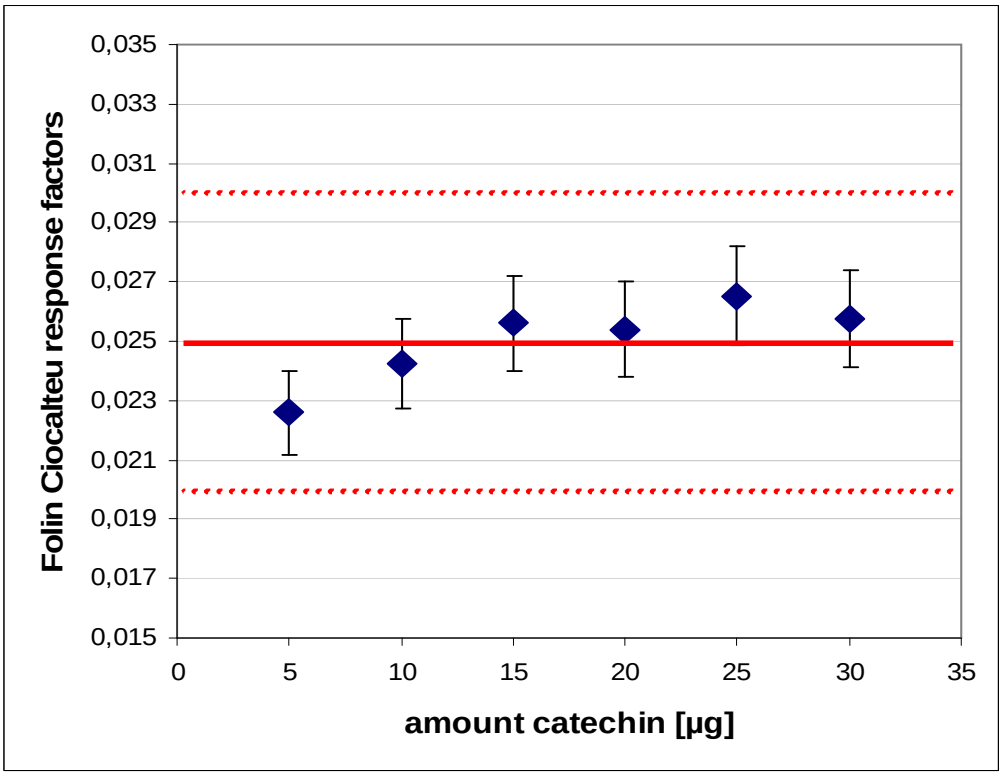


Figure 3.2b Linearity of Folin-Ciocalteu method – horizontal lines represent average +/- 20%, error bars represent 95% confidence intervals.

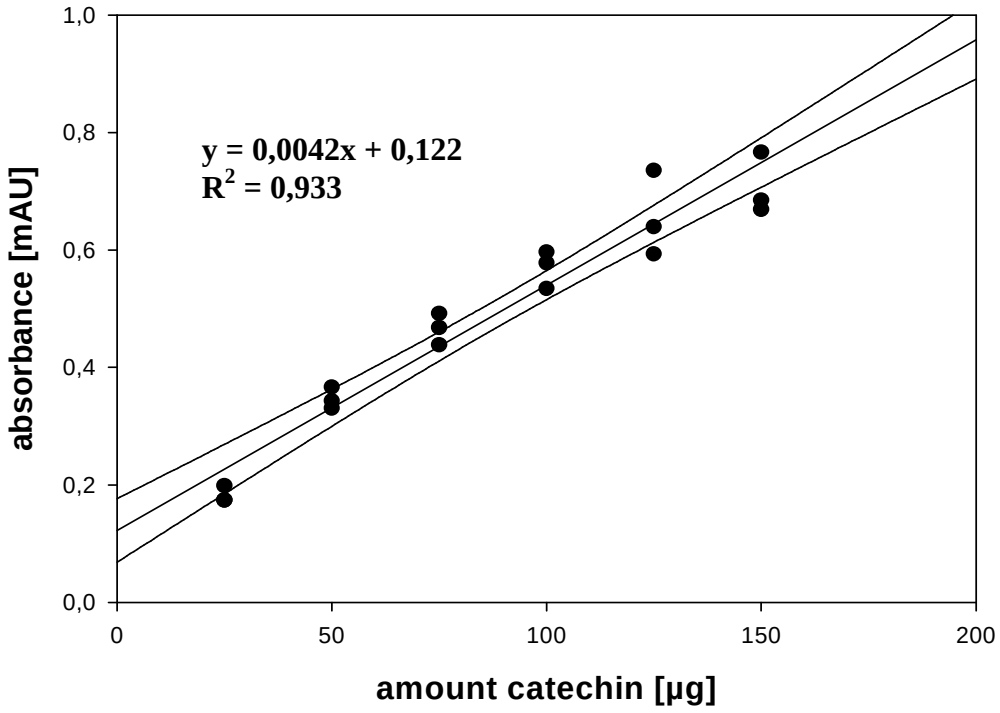


Figure 3.3a Standard curve for Vanillin-HCl assay (linear regression) - dashed lines represent 95% confidence intervall.

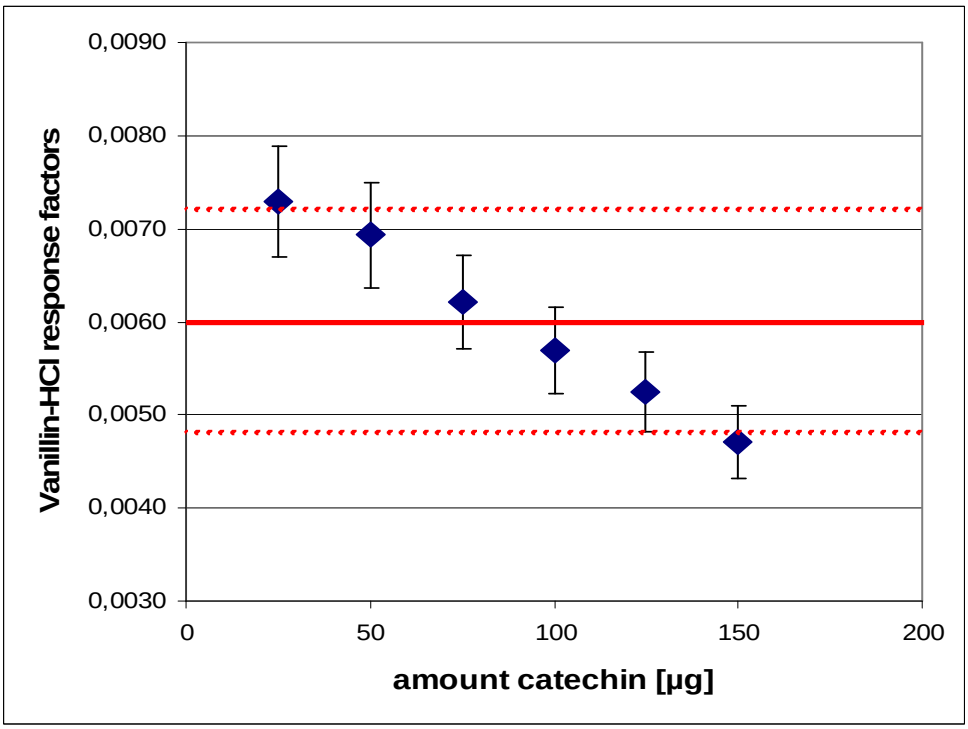


Figure 3.3b Linearity of Vanillin-HCl method – horizontal lines represent average +/- 20%, error bars represent 95% confidence intervals.

From the position of the single dots in the graphical presentation of the standard curves of the three assays and in the width of the 95% confidence intervals of the response factors, the high variation of the response of the Prussian-Blue method compared to the other two assays can be clearly seen. In addition, an evident trend of decreasing response factors with increasing concentration (or absolute amount of catechin in the cuvet) is evident for the Vanillin-HCl-assay in the tested range of 25 to 150µg. This shows a lack of linearity of the Vanillin-HCl assay, which is also confirmed by a significant difference ($p < 0.05$) for the intercept of the standard curve. In contrast, both other assays are linear over the tested concentration range, demonstrated graphically as well as by confirming significant differences from zero of the slopes and no significant differences for the intercepts of the standard curves. Nevertheless, the linear curve fitting achieved for the Folin-Ciocalteu-assay is clearly better than for the Prussian-Blue assay evidenced from their respective r-square values in the linear regression of 0.996 and 0.864, respectively. It is noteworthy that the linear regression performed for the Vanillin-HCl-assay reaches a reasonable curve fitting ($R^2 = 0.933$), which is even better than for the Prussian-Blue-assay despite the non-linear response. This highlights the importance of graphical presentations for interpreting assay linearity.

Finally, the "ease of use" of the assays for the analysis of cocoa polyphenols was compared. All assays are straightforward and easy to perform without the necessity of sophisticated instrumentation or technical skills of personal. If at all a difference between the assays is to be mentioned, it is the only little inconvenience of the need to prepare fresh reagent for every day for the Vanillin-HCl assay.

From the above it is clear that the Vanillin-HCl-assay is not applicable – at least in the present experimental design – to the analysis of cocoa polyphenols. Both the Prussian-Blue- and Folin-Ciocalteu assays are suitable for the fast quantitative estimation of polyphenols in cocoa, however, the latter is clearly superior in terms of repeatability. Table 3.1 summarises the findings of this assay comparison.

Table 3.1 Method performance characteristics of three commonly used colorimetric assays for the analysis of total polyphenol content in cocoa

	Prussian-Blue	**Folin-Ciocalteu**	**Vanillin-HCl**
Precision (catechin standard)	17.5%	4.6%	7.3%
Precision (real sample)	14.6%	9.9%	9.8%
Linearity	Acceptable	Good	Not linear
Ease of use	Straightforward	Straightforward	Daily reagent preparation
Final judgement	**Low repeatability**	**Good**	**Not linear**

The Vanillin-HCl assay may have given interesting information due to its selective reaction with the flavan-3-ols and procyanidins only, in particular when performed in combination with one of the other two methods (Price and Butler, 1977). However, due to the lack of linearity this method is unacceptable for quantitative analysis. Lack of linearity, low reproducibility, and variable reactivity of proanthocyanidin sub-units have been discussed elsewhere as limitations of the Vanillin-HCl assay and has led to various attempts in modifying the assay to overcome the shortcomings (Price et al., 1998; Desphande and Cheryan, 1985; Schofield et al., 2001).

The repeatability of the Prussian-Blue method has been shown to be very low compared to the Folin-Ciocalteu assay. This might be due to the low robustness of the method against small changes in the procedure. Graham (1992) reported formation of precipitates after short incubation periods, increases in colour density with time as well as dependence of the colour density to temperature, pH and even order in which reagents are added.

The Folin-Ciocalteu assay, or in its previous form the Folin-Denis assay, was originally designed and has been generally adopted by clinical laboratories for the analysis of

tyrosine (α-amino-β-(p-hydroxy)-phenylproprionic acid) in proteins and all phenols will react (Singleton and Rossi, 1965; Schofield et al., 2001). Slightly modified from the original procedure reported by Singleton and Rossi (1965) the Folin-Ciocalteu assay has become the official AOAC method for the analysis of total phenols in food (AOAC, 1975). Also in the comparison of three colorimetric assays as part of the present work, the Folin-Ciocalteu assay has been proved best in repeatability both with standard and real sample as well as linearity. In addition, this assay has been successfully applied to the analysis of total phenols in cocoa nibs, cocoa liquor, dark chocolate and milk chocolate (figure 3.4). The contents range form approximately 2 to 12 g/kg catechin equivalents. Since these values are accompanied by an analytical error of the methodology, the established precision of 9.9% has been used to calculate the uncertainty of these determined (mean) values in accordance with official analytical guidelines (EURACHEM, 2000; Alder et al., 2001).

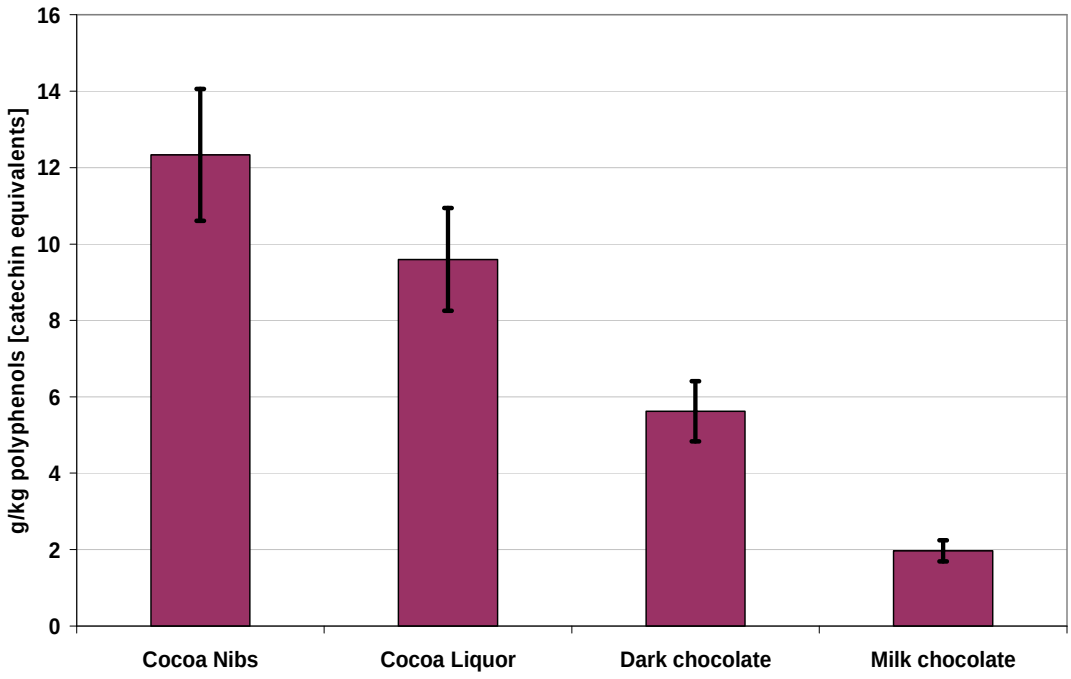


Figure 3.4 Polyphenols content in cocoa nibs, cocoa liquor, dark chocolate, and milk chocolate, respectively, as determined by the Folin-Ciocalteu assay – values represent average of 3 independent determinations ± expanded measurement uncertainty with a coverage factor of 2 corresponding to 95% confidence level.

3.1.2 Normal-phase versus reversed-phase high-performance liquid chromatography

A normal phase-high-performance liquid chromatographic method was established with identical conditions as described by Rigaud et al. (1993), who had first applied this methodology to cocoa polyphenols.

However, when it was set-up for this study, the method showed poor repeatability in retention times as well as peak height and area, respectively, in both the UV and electrochemical detectors. Peak shape was broad and sometimes asymmetric and neither the epimeric pairs catechin and epicatechin nor procyanidin dimers B2 and B5 reported by Porter et al. (1991) were resolved with this method (figure 3.5).

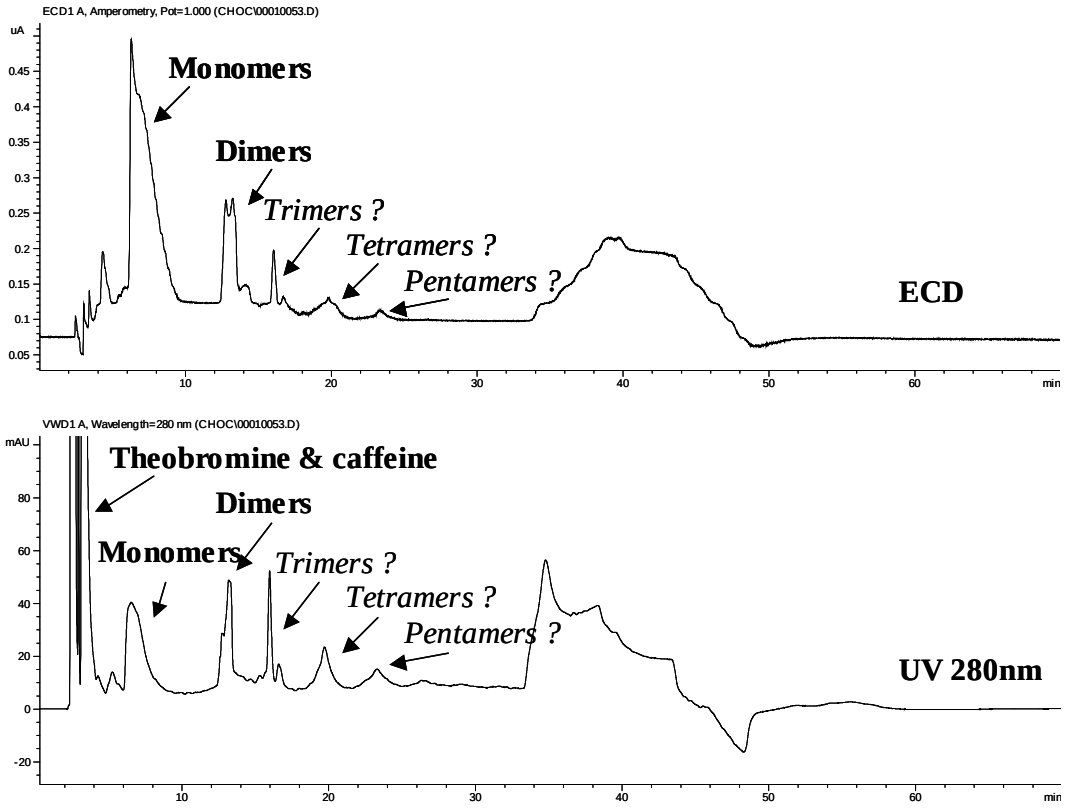


Figure 3.5 Normal-phase HPLC with UV/Vis (λ = 280 nm) (bottom) and electrochemical detection (ECD, top) (potential set at +1.0 V) of cocoa polyphenols in cocoa liquor – compounds in bold have been identified by comparison with authentic standards, compounds in italic are tentatively assigned.

Moreover, other more or less minor phenolic compounds, such as quercetin and quercetin glycosides in cocoa, were not separated from the major compounds including the catechins and the dimeric to tetrameric procyanidins and thus no information on these compounds can be obtained with this method. In addition, an important drawback of this method is the use of dichloromethane as main solvent compound, a highly toxic chemical. It is noteworthy in this context that chlorinated and fluorinated carbons are banned by the Montreal Protocol on substances that deplete the ozone layer (2000).

For comparison a reversed phase HPLC analysis method was set up using a binary gradient of methanol-water in the presence of 1% acetic acid optimised for the separation of major phenolic compounds. The system was optimised using standard solutions of commercially available compounds that can be found in cocoa, such as catechin, epicatechin and procyanidin B2 and the two xanthine alkaloids caffeine and theobromine. Subsequently, the developed system was also applied to extracts of cocoa liquor. Initially, only variable wavelength UV/Vis detection at 280 nm was used to identify cocoa polyphenols and xanthines. In order to improve selectivity of the method a combination in series of UV/Vis and electrochemical detection as well as UV/Vis and fluorescence detection were applied. Both fluorescence and electrochemical detection at optimised parameter settings have shown to be very specific for polyphenols greatly reducing the interferences originating from other matrix compounds (McMurrough and Byrne, 1992; Adamson et al., 1999). As shown in figure 3.6a, reversed phase HPLC with UV/Vis and electrochemical detection in series allowed good separation of catechin, epicatechin and procyanidin B2 as well as xanthines within 40 minutes, but no separation of higher procyanidin oligomers.

The presence of co-eluting procyanidins in this system was discovered only later transferring the HPLC-UV/Vis-fluorescence system to a different instrument maintaining the same column and solvent gradients but coupled to fluorescence and mass spectrometric detection in a parallel design using a three-way valve (figure 3.6b). Mass spectrometric detection allows identification of co-eluting polyphenols having similar UV absorbance, fluorescence, and electrochemical properties due to their common basic structure according to differences in their masses, or to be more precise, in their mass to charge ratios after ionisation.

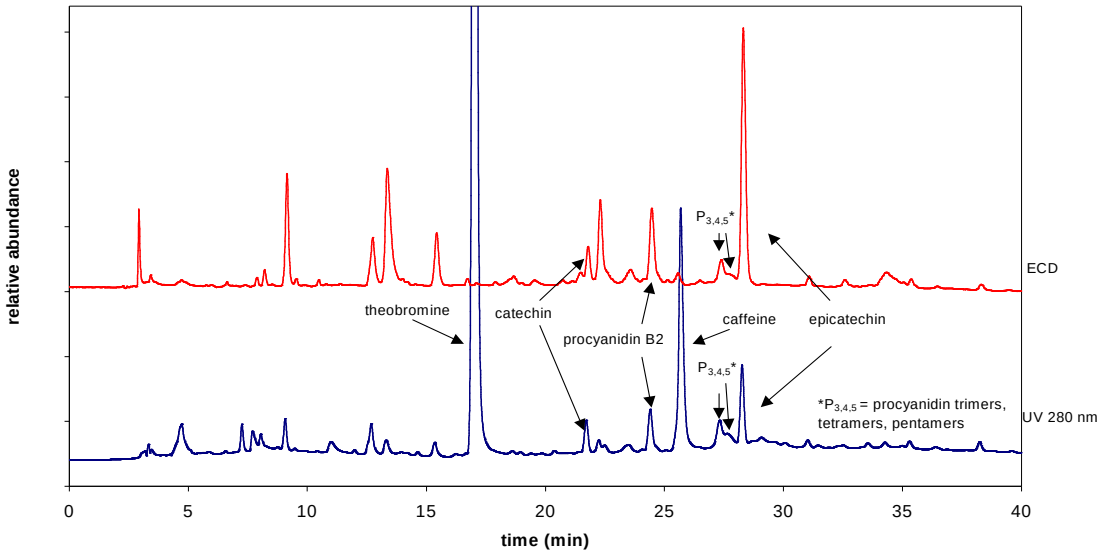


Figure 3.6a Reversed-phase HPLC with UV/Vis (λ = 280 nm) and electrochemical detection (potential set at +1.0 V) of cocoa polyphenols in cocoa liquor (for exact conditions see chapter 2).

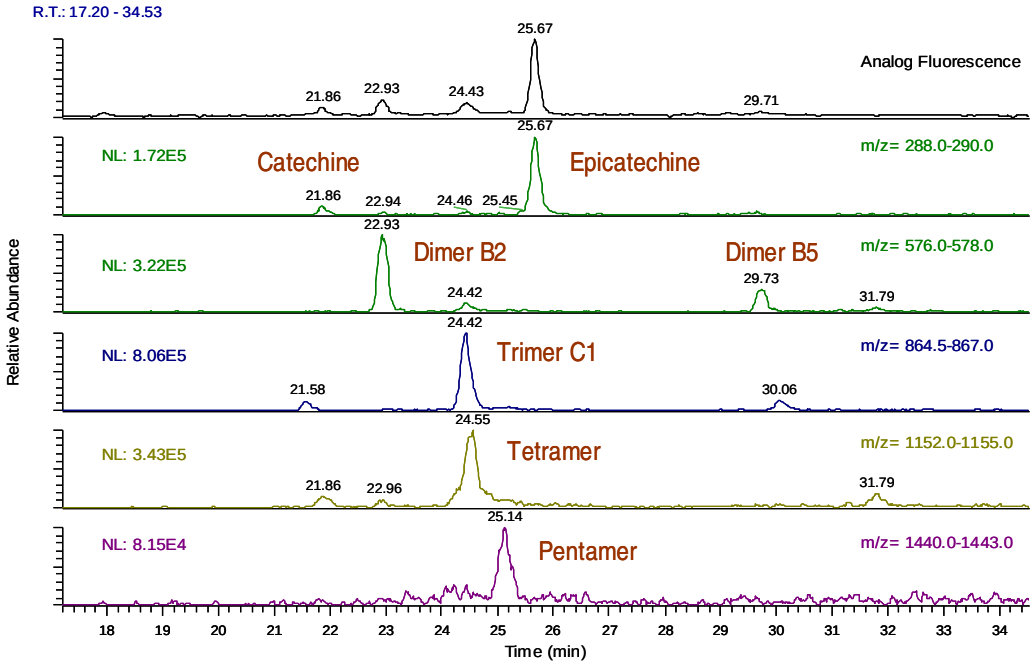


Figure 3.6b Reversed-phase HPLC as in figure 3.7a but transferred to a system with fluorescence (top chromatogram) and mass spectrometric detection (reconstructed mass chromatograms of deprotonated ions corresponding to catechins and dimeric to pentameric procyanidins).

In conclusion, reversed phase chromatography has been preferred as analytical separation technique for the following studies without further comparison of specific method performance criteria particularly in view of the reduced toxicity of solvents, in particular for the use of a mass selective detector, where the solvent is evaporated in the ion source.

3.1.3 Micellar electrokinetic capillary chromatography as alternative to high performance liquid chromatography

So far no methodology based on capillary electrophoresis (CE) for the analysis of cocoa polyphenols has been published. Therefore, this part of the study aimed at setting up a micelar electrokinetic capillary chromatographic (MEKC) method capable of separating the major phenolic compounds in cocoa and to compare the performance characteristics of this method with those of a common reversed phase HPLC method for the targeted determination of polyphenols in cocoa products as set up in section 3.1.2. The sample preparation was equal for both methods as described in chapter 2.1.2.

For the MEKC separation a mixture of boric acid and sodium dodecyl sulphate (SDS) was chosen as the buffer-surfactant system after having screened various other options, including carbonate, phosphate, 3-[N-morpholino]-propanesulfonic acid (MOPS), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), (Tris(hydroxymethyl)-aminomethane (TRIS), and (2-[N-cyclohexamino]-ethanesulfonic acid (CHES). In order to optimise separation of cocoa polyphenols several organic modifiers have been tested including ethanol, acetonitrile, acetone, isopropanol and methanol. Methanol showed the greatest improvement in analytes separation without increasing too much the total analysis time. The instrument was operated in the positive voltage mode and spectral data were collected with an UV-DAD detector. The effect of applied voltage and injection technique were studied for further method optimisation of pH, borate, SDS and methanol concentrations.

The difficulty here derives from the fact that these four parameters interact with each other and thus, optimising one at a time might not lead to the best result. For the simultaneous optimisation of all parameters a simplex protocol has been applied as previously described for optimisation of gas chromatography conditions (Dose, 1987; Guillaume and Guinchard, 1996; Snijders et al., 1996). The simplex method is a

multifactor optimisation strategy using a hill-climbing algorithm that moves a pattern of (k + 1) experimental points (for k factors) away from regions of worse response toward convergence on an optimum in the response surface (Walters et al., 1991). Before applying an optimisation scheme, criteria for the evaluation of the separation quality of cocoa polyphenols had to be defined. Since cocoa contains various phenolic compounds for which no standards are commercially available, such as the oligomeric procyanidins, the optimisation has been conducted using a real sample, i.e., an extract of cocoa liquor polyphenols. Two criteria have been defined as follows:

Separation efficiency, being a qualitative judgement of the separation of xanthine alkaloids as well as known (standard available) and unknown polyphenols (The identity and peak purity of compounds has been confirmed by comparing electrophoretic mobility with authentic standards and UV-spectra, respectively. For unknown compounds UV-spectra similar to those of catechins and procyanidin dimer B2 have been considered as confirmation for the presence of other unknown procyanidins).

Velocity of analysis time, with analysis time exceeding 50 minutes being rejected.

Before starting the optimisation procedure the upper and lower borderline levels of each parameter were determined by practical considerations, such as buffer pH range or critical micelle concentration (CMC) of SDS (8mM) and maximal solubility. The simplex method starts with the definition of initial (k + 1) vertexes. The location of the initial simplex should be carefully chosen, as a problem associated with this procedure is that the resulting optimum may not be the global but only a local optimum (Walters et al., 1991; Snijders et al., 1996). However, the determined optimum can be and was here confirmed by starting one or more new simplex procedures with different starting locations (Walters et al., 1991). In one approach the four parameters of the initial simplex were set from previously performed experiments and selected additional conditions between the upper and lower level of the n + 1 variables giving five different combinations representing the vertexes of the starting simplex. In the second approach initial levels of parameters were calculated by selecting a set of base levels (c_1-c_4) followed by calculation of the five variable combinations according to the algorithm outlined in table 3.2 after having set a practical step width for each parameter (a_1-a_4).

Table 3.2 Scheme for calculation of initial simplex

Vertex-no.	pH	Methanol	SDS	Boric acid
1	c_1-a_1	c_2-a_2	c_3-a_3	c_4-a_4
2	$c_{1+}a_1$	c_2-a_2	c_3-a_3	c_4-a_4
3	c_1	c_2+2a_2	c_3-a_3	c_4-a_4
4	c_1	c_2	c_3+3a_3	c_4-a_4
5	c_1	c_2	c_3	c_4+4a_4

With: c_1 = 8.5 and a_1 = 0.2; c_2 = 20% and a_2 = 5%; c_3 = 50mM and a_3 = 10mM; c_4 = 200mM and a_4 = 25mM.

MEKC separations of cocoa extracts have been conducted for all initial and subsequently calculated conditions in triplicate. The optimisation continues then by rejecting the least desirable case followed by calculation of the conditions of the replacing vertex (R) guided by the algorithm R = C + (C – W), with C being the average value of the four not rejected vertexes and W being the conditions of the worst and therefore rejected vertex. The next simplex consists then of the remaining four combinations of conditions of the parameters and the one new calculated parameter combination. In one approach, the first new calculated vertex R was found to be the least desirable. In order not to oscillate between two vertexes the second worst case has been rejected and the algorithm applied as described above. The process of evaluation is repeated with the new simplex until the calculated new fifth vertex is judged to be the least desirable. Here, the resulting optimised MEKC conditions for the separation of cocoa polyphenols were in agreement for both initial simplexes: 175 mM boric acid with 40 mM SDS and 30 % methanol, pH 7.9. It should be emphasised that with this optimisation method less than 10 trials for parameter combinations including the 5 initial ones for each starting simplex had to be conducted for finding the optimised conditions. This is considered to be a quick and systematic optimisation procedure and a

useful practical approach for optimisation of more than two parameters. Liang et al. (1997) used a tedious determination of pK_a values of analytes followed by mathematic modelling, whereas Ng et al. (1992) applied a rectangular optimisation scheme for two parameters at 3 levels (lowest, medium, and highest practical levels) needed $3^2 = 9$ trials, which would have increased to $3^4 = 81$ trials in the present case of four parameter optimisation. Figure 3.7 shows the electropherogram of a cocoa liquor extract under optimised MECC conditions.

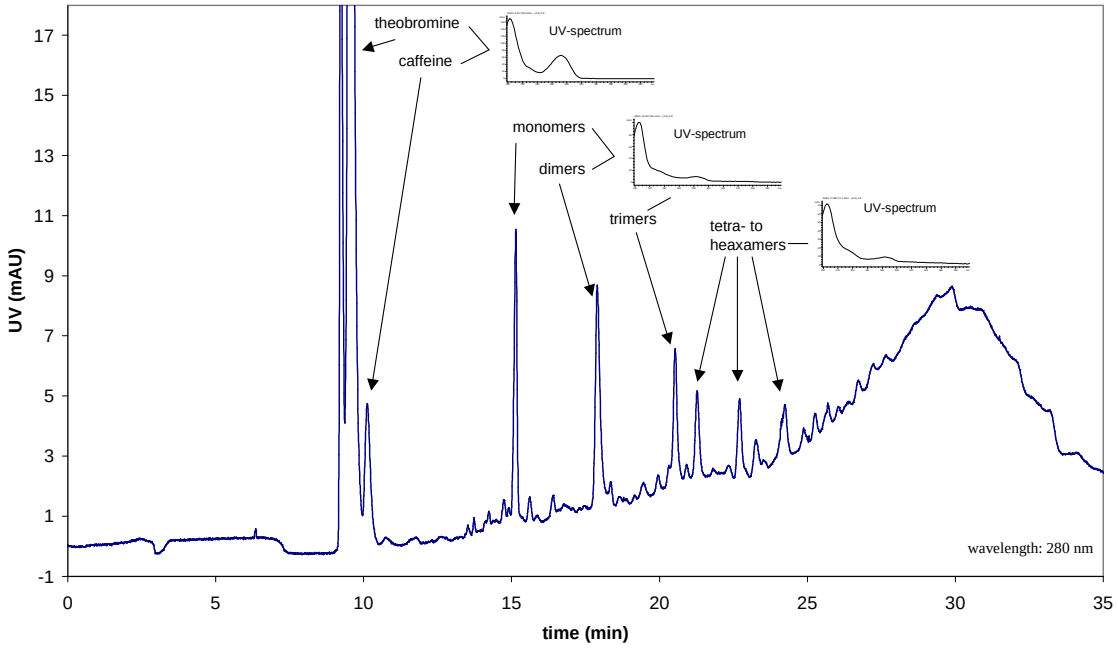


Figure 3.7 Micellar electrokinetic capillary chromatography (MEKC) with UV-DAD detection of cocoa polyphenols in cocoa liquor.

The developed MEKC-UV-DAD method allowed separation of theobromine, caffeine, catechins, and procyanidin oligomers with a very good resolution within 35 minutes. However, epimeric compounds like catechin and epicatechin were not separated under the chosen conditions. The addition of urea, cyclodextrins or combinations thereof, as proposed by Nelson et al. (1998) for the separations of chiral isomers, did not show any improvement in separation of catechin and epicatechin. DAD allows identification of polyphenols against authentic standards (caffeine, theobromine, catechin, epicatechin, and procyanidin dimer B2) and confirmation of peak purity according to the UV-spectra. Peaks migrating slower than the procyanidin dimer had the same UV spectra as

the dimer and were believed to be higher procyanidin oligomers. Confirmation of the procyanidin trimer C1 was done after isolation from cocoa powder by a combination of various chromatographic techniques and subsequent identification by LC-MS and NMR as described later in this chapter. Higher procyanidin oligomers (tetramer, pentamer, and hexamer) were tentatively identified by repeated injections of various procyanidin-rich fractions from semi-preparative HPLC having different ratios of tetramer, pentamer and hexamer as characterised by simultaneous LC-MS experiments of these fractions. Despite the separation capacities of this method for oligomeric procyanidins representing a valid complementary technique to reversed-phase HPLC, the baseline increase between 15 and 30 minutes of the electropherogram suggest the presence of underlying co-eluting matrix compounds with UV-spectra (figure 3.8) most similar to catechins and procyanidin dimer B2 that have not been possible to be resolved by variations in SDS, borate or methanol concentrations.

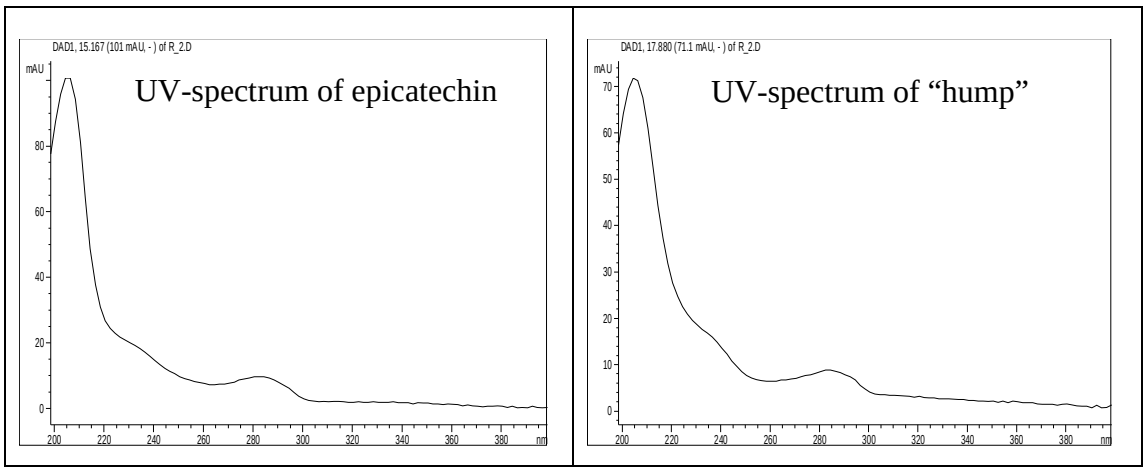


Figure 3.8 UV-spectra of epicatechin standard (left) and unresolved compounds of the electrphoretic or chromatographic “hump” (right) in figures 3.7 and 3.9, respectively.

This effect can also be seen in reversed-phase HPLC as being demonstrated in the UV-DAD trace at 280 nm of the HPLC-UV-DAD-ESI-MS method discussed more in detail later in this chapter (figure 3.9).

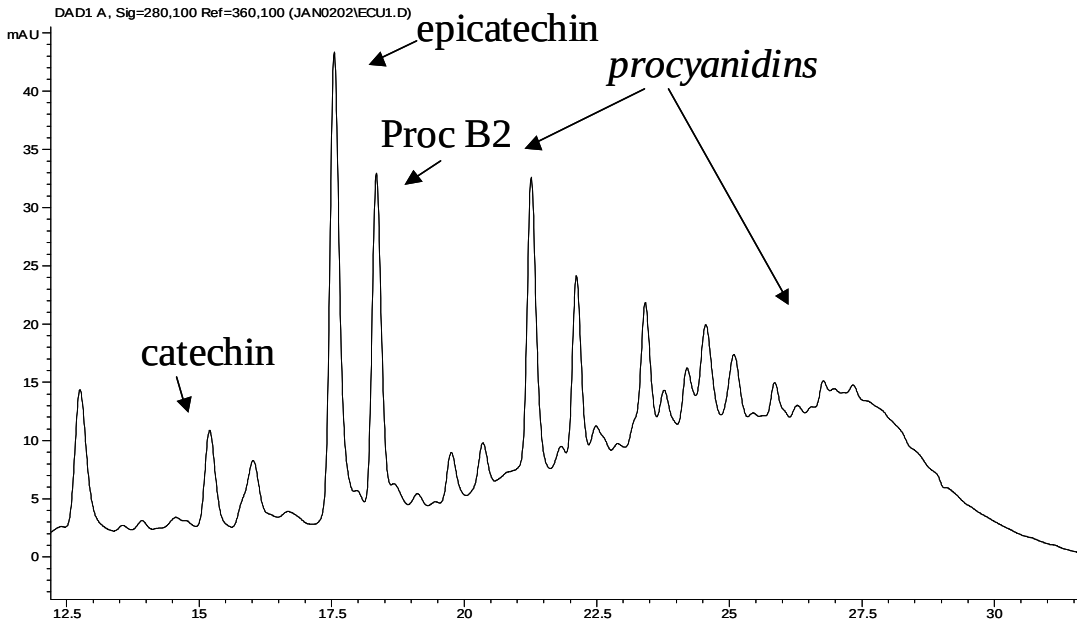


Figure 3.9 Chromatogram of cocoa liquor extract – UV at 280nm (conditions see HPLC-DAD-ESI-MS method in chapter 2.1.6).

The presence of an underlying unresolved hump has been observed as well by Larger et al. (1998) for the analysis of black tea polyphenols by MEKC. They suggested either adsorption or degradation of condensed tea tannins (theaflavins, thearubigens) by the capillary wall and proposed the use of physical or dynamic coating of the inner capillary wall or the introduction of a liquid-liquid extraction (LLE) step during sample preparation. However, polyphenols were not distributed quantitatively between fractions neither in the three black tea polyphenol fractions described by Larger et al. (1998) nor when applying LLE with ethyl-acetate and n-butanol for cocoa polyphenols for the method presented here. Thus, the introduction of this sample preparation step would represent a limitation for quantitative use. Nevertheless, the elimination of these underlying matrix compounds in the most apolar ethyl-acetate fraction together with the similarity of UV-spectra with those of procyanidins and catechins, respectively, as well as the late elution suggest the presence of large hydrophilic phenolic compounds, either associated with the protein or fibre matrix of cocoa or simply high polymeric or possibly partly oxidised. More evidence for this can be deduced from Guyot et al. (1997) characterising polymeric procyanidins in cider apple skin and pulp as well as Le Roux et al. (1998) characterising those of litchi pericarp. They describe such an

unresolved chromatographic “hump” with a single UV absorbance band around 278 nm corresponding to those of catechins by combinations of chromatographic isolation procedures, partial hydrolysis and subsequent structure elucidation by NMR and MS as polymeric procyanidins with degrees of polymerisation between 4 and 22 with more complicated bonding between sub-units.

The results from this part of the study indicate that MEKC is a well-suited technique complementary to HPLC for the analysis of polyphenols in cocoa products. It offers the advantage of relatively short analysis times in combination with good resolution of compounds. In addition and unlike HPLC, only tiny amounts of organic solvent are consumed thereby reducing costs and environmental burden. Direct injection of complex matrices, such as food extracts or biological fluids is possible with CE in general without the risk of column obstruction (Torres-Lapasio et al., 2000). The presented reversed-phase HPLC method with UV/Vis and electrochemical or fluorescence detection in series (see section 3.1.2) allowed good separation of caffeine, theobromine, catechin, epicatechin and procyanidin B2 within about the same time frame of 35 to 40 minutes. However, unlike the presented MEKC method the separation of higher procyanidin oligomers was not possible under these HPLC conditions.

3.1.4 High-performance liquid chromatography with mass spectrometric detection

Although technically possible the present mass-selective detection systems were not available for coupling with the CE-system for the period of this study and consequently, only liquid chromatography-mass spectrometry (HPLC-MS) was possible to apply for additional studies. Thus, the objective here was to develop a reversed-phase HPLC-MS method capable of separating and tentatively identifying catechins, procyanidins, and other minor phenolic compounds in chocolate in a reasonably short analysis time and with a minimum of sample preparation. Subsequently, this method should have been optimised and validated for quantitative use, as the application of UV or fluorescence detection for quantitation and the sole use of MS for compound confirmation did not suffice for quantifying the whole range of polyphenols in cocoa.

Two HPLC-MS systems were available: one was based on a single quadrupole mass analyser that allowed the tentative identification of compounds according to the mass-

to-charge ratio of their (de)protonated ions as well as some fragmentation by application of an accelerating field inside the mass analyser creating suitable collision-induced dissociation (CID) in the intermediate pressure region between source and mass analyser. Whereas this system has proven to be valid for the quantification of catechin and epicatechin in a proficiency testing exercise (see section 3.3.2), it did not permit the unequivocal assignment of mass spectra to a number of cocoa polyphenols due to their structural similarity. This was achieved with the second system, a modern ion trap mass analyser that allows not only the detection of pseudomolecular ions but also of specific fragments of these ions that are first isolated and in a second step subject to single and multiple CID reactions with helium in the mass analyser. This study is described in section 3.2.1 and includes MS and MS^n analysis of cocoa polyphenols mainly in the negative ion mode as well as the investigation of the presence of multiple-charged species by determination of the isotopic distribution of the signals. Moreover, this HPLC-MS method has been optimised and validated for quantitative use and subsequently applied to the determination of polyphenol contents in various chocolate and chocolate raw products. Finally, the system was set to be used for identifying cocoa polyphenols and their in-vivo metabolites in human plasma and urine after the consumption of dark and milk chocolate (section 3.5).

3.1.5 Non-extractable procyanidins through hydrolysis with a modified proanthocyanidin assay

It is well known that despite all efforts, proanthocyanidins cannot be extracted exhaustively from plant material (Rohr, 1999). Nevertheless, this polyphenols could still exert some biological effects, at least in the gastrointestinal tract. Thus, the objective was to set up a methodology to determine the amount of non-extractable polyphenols (NEPP), i.e., higher polymeric and matrix-bound procyanidins in the residue of the extraction of fat and polyphenols from cocoa matrices according to the procedures described in 2.1.2 and 2.3.2, respectively.

The proanthocyanidin assay as described by Porter et al. (1986) was used in a slightly modified form. Commonly, after a hydrolysis time of 40 to 120 minutes the amount of resulting anthocyanidin is measured spectrophotometrically at a detection wavelength between 545 and 555 nm. Here in a slightly modified form, the analytical technique used is a quick (less than 10 minutes) isocratic HPLC method allowing detection of

both cyanidin and delphinidin as the resulting anthocyanidin after depolymerisation of bound procyanidins and prodelphinidins, respectively. Nevertheless, when applied to cocoa in this study, the resulting anthocyanidin from depolymerisation of proanthocyanidins was exclusively cyanidin suggesting the presence of exclusively catechin- and epicatechin-based procyanidins in cocoa.

Despite the shortcomings outlined in chapter 1.4.4 this modified proanthocyanidin assay has been further used in this study as it appears to be a simple estimate of non-extractable cocoa polyphenols that could still exert some biological activity in the human gut. The repeatability of the assay was calculated to be 15% (coefficient of variation) from duplicate analysis of samples of cocoa beans, cocoa nibs, cocoa liquor, dark chocolate and milk chocolate and subsequent analysis of variance. This is an acceptable precision considering the errors from multiple mechanical extractions of polyphenols as well as the 75 minutes hydrolysation procedure prior to HPLC analyses. Nevertheless, the results obtained with this assay should be considered indicative and probably an underestimation mainly due to the formation of side-reaction products (Rohr, 1999; Schofield et al., 2001).

3.2 Identification of phenolic compounds in chocolate

In the following the identification of phenolic compounds present in chocolate, cocoa liquor, and cocoa nibs, respectively, is described. Firstly, this has been achieved tentatively according to the mass-to-charge ratio of their protonated or deprotonated ions as well as the product ions thereof followed by interpretation of fragmentation pathways using high-performance liquid chromatography-electrospray ionisation-mass spectrometry (HPLC-ESI-MS). Further evidence was deduced by comparison of their retention times and mass spectra with those of commercially available authentic standards. Finally, several cocoa polyphenols that cannot be found on the market have been isolated and purified by a combination of various chromatographic techniques, and their structure has been elucidated by means of various nuclear magnetic resonance (NMR) experiments, as well as HPLC-MS and hydrogen/deuterium exchange mass spectrometry (H/D-MS).

3.2.1 Identification of the major phenolic cocoa compounds

Prior to high-performance liquid chromatographic separation of cocoa polyphenols, direct infusion experiments were carried out with cocoa liquor extract in order to obtain an overview of ionisable compounds and to identify the most relevant ions for further investigation. However, the presence of several matrix compounds in the extract resulted in mass spectra that were difficult to read. Therefore, the mass spectra were gained by summing up all single recorded mass spectra over the whole chromatogram region. It must be notified that the first 5 minutes of the column effluent containing non-phenolic matrix compounds that were not retained by the column were diverted to waste in order to avoid rapid quick decrease in sensitivity of MS analysis.

Figure 3.10 and 3.11 show the full scan negative and positive ionisation mass spectra, respectively. The mass-to-charge ratios of ions corresponding to major polyphenols in cocoa have been highlighted in both spectra. Signals having the same Roman numerals correspond to deprotonated ions and protonated ions of the same polyphenol in the negative and positive ion mass spectra, respectively.

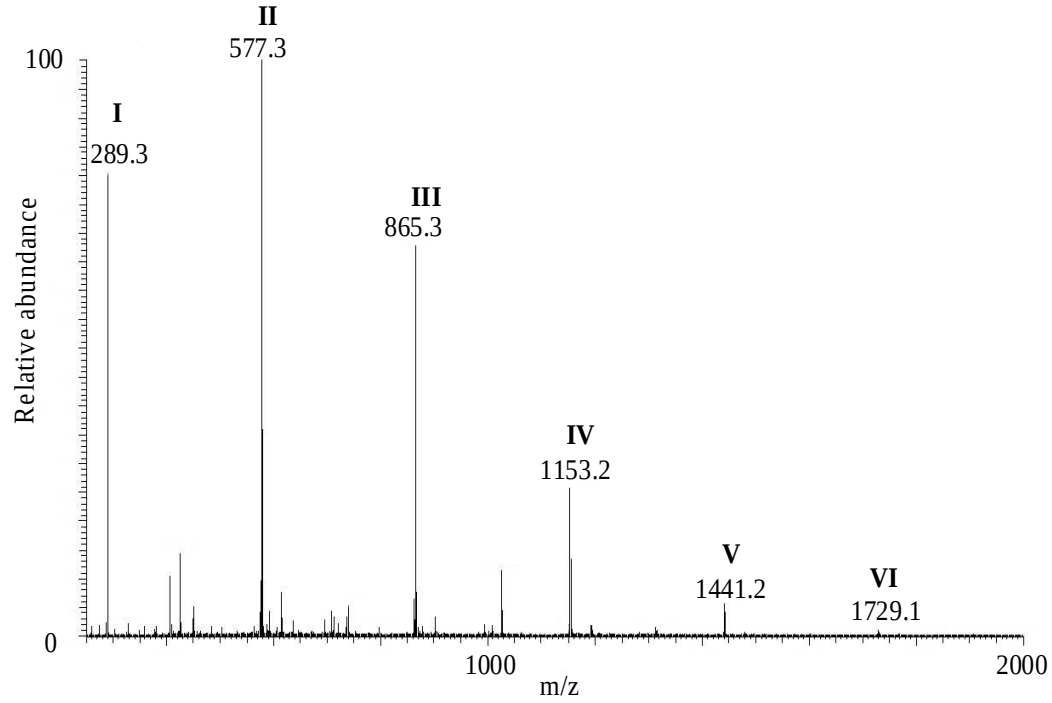


Figure 3.10 Negative ion mass spectrum of cocoa liquor extract. Compound I: catechin monomers, compounds II-VI: procyanidin oligomers (dimer through hexamer).

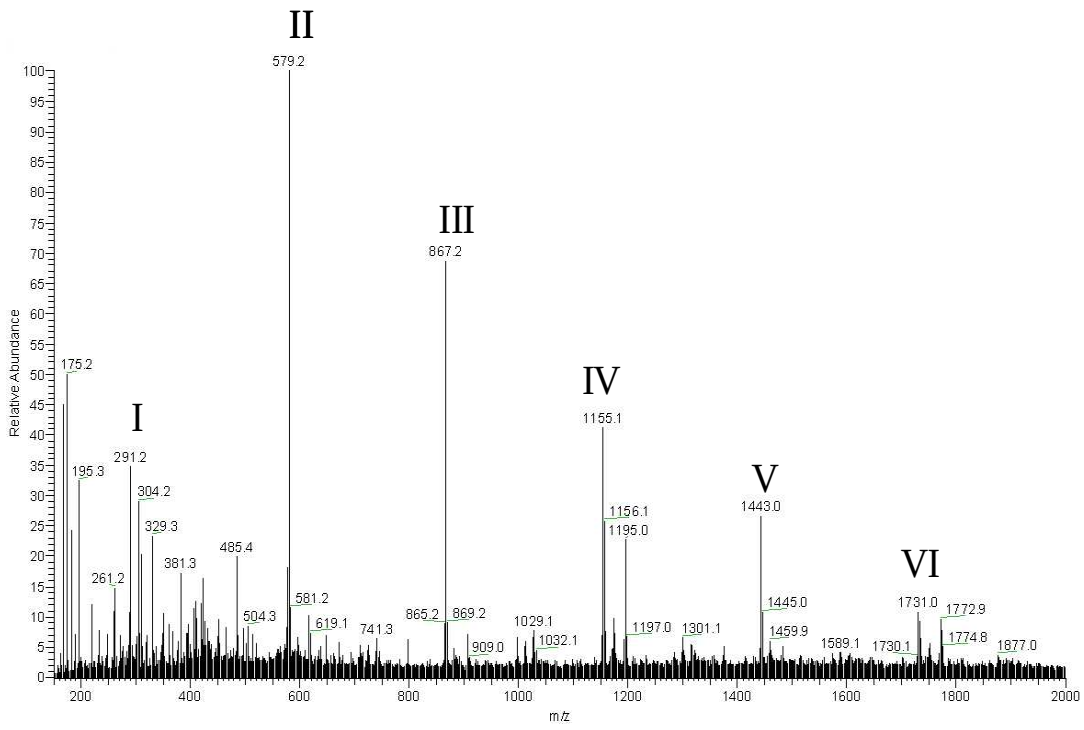


Figure 3.11 Positive ion mass spectrum of cocoa liquor extract. Compound I: catechin monomers, compounds II-VI: procyanidin oligomers (dimer through hexamer).

The monomeric catechin and epicatechin with a molecular weight of 290 Da show pseudomolecular ions ($[M-H]^-$) (deprotonation) at m/z 289 and ($[M+H]^+$) (protonation) at m/z 291, respectively (compound I in figures 3.10 and 3.11). The subsequent oligomeric procyanidin dimers, trimers, tetramers and so forth being built up by epi(catechin) subunits have molecular weights of 578 (290+288), 866 (578+288), 1154 (866+288) Da etc., and give deprotonated ions at m/z 577, 865, 1153, 1441, 1729 as well as protonated ions at m/z 579, 867, 1155, 1443, 1731 for dimers through hexamers (compounds II-VI).

Comparing negative and positive mass spectra it can be clearly seen by the presence of high background noise that the negative ion spectra is more selective towards polyphenols, which are weak acids that are more easily deprotonated than protonated. However, the preference for the negative ion mode is counteracted by improving chromatographic separation in reversed-phase HPLC through decrease in pH because deprotonation is more difficult at low pH. Ammonium acetate (pH 6) was tested as compromise between chromatographic resolution and pH for negative ion mode in the ion source. Surprisingly, very low presence of acetic acid (0.2%, pH 4) has proven to work better in terms of ion intensity and background noise. Another option was the addition of ammonia via a three-way valve after the chromatographic column prior to electrospray ionisation. However, ion intensities did not increase significantly, whereas background noise did. This can be probably attributed to signal suppression effect due to the introduction of matrix compounds via the ammonia solution. Finally, negative ion mode was preferred for further use and additional positive ion spectra were solely used for obtaining better confidence concerning the molecular mass of the unknown.

Figure 3.12 shows the reconstructed mass chromatograms of compounds I-VI. From these findings the presence of monomeric catechins and dimeric through hexameric procyanidins in chocolate can already be suggested. Moreover, due to the separation power of RP-HPLC the presence of several stereoisomers can be anticipated according to the number of peaks per mass chromatogram. As for example, there are two dimers (m/z 577) with retention times of 11.53 and 17.37 minutes, respectively.

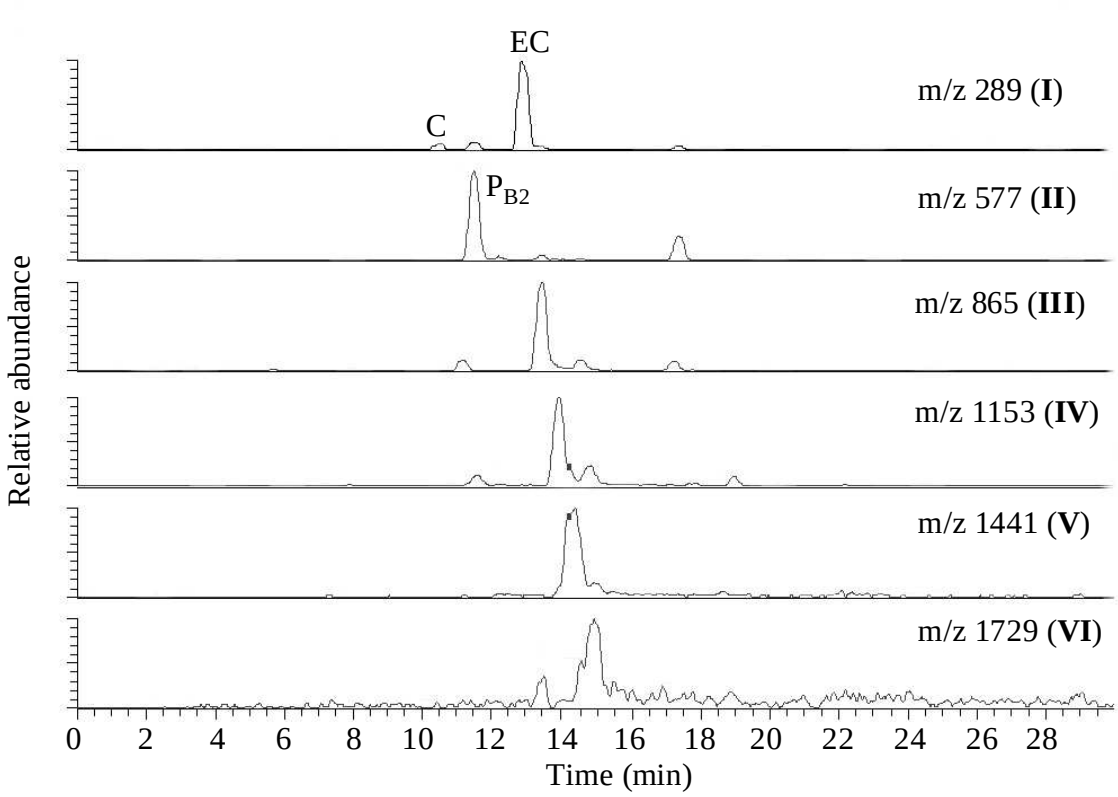


Figure 3.12 Reconstructed mass chromatograms of catechin monomers (m/z 289) and procyanidin oligomers (dimers through hexamers m/z 577 to m/z 1729). C = catechin, EC = epicatechin, and P_{B2} = procyanidin B2 as identified by the retention times of authentic standards.

In order to get more confidence in identifying cocoa procyanidins additional structural information was obtained by CID-MS-MS experiments on the detected deprotonated ions leading to specific fragmentation of the compounds. Figure 3.13 (bottom) shows the CID-MS-MS spectrum of deprotonated epicatechin from cocoa liquor. The identity of epicatechin has been confirmed additionally by comparing the obtained spectrum with that of an authentic epicatechin standard. The assignment of observed product ions as indicated by arrows and neutral losses in brackets in figure 3.13 (bottom) follows the suggestions made by Cuyckens et al. (2000) and Wolfender et al. (2000). However, assignment of the product ions of epicatechin in the negative ion mode is problematic as neutral losses correspond to rather unspecific losses of water (-18 Da), CO_2, or C_2H_2O-2H (-44 Da) as well as $C_4H_4O_2$ (-84 Da), respectively.

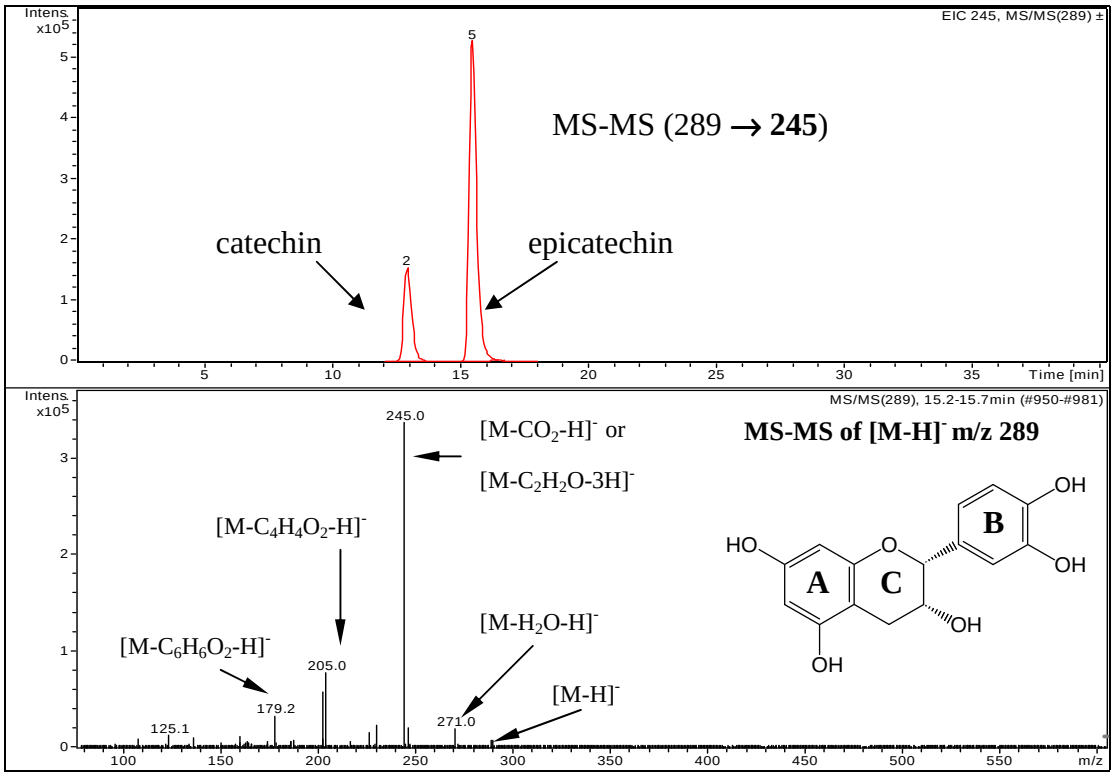


Figure 3.13 Reconstructed MS-MS mass chromatogram (m/z 289 → 245) and CID-MS-MS spectrum of catechin and epicatechin monomer in the negative ion mode ([M-H]$^-$ of m/z 289).

Therefore, CID-MS-MS experiments were also carried out on the protonated ions of epicatechin and catechin, respectively, showing typical retro Diels-Alder (RDA) fragmentation pathways resulting in characteristic product ions (figure 3.14). Since catechin and its epimer epicatechin, eluting later from the column, both have identical mass-to-charge ratios of the deprotonated ion (m/z = 289) and mass spectra of product ions from CID of the parent, respectively, they can only be distinguished by their different retention times and be confirmed by authentic standards. This shows clearly the importance of at least some chromatographic separation prior to MS detection of cocoa polyphenols.

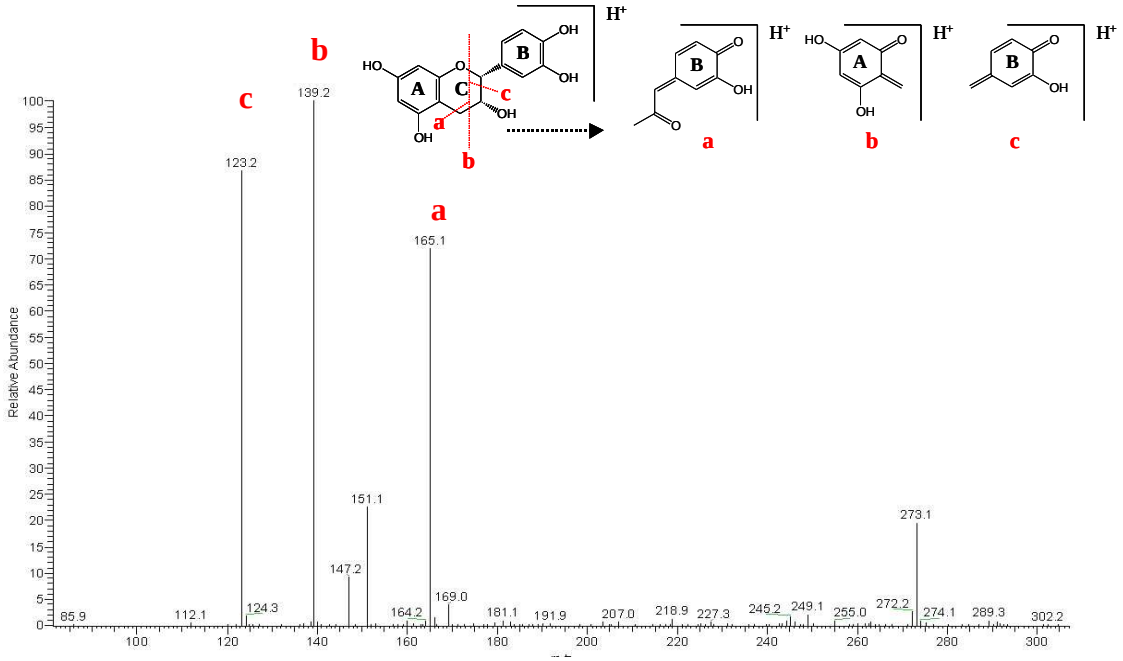


Figure 3.14 CID-MS-MS spectrum of catechin and epicatechin monomer in the positive ion mode ($[M+H]^+$ of m/z 291) and proposed main fragmentation pathways.

The mass spectrum resulting from CID-MS-MS experiments of procyanidin dimer in the negative ion mode shows main product ions at m/z 451, 425, 407, 289, and 287 (figure 3.15).

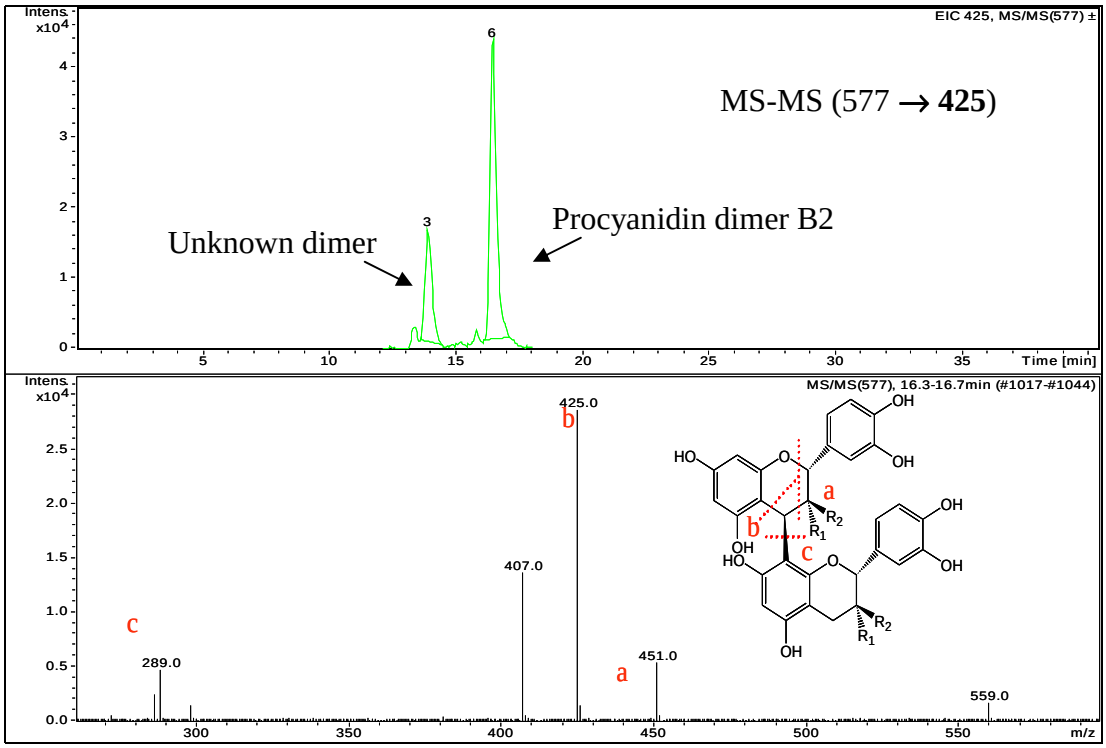


Figure 3.15 Reconstructed mass chromatograms (m/z 577 → 425) and CID-MS-MS spectrum of procyanidin dimers ($[M-H]^-$ of m/z 577) of a highly processed cocoa liquor extract – red dashed lines and letters indicate proposed fragmentation pathways.

The most obvious fragmentation pathway is the breakage of the interflavan bond indicated by the letter "c" in the mass spectrum of figure 3.15 leading to one fragment of m/z 289 (corresponding to epicatechin or catechin monomer) and another of m/z 287 (the same fragment desaturated, - 2 hydrogen atoms). The product ions with m/z of 451, 425, and 407 indicate different fragmentation patterns of procyanidin dimer in the negative ion mode compared to the monomer. However, two of these ions, namely m/z 451 and 425, correspond to the same neutral loss from the parent ion as described for epicatechin and catechin monomers in the positive mode (m/z 291 → 165 and m/z 291 → 139, respectively) suggesting ring fission as indicated by the letters a and b in the mass spectra of figure 3.15. The third ring breakage seen in the monomer in the positive ion mode would correspond to an ion of m/z 409 in the spectra of the procyanidin dimer in the negative mode, which, however, is absent. A neutral loss of water (-18 Da) from the product ion at m/z 425 is suggested for the daughter m/z 407, as well as for the one at m/z 559 from the parent ion (m/z 577). Nevertheless, the loss of water (18 Da) from a

parent ion is relatively unspecific as most deprotonated ions show this loss and thus, no additional information on the chemical structure is gained.

Figure 3.16 outlines the fragmentation pathway of the procyanidin dimer B2 (epicatechin 4β→8 epicatechin), the presence of which in cocoa was confirmed by performing an HPLC-MS-MS analysis of an authentic standard. As proposed for the monomer in the positive mode, the main fragmentation pathways of the dimer in the negative mode include retro Diels-Alder reactions (RDA) and the neutral loss of gallic acid through breakage in the heterocyclic C-ring of the upper flavanols-monomer. In addition, cleavage of the interflavanoid linkages can be observed leading to an upper unit corresponding to (epi)catechin monomer and a lower unit corresponding to a monomer with additional desaturation, respectively (figure 3.16).

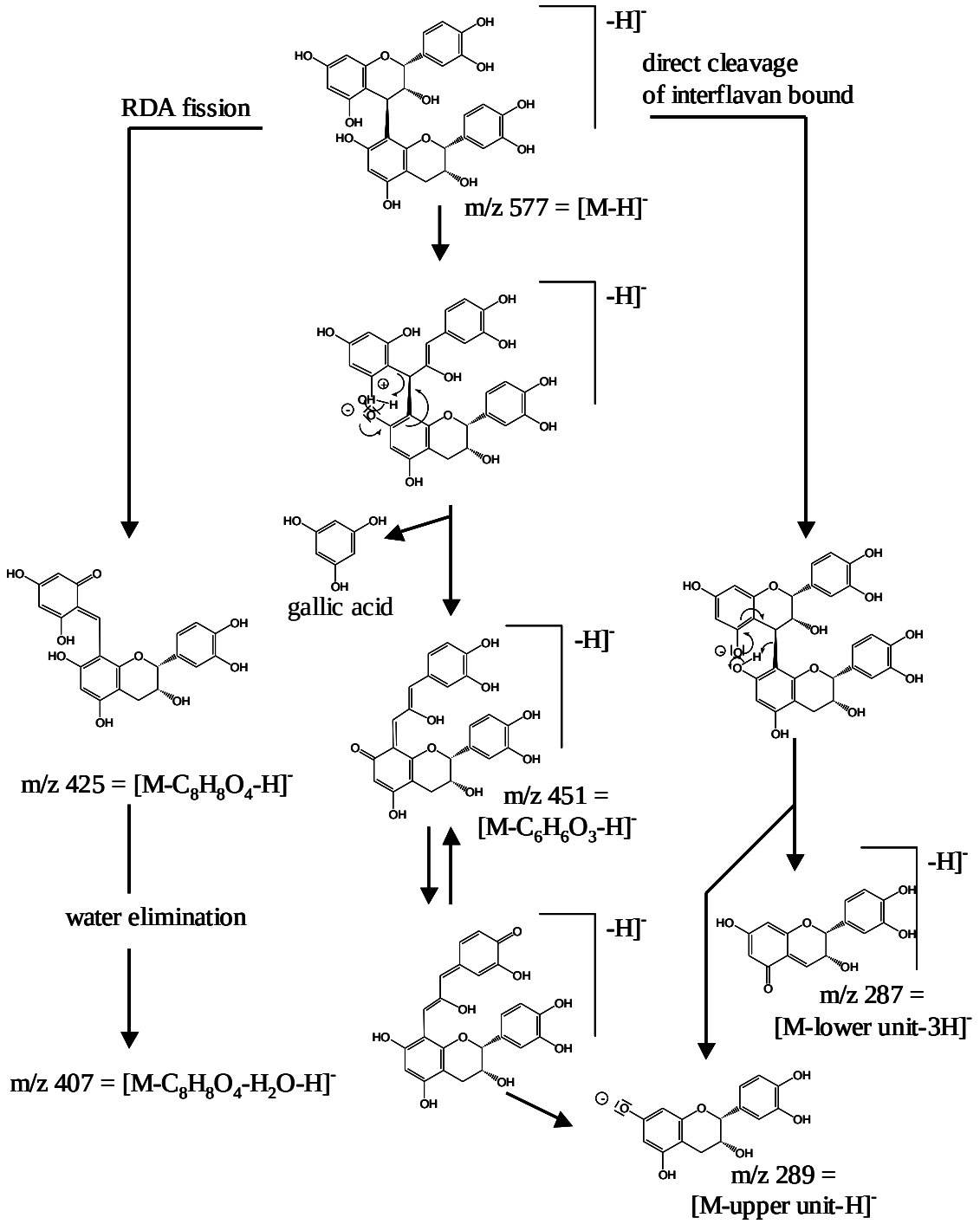


Figure 3.16 Outline of the postulated fragmentation pathway of deprotonated procyanidin dimer B2 after CID in the ion trap of the mass analyser.

Mass spectra following CID-MS-MS experiments for higher oligomeric procyanidins were getting increasingly complicated. Figure 3.17 shows for instance the MS-MS spectrum of tentatively assigned procyanidin tetramer after CID of $[M-H]^-$) m/z 1153.

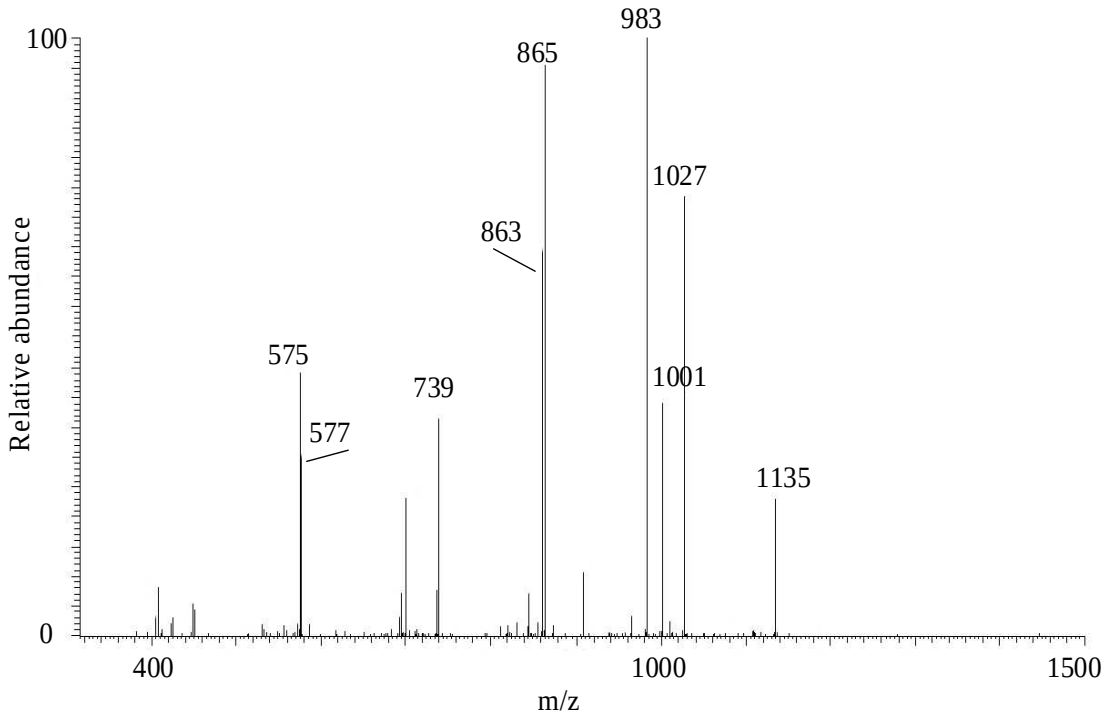


Figure 3.17 CID-MS-MS spectrum of procyanidin tetramer ($[M-H]^-$ of m/z 1153).

Nevertheless, the product ions at m/z 1027, 1001, and 983 result from the same neutral loss in the most upper monomeric unit corresponding to the retro Diels-Alder reaction (loss of $C_8H_8O_4$, molecular weight (MW) of 168 Da) and subsequent loss of water as well as loss of gallic acid ($C_6H_6O_3$, MW 126 Da) as described for the dimer. Moreover, stepwise interflavan cleavages of a tetramer result in product ions corresponding to lower oligomers (trimer, dimer) and monomers as well as those compounds desaturated. Finally, these lower oligomers appear to be readily fragmented by cleavage of their respective upper monomer C-ring.

In order to distinguish the flavanol units in proanthocyanidins Porter (1980) developed a nomenclature depending on the positions of the interflavanoid bonds. In accordance

therewith, a T-unit (top) has only one interflavanoid linkage at C-4, the M-units (middle) have an additional linkage at C-6 or C-8, and the B-unit (base) has one interflavanoid bond at C-8 or C-6 (figure 3.18). The configuration of the bonds at C-4 is indicated by the αβ nomenclature.

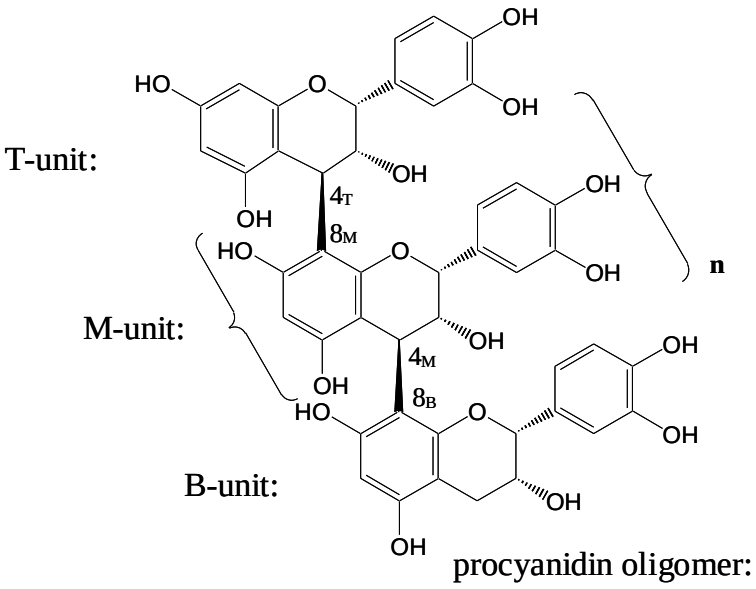


Figure 3.18 Structure of procyanidin 4β-8 oligomers; nomenclature according to Porter (1980).

According to this nomenclature system two pathways can be distinguished for the cleavage of the interflavanoid linkage. One is based on the loss of neutral fragments containing the T-unit that leads to fragment ions containing the B-unit corresponding to already observed lower deprotonated procyanidins, e.g., in the case of the tetramer a dimer $[M_B\text{-}H]^-$ at m/z 577 and a trimer at $[M_B\text{-}H]^-$ at m/z 865, respectively. The other possibility is the loss of neutral fragments containing the B-unit leading to fragment ions containing the T-unit corresponding to lower procyanidins minus 2 H, e.g., here a dimeric ion $[M_T\text{-}3H]^-$ at m/z 575 and a trimeric ion $[M_T\text{-}3H]^-$ at m/z 863, respectively. These findings are summarised in Table 3.3 for the tetramer but they have also been confirmed for procyanidin dimers, trimers, pentamers, hexamers and heptamers.

Table 3.3 Main masses in the CID-MS-MS spectrum of deprotonated procyanidin tetramer (m/z 1153 = $[M-H]^-$)

m/z		comment*
1135	$[M-H_2O-H]^-$	Ions present after water elimination
1027	$[M-C_6H_6O_3-H]^-$	Ions after loss of gallic acid
1001	$[M- C_8H_8O_4-H]^-$	Ions of RDA fission product
983	$[M- C_8H_8O_4-H_2O-H]^-$	Ions of RDA fission product and subsequent water elimination
865	$[M- C_{15}H_{12}O_6-H]^-$	Ions consisting of 1 B-unit and 2 M-units after interflavanoid cleavage and loss of neutral T-unit (MW 288)
863	$[M- C_{15}H_{14}O_6-H]^-$	Ions consisting of 1 T-unit and 2 M-units after interflavanoid cleavage and loss of neutral B-unit (MW 290)
739	$[M- C_{15}H_{12}O_6- C_6H_6O_3-H]^-$	Ions consisting of 1 B-unit and 2 M-units minus 1st RDA fission product
577	$[M- C_{30}H_{24}O_{12}-H]^-$	Ions consisting of 1 B-unit and 1 M-units after interflavanoid cleavage and loss of neutral T- and M-unit (MW 576)
575	$[M- C_{30}H_{26}O_{12}-H]^-$	Ions consisting of 1 T-unit and 1 M-units after interflavanoid cleavage and loss of neutral B- and M-unit (MW 578)

* analogous to the postulated fragmentation pathway in figure 3.16.

It is known that multiple-charged species like $[M-2H]^{2-}$ or $[M-3H]^{3-}$ are often observed with ESI (Guyot et al., 1997; Le Roux et al., 1998). In order to investigate the existence of multiple charges, a special "high-resolution" mass scan was applied in this study. This instrumental possibility of the mass analyser does not allow and is not comparable with the accurate mass determination of a quadrupole-time-of-flight hybrid instrument (Q-TOF) as used by Wolfender et al. (2000) or a MALDI-TOF applied by Ohnishi-Kameyama et al. (1997) and Yanagida et al. (2000a). However, it allowed the determination of the carbon isotope distribution in the range near the ions of interest.

In nature, carbon consists of 98.89% of ^{12}C and of 1.11 of its stable isotope ^{13}C (Budzikiewicz, 1992). Thus in the mass spectrum of a deprotonated molecule with 90 carbon atoms, corresponding to a procyanidin hexamer the first isotope peak ($^{12}C_{89}$ ^{13}C) is more intense than $^{12}C_{90}$, which corresponds to the nominal mass minus one for $[M-H]^-$. In the case of a single-charged ion the distance between two carbon isotope ions would be m/z 1, in the case of a double-charged ion m/z 0.5 and so forth. In addition, the relative distributions of the ions of the carbon ^{12}C and ^{13}C isotopes, respectively, can be calculated and compared to the measured intensities for further evidence of the presence of procyanidin oligomers.

In this study there was evidence for the presence of double-charged ions of procyanidins higher than tetramers. Although the intensity was very low, the distance of approximately m/z 0.5 suggested the presence of double-charged ions of pentamers at m/z 720, hexamers at m/z 864 and heptamers at m/z 1008. An example of the high-resolution spectrum of the double-charged heptamer (m/z 1008) compared to the spectrum of a single-charged trimer (m/z 865) is shown in figure 3.19.

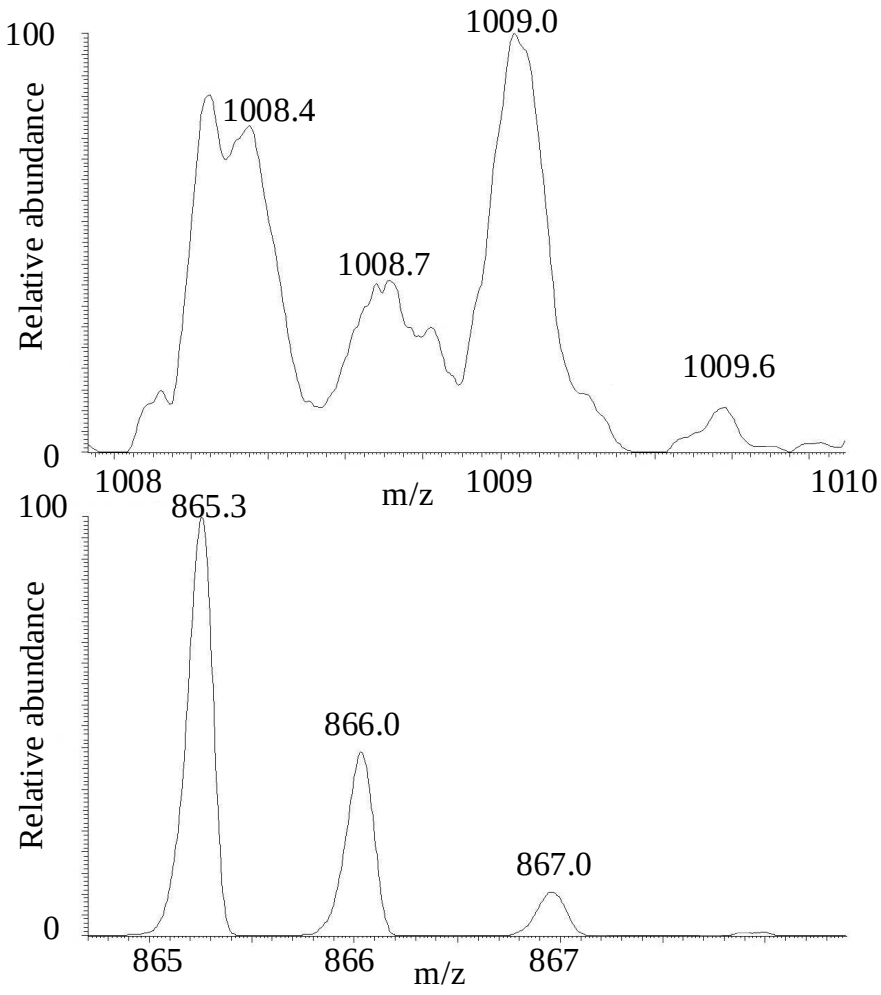


Figure 3.19 Negative ion mass spectra (profile format) of double-charged procyanidin heptamer ($[M-2H]^{2-}$ of m/z 1008) (top) and of single-charged procyanidin trimer ($[M-H]^{-}$ of m/z 865) (bottom).

It is evident that due to the low intensity of a double-charged ion of the procyanidin heptamer at m/z 1008 the relative abundance (RA) of the carbon isotopic distribution of ions at m/z 1008.4 (RA = 80), m/z 1009.0 (RA = 100), and m/z 1009.6 (RA = 15), respectively, does not correspond well to the calculated carbon isotope distribution for a heptamer ($C_{105}H_{86}O_{42}$), possibly due to interferences of other compounds from the matrix. Considering only the contribution of the isotopic distribution of ^{12}C and ^{13}C (and not the very low contribution of the isotopic distributions of ^{1}H and ^{2}H or ^{16}O and ^{17}O, respectively) the calculated distribution of the heptameric ions would be m/z 1008.3 (RA = 85), m/z 1008.8 (RA = 100), and m/z 1009.3 (RA = 58). However, the

approximate distance of m/z 0.5 suggests the presence of double-charged heptameric ions and justifies further MS-MS experiments as discussed below. In contrast, for the trimer the calculated distributions of the carbon isotope ions would be m/z 865.2 (RA = 100), m/z 866.2 (RA = 50), and m/z 867.2 (RA = 12), which corresponds very well to the measured relative intensities in this work (figure 3.19).

In order to assure that these ions found in the HPLC-MS analysis of cocoa extract correspond to double-charged ions of procyanidins CID-MS-MS experiments were carried out in subsequent analyses on the ions at m/z 720, m/z 864 and m/z 1008, respectively. The spectra were compared to the MS-MS spectra of the single-charged pentamer and hexamer and in all three cases investigated for the fragmentation patterns due to RDA fission, gallic acid loss, and breakage of interflavanoid linkages as discussed for the tetramer.

Figure 3.20 (bottom) represents the MS-MS spectrum of the single-charged pentameric ion $[M-H]^-$ at m/z 1441, figure 3.21 (bottom) the MS-MS spectrum of the double-charged pentameric ion $[M-2H]^{2-}$ at m/z 720. Both spectra show the typical fragmentation pattern of procyanidins as discussed before, although, in the MS-MS spectrum of the double-charged ion there are other non-specific ions present (e.g., m/z 635.3, m/z 644.2). However, this is probably due to matrix interferences as the intensity of the double-charged pentameric ion was relatively low. In addition, evidence for the presence of a double-charged pentameric ion is shown by the sum of the two reconstructed mass chromatograms of m/z 863 and m/z 865, figure 3.21 (top) showing a nearly identical chromatogram as for the single-charged pentameric ion, figure 3.20 (top).

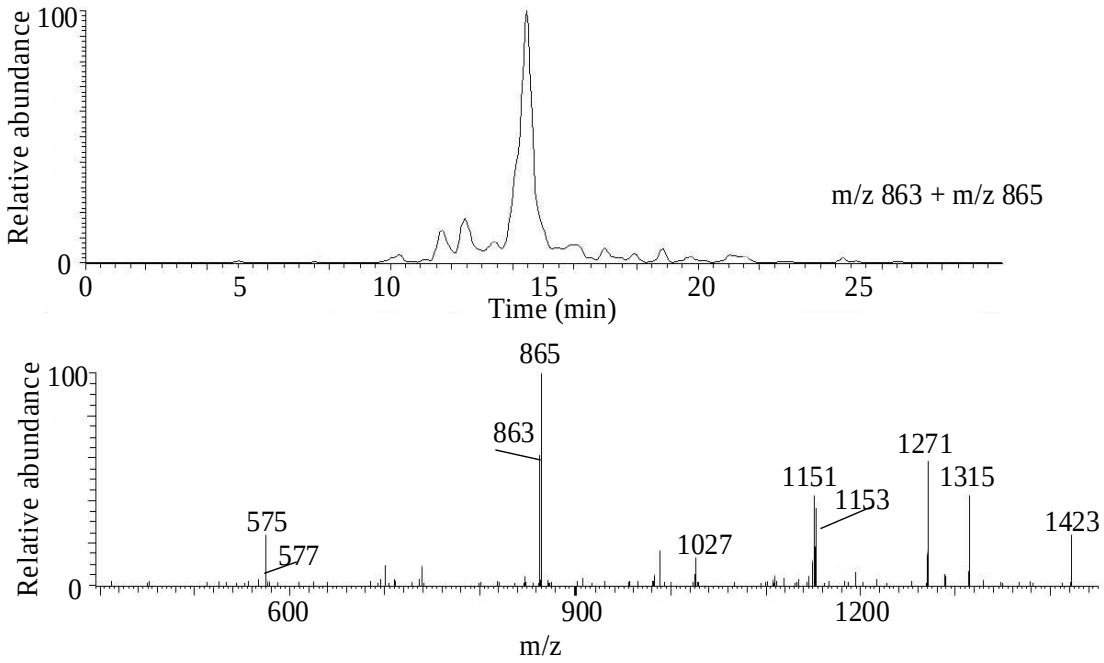


Figure 3.20 CID-MS-MS spectrum of single-charged procyanidin pentamer ($[M-H]^-$ of m/z 1441) (bottom) and sum of reconstructed mass chromatograms of two of the product ions ($[M-H]^-$ of m/z 863 and m/z 865) (top).

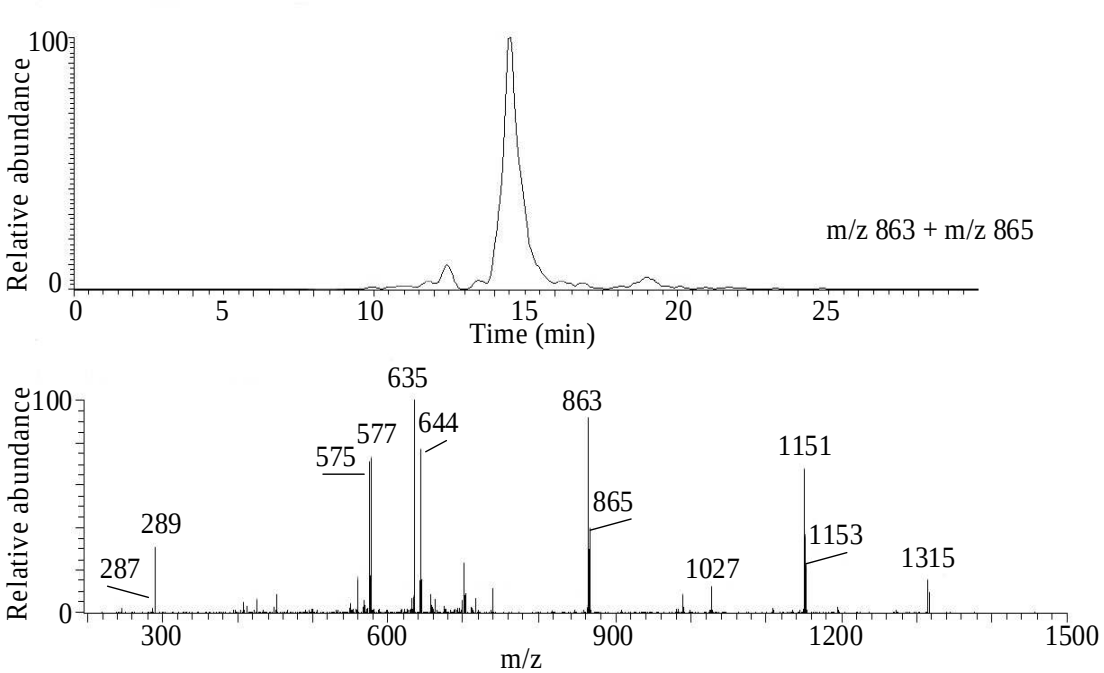


Figure 3.21 CID-MS-MS spectrum of double-charged procyanidin pentamer ($[M-2H]^{2-}$ of m/z 720) (bottom) and sum of mass chromatograms of two of the product ions ($[M-H]^-$ of m/z 863 and m/z 865) (top).

The presence of fragment ions at higher mass-to-charge ratios than the parent ion (figure 3.21) indicates double or multiple charges of the latter and single charge or lower charge than the parent of the product ions, as product ions cannot derive from compounds with a higher mass than the parent compound.

Finally, in figure 3.22 the presence of a double-charged heptameric ion at m/z 1008 is indicated by a CID-MS-MS spectrum showing the typical fragmentation pattern of a procyanidin ion and a sum of reconstructed mass chromatograms of unique fragment ions at m/z 863 and m/z 865, respectively. In this work procyanidin heptamer was the procyanidin compound with the highest molecular weight that was detected in the cocoa liquor extract.

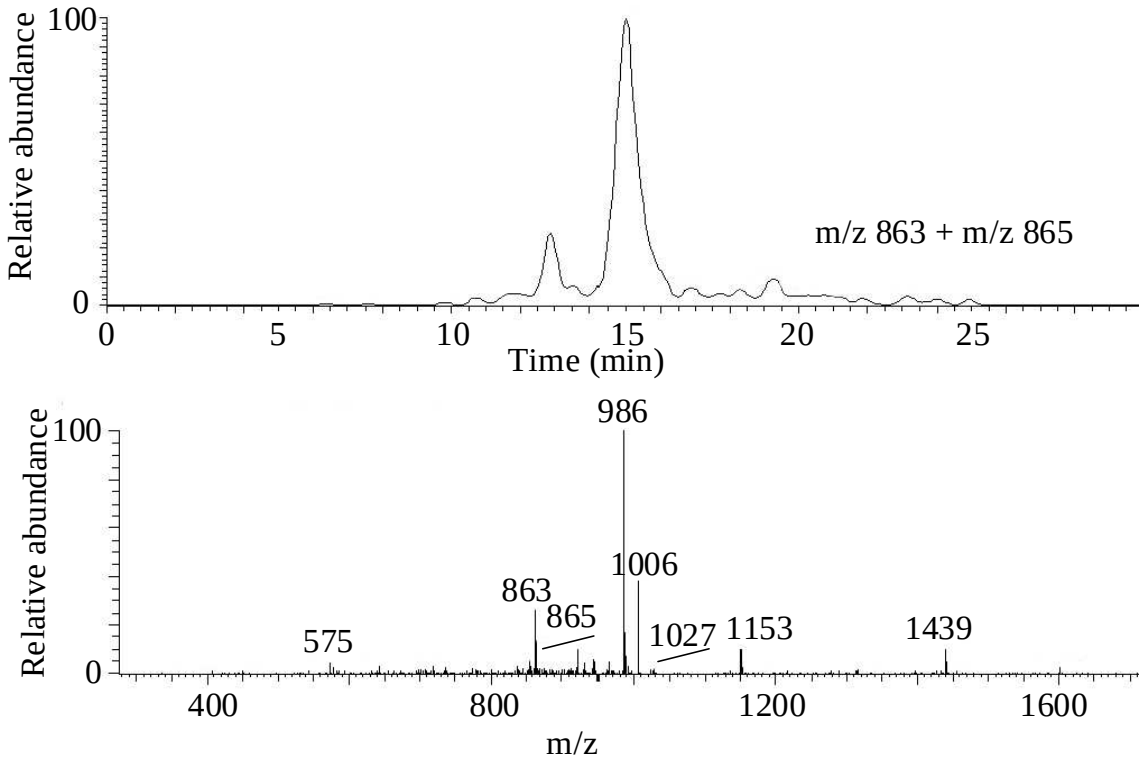


Figure 3.22 CID-MS-MS spectrum of double-charged procyanidin heptamer ($[M-2H]^{2-}$ of m/z 1008) (bottom) and sum of reconstructed mass chromatograms of two of the product ions ($[M-H]^{-}$ of m/z 863 and m/z 865) (top).

In summary, catechin and epicatechin monomers as well as procyanidin oligomers from dimers to heptamers were identified in this work as major polyphenolic compounds in cocoa liquor. Stereoisomers having the same molecular weight were separated well applying this reversed-phase HPLC method. The major peaks in each mass chromatogram are assumed to be procyanidin homologues built up of only epicatechin sub-units as confirmed by comparing the retention time of authentic standards of epicatechin monomer and procyanidin B2 dimer, respectively. In addition, the presence of almost exclusively epicatechin based procyanidin oligomers has been reported in an early publication applying a combination of several isolation and identification procedures (Porter et al., 1991). Nevertheless, the position and stereochemistry of the interflavanoid linkage cannot be elucidated by means of mass spectrometry. Isolation of procyanidins and subsequent structure elucidation are necessary to confirm the full chemical structure. In this work, the isolation of procyanidin dimer B2 and trimer C1 followed by structure elucidation by NMR is described in section 3.2.3.

3.2.2 Identification of minor phenolic compounds

The method developed and described in section 2.1.6, that had been applied for the identification of major cocoa compounds including catechins and procyanidins (section 3.2.1) was applied also for characterisation of minor phenolic compounds. This exploratory approach included the search for already described compounds in the literature (chapter 1.3), other polyphenols commonly present in plants as well as unknown substances, which showed some retention in reversed phase HPLC and which were ionised in the negative ion mode in the mass selective detector. Initially, these phenolic substances were tentatively identified by HPLC-MS according to retention behaviour, mass-to-charge ratios, and CID fragmentation. For some compounds authentic standards were commercially available and were used for confirmation of tentative identification. In addition, three of the quantitatively more interesting ones were isolated and characterised by NMR as described later in section 3.2.3.

3.2.2.1 Quercetin and quercetin glycosides

The molecular weight of quercetin and its putatively present glycosides (figure 3.23) can be easily calculated from the sum formulae.

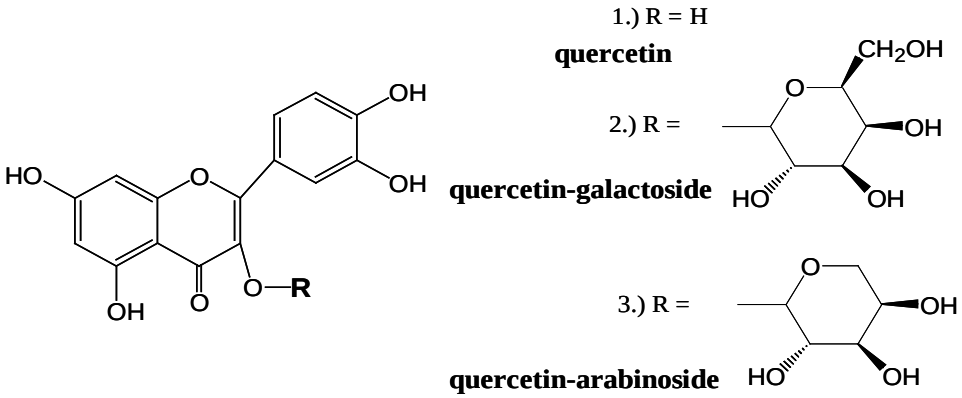


Figure 3.23 Flavonols found in cocoa: quercetin, quercetin-galactoside, and quercetin-arabinoside.

The presence of compounds with deprotonated ions at m/z 301, 433, and 463 (figure 3.24) produce evidence for the existence of cocoa quercetin (MW of 302 Da), quercetin-arabinoside (MW of 434 Da), and quercetin-galactoside and/or quercetin-glucoside (both having a MW of 464 Da).

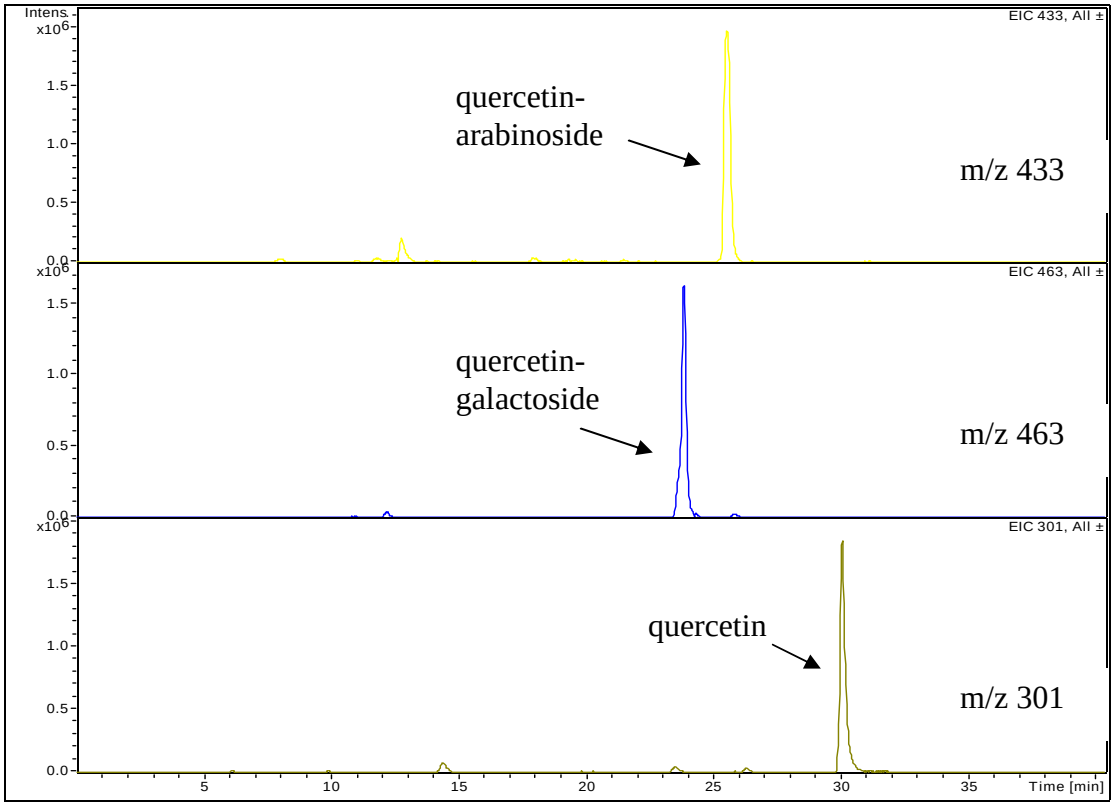


Figure 3.24 Reconstructed mass chromatograms of quercetin (bottom) and its two glycosides arabinoside (top) and galactoside (middle).

In addition, performing mass spectrometric detection in the positive ion mode resulted in mass chromatograms for protonated ions with the same retention times at m/z 303, 435, and 465 (mass chromatograms not shown), which indicates the presence of substances with molecular weights of 302, 434 and 464 Da, respectively.

Further evidence was gathered by applying single CID experiments (MS-MS) to all three ions. Figure 3.25 shows the CID-MS-MS spectrum of quercetin in a cocoa liquor extract and its presence was confirmed by comparison of mass spectra and retention time with those of an authentic quercetin standard showing identical retention behaviour and CID fragmentation pattern.

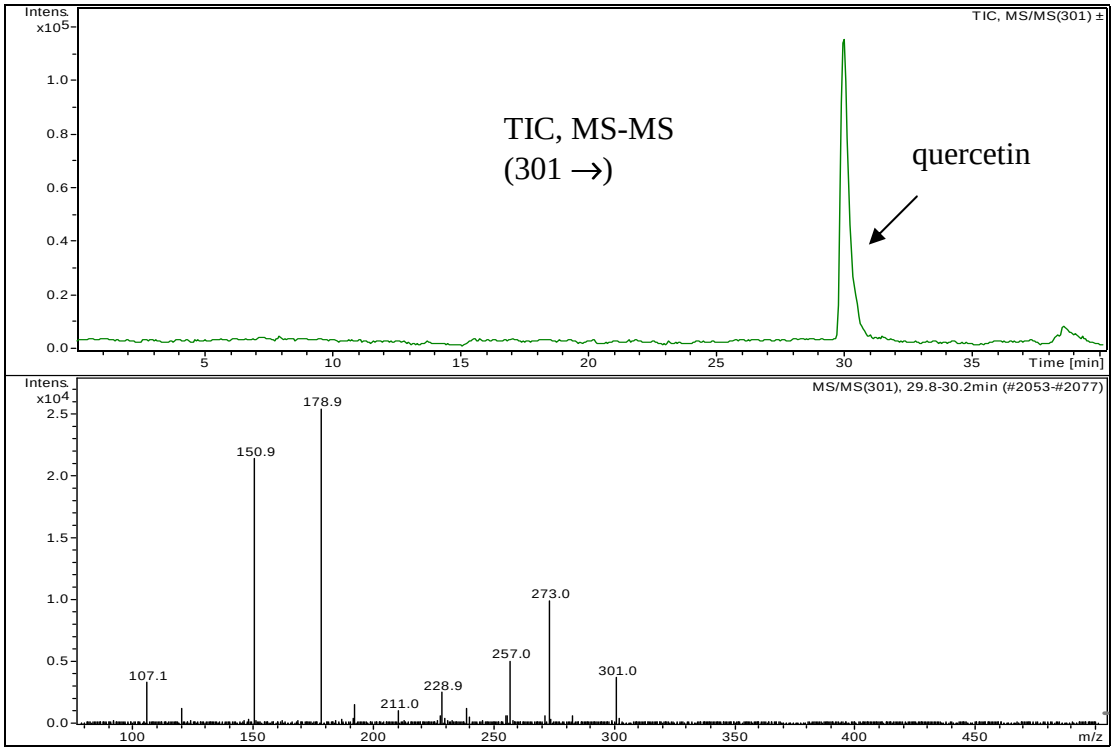


Figure 3.25 Reconstructed MS-MS mass chromatogram (total ion current (TIC) m/z 301 →) and CID-MS-MS spectrum of quercetin ($[M-H]^-$ of m/z 301).

Deprotonated quercetin-arabinoside (data not shown) as well as quercetin-galactoside or quercetin-glucoside (shown in figure 3.26) gave m/z 301 corresponding to deprotonated quercetin in their CID-MS-MS spectra. The presence of quercetin-arabinoside was additionally confirmed by comparison with an authentic standard. For distinction between quercetin-galactoside and quercetin-glucoside, the retention times of the

suspected candidate compounds in cocoa liquor extract were compared to those of authentic standards. Initially only quercetin glucoside was commercially available. This compound showed slightly shorter retention on the column and thus eluted earlier than the compound in cocoa. Towards the end of the study also quercetin-galactoside was available and the presence of this quercetin derivate was finally confirmed in cocoa by their identical retention times and behaviour in the mass selective detector.

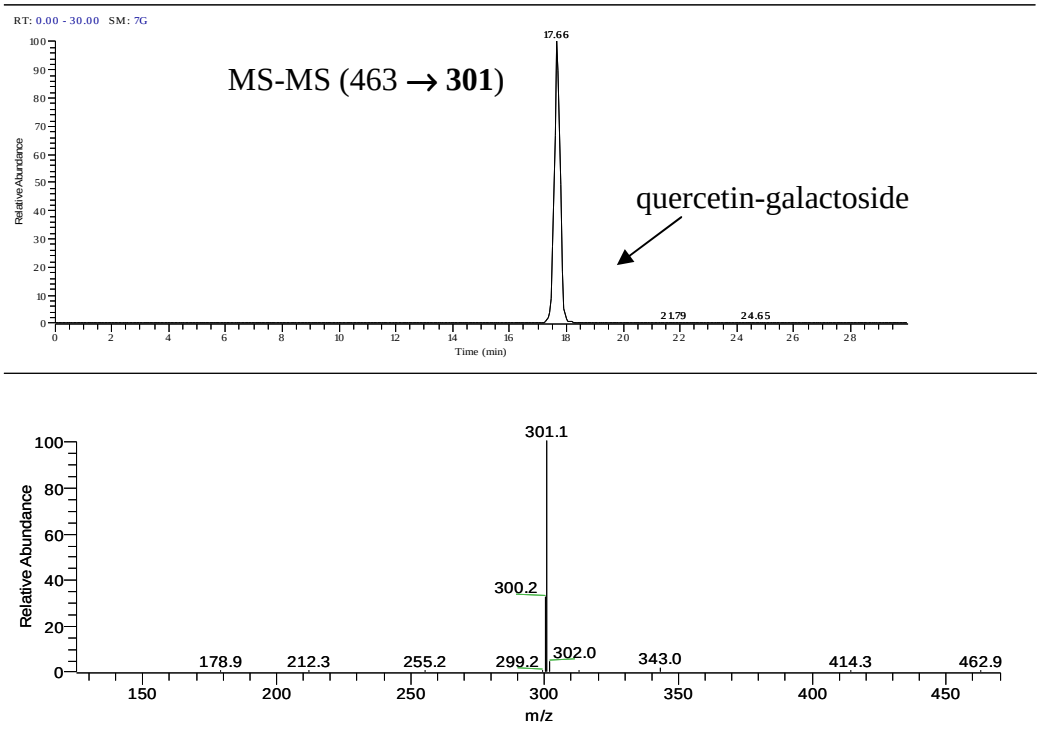


Figure 3.26 Reconstructed MS-MS mass chromatogram (463 → 301) of quercetin-galactoside (top) and its CID-MS-MS spectrum (bottom).

Nevertheless, the presence of only one product ion that is likely due to a neutral loss of the sugar residue, allows only the identification of quercetin in the molecule according to the identical mass of the product ion with the deprotonated ion of quercetin aglykon. Since the ion trap technique of the mass analyser used in the analyses within this work allows for further isolation of product ions and subsequent CID experiments, double CID experiments (MS-MS-MS or MS^3) were carried out on the presumed quercetin-

glycosides in cocoa liquor extract. Further fragmenting the observed product ion (m/z 301) produced spectra with typical product ions of authentic quercetin standard for the respective quercetin-glycoside, both for the compounds in the cocoa extract and the authentic standard compounds. For instance, figure 3.27 shows the CID-MS3 spectrum of quercetin-galactoside.

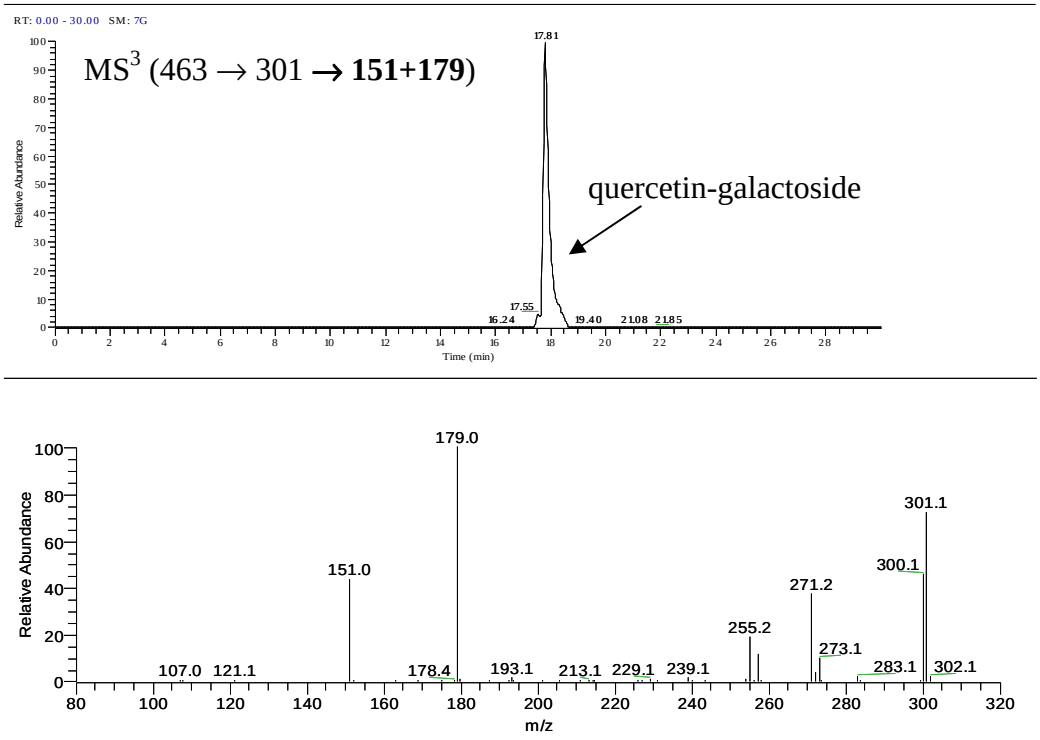


Figure 3.27 Sum of reconstructed MS^3 mass chromatograms (463 → 301 → 151) and (463 → 301 → 179) of quercetin-galactoside (top) and its CID-MS^3 spectrum (bottom).

Finally, the presence of quercetin-glucuronide in cocoa has been suggested by Andres-Lacueva et al. (2000). However, no evidence for its presence was gathered from reconstructed mass chromatograms of its putative deprotonated (m/z 477) or protonated (m/z 479) ions in the present work as for the above discussed quercetin compounds. Nevertheless, exploratory CID-MS-MS experiments of deprotonated ions of m/z 477 were carried out in search for a product ion of m/z 301 corresponding to free deprotonated quercetin after neutral loss of glucuronic acid allowing enhanced

selectivity of the detection. Still these analyses revealed several compounds giving product ions of m/z 301 making the basis for identification of a quercetin-glucuronide yet insufficient. Further isolation of these product ions followed by a second step CID exploring for deprotonated quercetin among these ions finally resulted in one compound that gives the two characteristic product ions of quercetin m/z 151 and m/z 179 (figure 3.28).

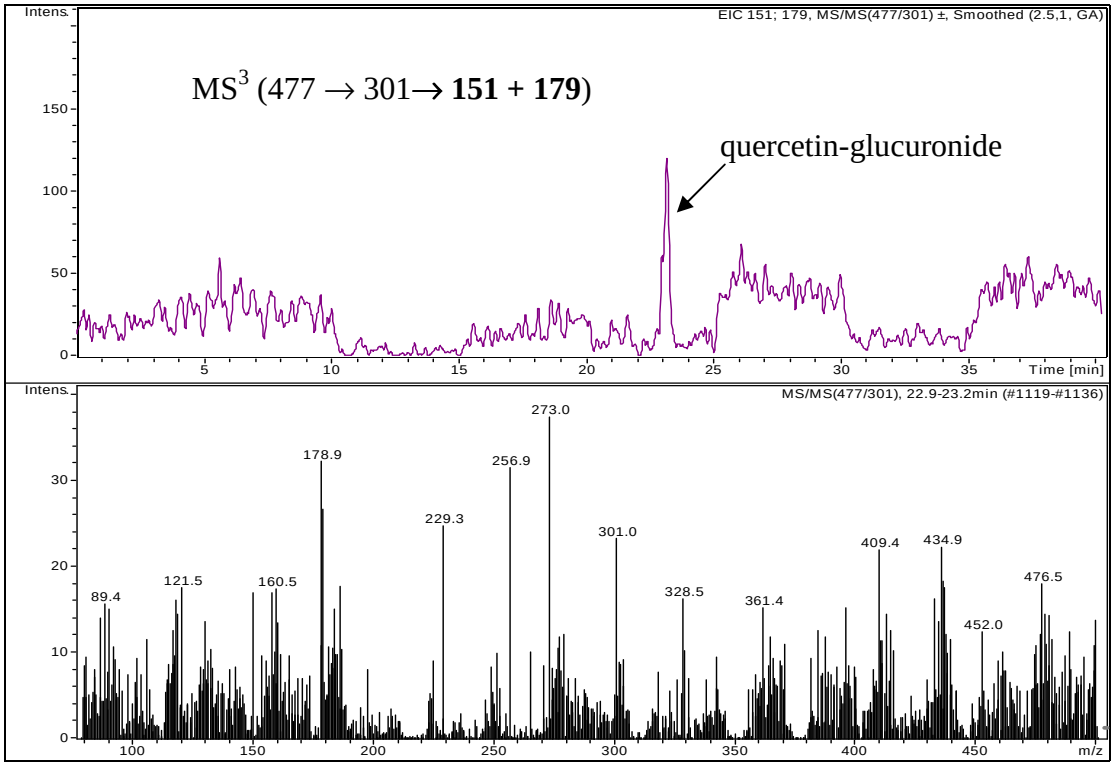


Figure 3.28 Sum of reconstructed MS^3 mass chromatograms (477 → 301→ 151) and (477 → 301→ 179) of quercetin-glucuronide (top) and its CID-MS-MS spectrum (bottom).

Nevertheless, the very low intensity of the ions in MS, MS-MS, and MS^3 spectra in the experiments carried out in this work does not allow the confirmation of the presence of quercetin-glucuronide in absence of an authentic standard compound. In any case quercetin-glucuronide, if at all, appears to be present only in trace amounts being several times lower than those of the above presented quercetin compounds. Consequently, quercetin-glucuronide has not been considered for quantitative analysis

(section 3.3 and 3.4) but only quercetin, quercetin-arabinoside, and quercetin-galactoside.

In summary, the presence of quercetin-arabinoside and quercetin-galactoside as well as traces of quercetin-glucuronide was confirmed in this work. In contrast, from the experiments carried out there is no evidence for the presence of a quercetin-glucoside.

3.2.2.2 Hydroxycinnamic acid derivates

In this part of the work the objective was to confirm the presence of two rosmarinic acid derivates in cocoa as suggested by Sanbongi et al (1998). These two compounds, namely clovamide (caffeoyl-DOPA) and its analogue dideoxyclovamide (p-coumaroyltyrosine) have molecular masses of 359 and 327 Da, respectively. Evidence for the presence of these two compounds in cocoa liquor was gathered in the present work by carrying out HPLC-MS analyses of cocoa liquor extract in the negative ion mode as described for the compounds in section 3.2.1 and 3.2.2.1 revealing one major compound for each corresponding deprotonated ion of m/z 358 and 326 (figure 3.29 top and bottom mass chromatograms). As clovamide and a *di*-deoxy analogue were present, a reconstructed mass chromatogram of m/z 342 was produced as well, thereby exploring the additional presence of a *mono*-deoxy analogue. In fact, the presence of one major compound, the deprotonated ion of which fits with the anticipated mass-to-charge ratio, and that elutes later than clovamide but earlier than dideoxyclovamide from the reversed-phase HPLC column suggests the presence of monodeoxyclovamide in the cocoa liquor extract used in this study (figure 3.29).

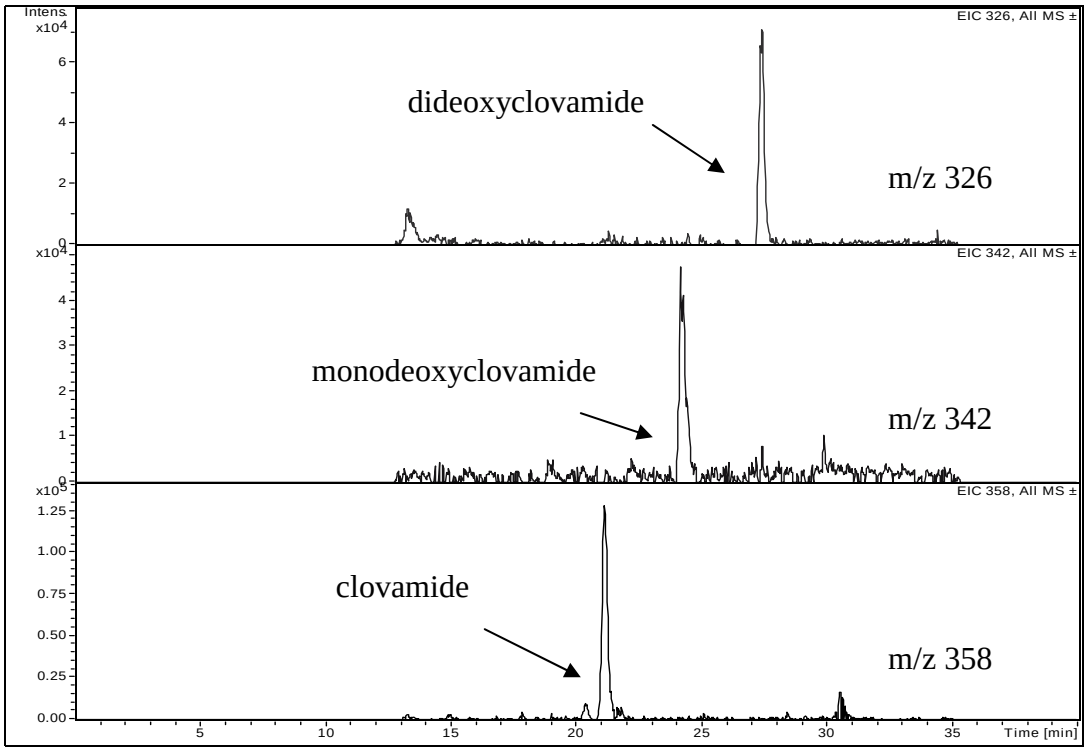


Figure 3.29 Reconstructed mass chromatograms of clovamide (bottom), monodeoxyclovamide (middle) and dideoxyclovamide (top).

The respective presence of protonated ions at plus one, i.e. m/z of 360, 344, and 328, respectively (data not shown) as well as CID mass spectra with product ions differing in 16 Da (oxygen) (figures 3.30a-c) provide further evidence for the presence of these three compounds (formulae of each clovamide shown in the respective CID mass spectrum in figures 3.30a-c). Following the discovery of a new compound, tentatively identified as mono-deoxygenated clovamide, additional exploratory experiments were carried out for other higher deoxygenated clovamide compounds. However, the experiments carried out in this work did not suggest any tri- or tetra-deoxygenated clovamide derivative.

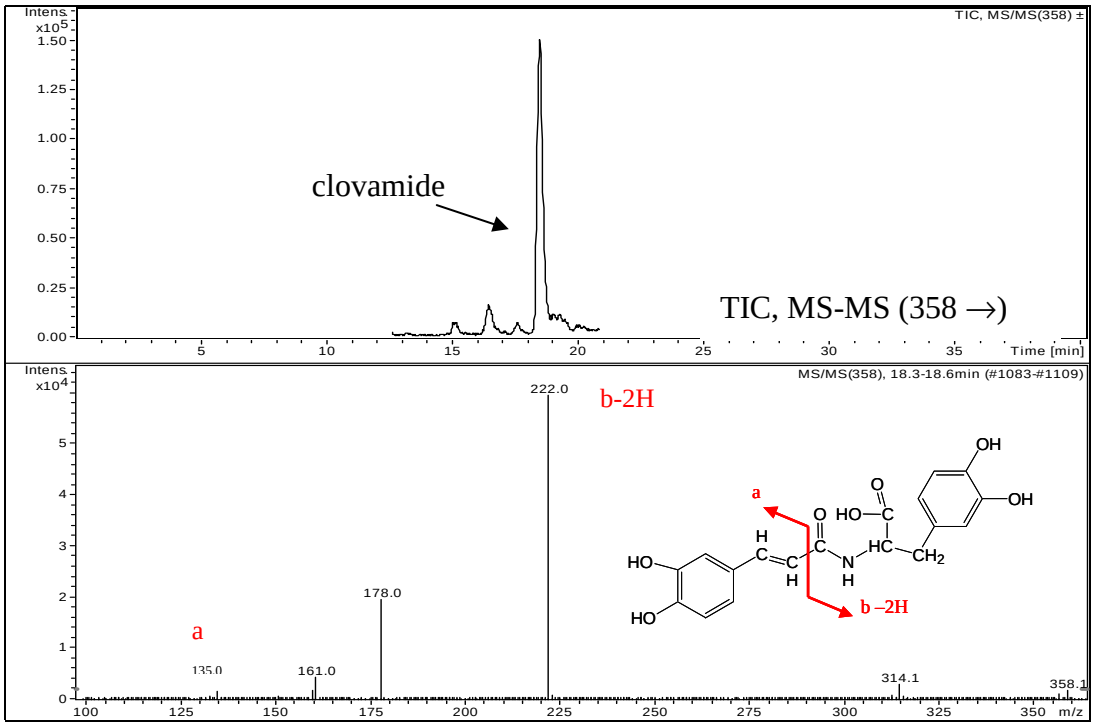


Figure 3.30a Reconstructed MS-MS mass chromatogram (total ion current (TIC) m/z 358 →) and CID-MS-MS spectrum of clovamide ($[M-H]^-$ of m/z 358) - red lines and letters indicate postulated fragmentation pathways.

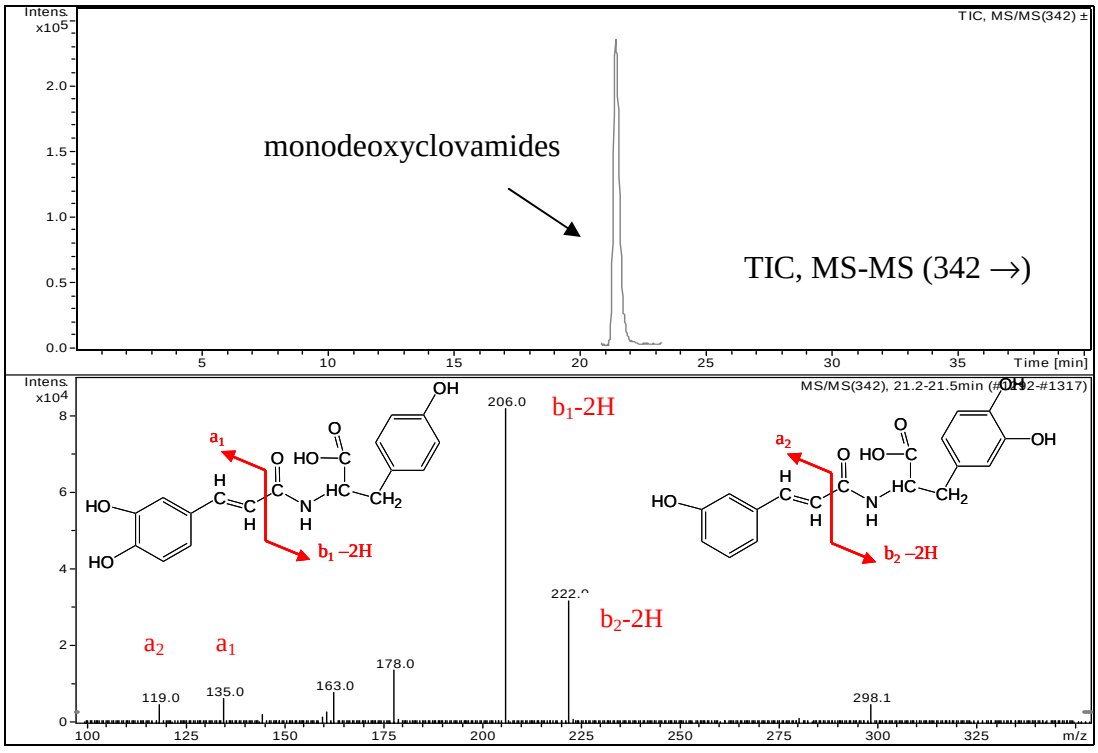


Figure 3.30b Reconstructed MS-MS mass chromatogram (total ion current (TIC) m/z 342 →) and CID-MS-MS spectrum of monodeoxyclovamides ($[M-H]^-$ of m/z 342) - red lines and letters show postulated fragmentation pathways.

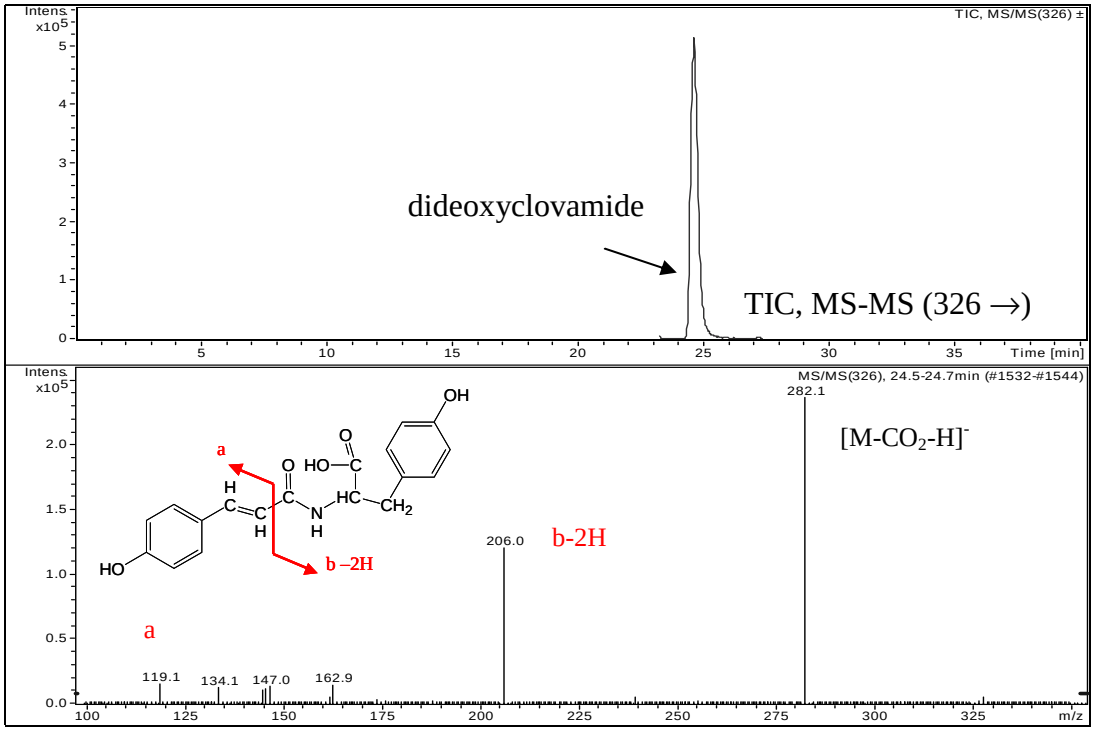


Figure 3.30c Reconstructed MS-MS mass chromatogram (total ion current (TIC) m/z 326 →) and CID-MS-MS spectrum of dideoxyclovamide ($[M-H]^-$ of m/z 326) - red lines and letters indicate postulated fragmentation pathways.

Monodeoxygenation can occur on each of the two aromatic rings resulting in two monodeoxyclovamide isomers, namely caffeoyltyrosine and p-coumaroyl-DOPA. The contemporary presence of all four possible product ions of two deprotonated monodeoxyclovamide isomers in the performed experiments corresponding to the postulated fragmentation pathway (a_1 and b_1 as well as a_2 and b_2 in figure 3.30b) suggest that both isomers occur at the same time and elute together from the column. In addition, it may be hypothesised from the ion intensities of the two pairs of product ions that caffeoyltyrosine leading to fragments a_1 and b_1 is present in higher concentrations in the cocoa liquor extracted and analysed in this work. However, unknown differences in the stability of product ions may lead here to erroneous interpretation. Nevertheless, despite the co-elution of the two isomers from the column they can be separately detected in the mass analyser performing CID-MS-MS analyses on the deprotonated ion having the common mass-to-charge ratio of 342 followed by selection of the respective specific product ions of the isomers, i.e., m/z 135 and/or m/z 206 for caffeoyltyrosine and m/z 119 and/or m/z 222 for p-coumaroyl-DOPA, respectively.

Finally, confirmation of the presence of the four clovamide derivatives was gained by isolation of the single compounds and subsequent structure elucidation by NMR and hydrogen/deuterium exhange mass spectrometry as described later in chapter 3.2.3.

In addition to the hydroxycinnamic acid amides, the presence of another hydroxycinnamic acid derivative, namely 3-O-caffeoyl D-quinic acid (chlorogenic acid) has been proposed by Jalal and Collin (1977). In the set of HPLC-MS experiments carried out in the present study chlorogenic acid in cocoa liquor was confirmed by comparison of retention time behaviour, presence of one substance giving the anticipated deprotonated (m/z 353) and protonated (m/z 355) ions in the mass selective detector, as well as by comparing CID-MS-MS spectra of both parent ions in either negative or positive ion mode with those of an authentic chlorgenic acid standard. Figure 3.31 shows the result of the CID-MS-MS of deprotonated chlorogenic acid including the formula and the postulated fragmentation.

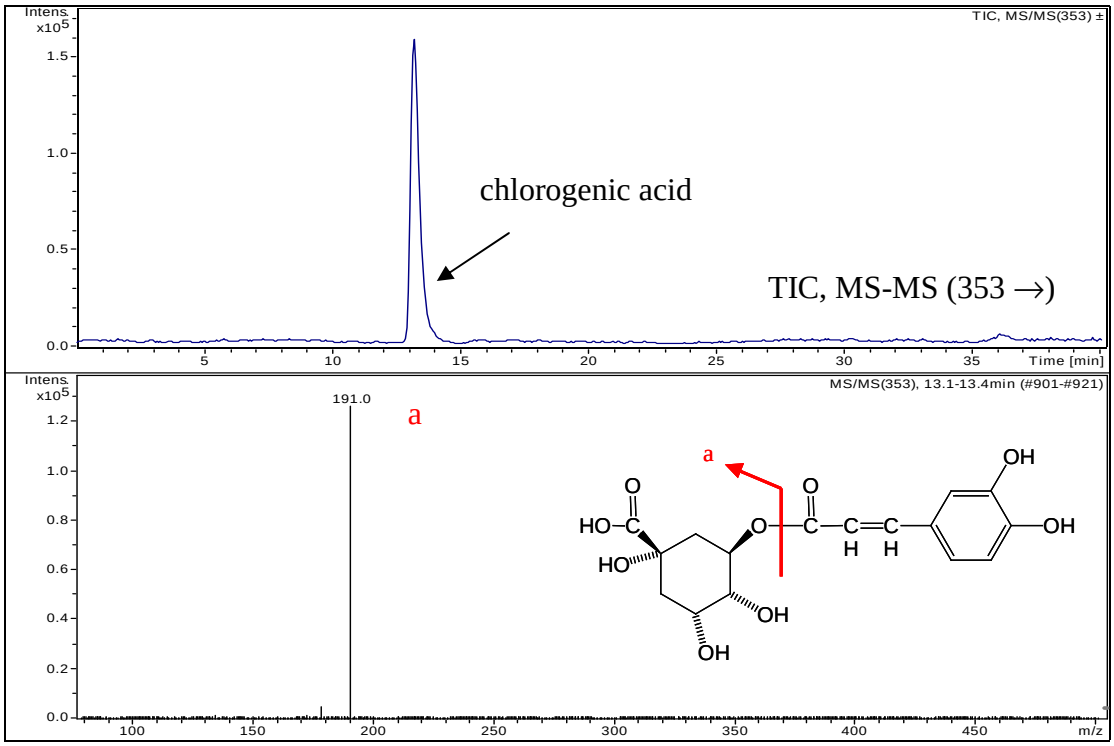


Figure 3.31 Reconstructed total ion current (TIC) MS-MS mass chromatogram (m/z 353 →) and CID-MS-MS spectrum of chlorogenic acid ($[M-H]^-$ of m/z 353) - red line indicates postulated fragmentation pathway.

3.2.2.3 Minor flavan-3-ols (catechins and procyanidins)

The objective in this part of the experimental studies was to confirm the presence of additional flavan-3-ols and procyanidins in cocoa liquor as suggested in the literature as well as to explore for unknown procyanidins.

Occurrence of gallocatechin and epigallocatechin was confirmed in the performed HPLC-MS experiments by comparison of column retention times, deprotonated (m/z 305) and protonated (m/z 307) ions as well as CID-MS-MS experiments of deprotonated ions with those of authentic gallocatechin and epigallocatechin standards. Figure 3.32 shows the reconstructed mass chromatogram after CID-MS-MS indicating the presence of both catechins.

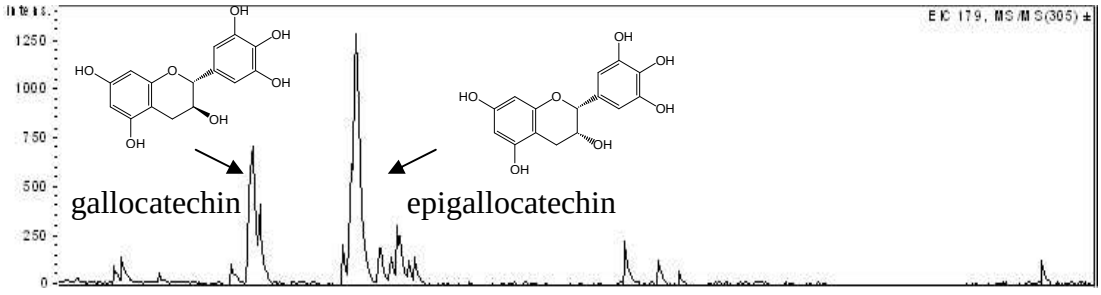
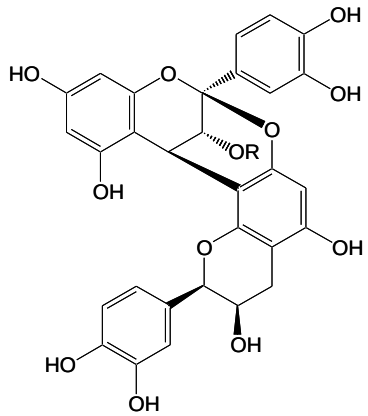
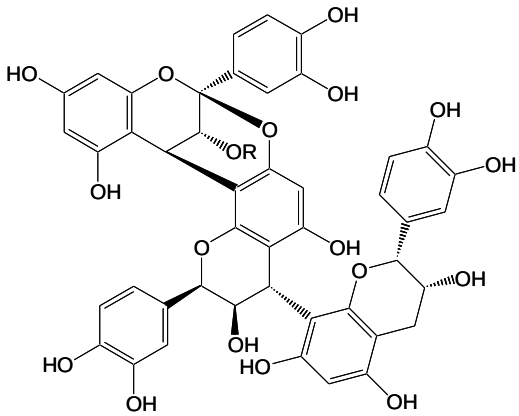


Figure 3.32 Reconstructed MS-MS mass chromatogram (305 → 179) of gallocatechin and epigallocatechin ($[M-H]^-$ of m/z 305)

Nevertheless, compared to catechin and epicatechin, the ion intensities of gallocatechin and epigallocatechin were 3 to 4 orders of magnitude lower and thus, their quantities were considered to be of little relevance for quantitative studies (chapters 3.3 and 3.4).

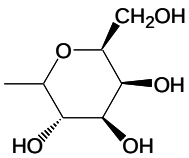
In addition to B-type procyanidins studied in section 3.2.1, exploratory experiments were carried out searching for the presence of A-type procyanidins having a second ether linkage between an A-ring hydroxyl function of the bottom unit to C(2) of the T-unit. As both aglykons and sugar-bound A-type procyanidins have been described in literature (e.g., Hatano et al., 2002), the experiments were designed to search for dimeric and trimeric procyanidins in the free form and bound to the cocoa glycosides arabinose or galactose, respectively (figure 3.33).





procyanidin A-type dimers: **procyanidin A-type trimers:**

II = aglykon 1.) R = H **III = aglykon**

II-g = galactoside 2.) R =  **III-g = galactoside**

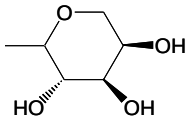
II-a = arabinoside 3.) R =  **III-a = arabinoside**

Figure 3.33 A-type procyanidins.

As molecular weight of these proposed cocoa compounds can be easily calculated, it is straightforward to search for the respective single-charged deprotonated and protonated ions in the mass selective detector of the HPLC-MS system set in the negative or positive ion mode. HPLC-MS analyses of cocoa liquor extract in the present work showed all of the anticipated ions in the mass spectrum (figure 3.34) summed over the expected retention time region of the compounds observed as unresolved “hump” in the UV trace (figure 3.8). Figure 3.35 shows the respective reconstructed mass chromatograms of the six deprotonated ions. The ion intensities and the presence of more than one isomer suggest that these compounds, tentatively identified as A-type procyanidins, may be of quantitative relevance in cocoa liquor if their identity can be confirmed, in particular the two dimeric glycosides.

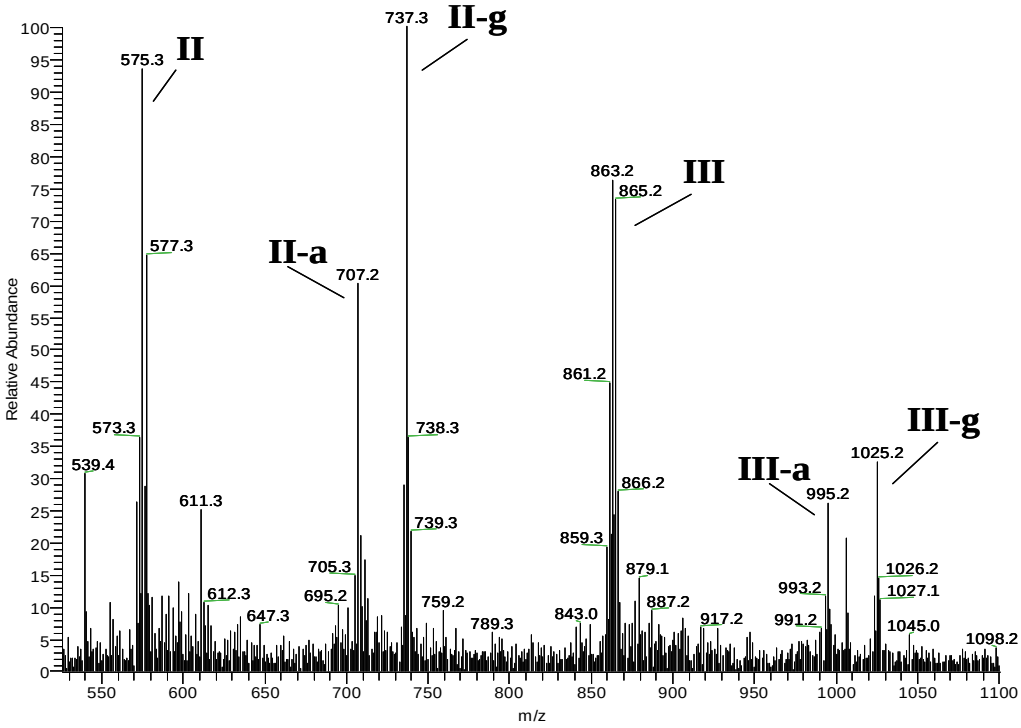


Figure 3.34 Sum of mass spectra over the chromatogram region where A-type procyanidins are assumed to elute from the column (compound identities II to III-g as in figure 3.33).

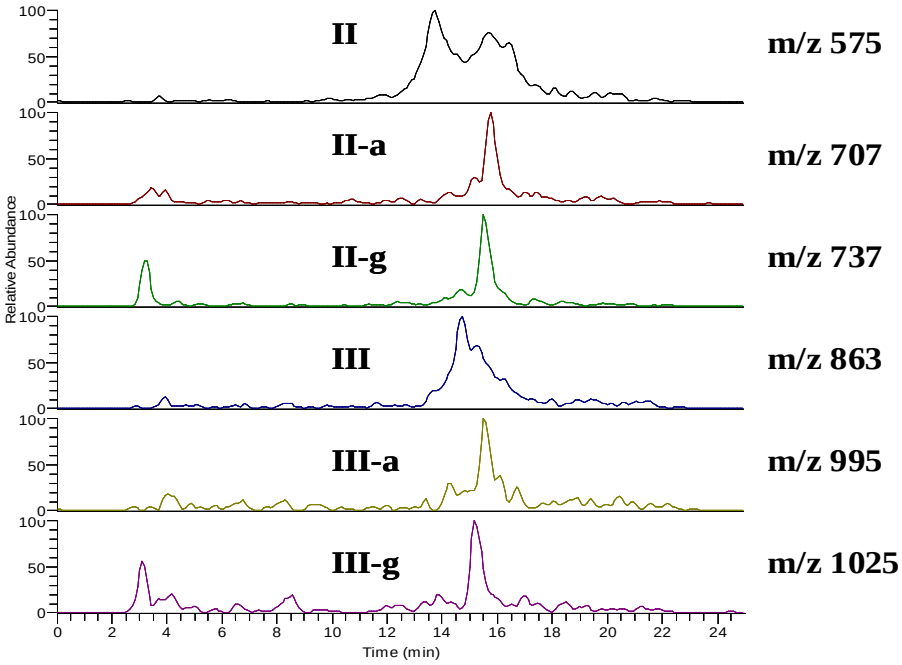


Figure 3.35 Reconstructed mass chromatograms of ion traces corresponding to deprotonated A-type procyanidins (compound II to III-g as in figure 3.34).

Mass chromatograms of the putative A-type procyanidin glycosides show good peak-shape. In contrast, those of m/z 575 and m/z 863 have broad shapes with more than one peak indicating the presence of two or more dimeric and trimeric A-type procyanidins aglykons or the presence of other compounds with such deprotonated ions, respectively. The interpretation gets more difficult due to the likely presence of oligomeric and polymeric procyanidin ions composed of solely epicatechin and catechin sub-units that can have double or multiple charges, because their deprotonated ions have the same mass-to-charge ratio as those of lower oligomers with fewer charges. Ions of such higher molecular weight compounds or A-type procyanidins glycosides might also undergo partly fragmentation in the ion source resulting in interflavan breakages as described in section 3.2.1, where the loss of a B-unit or a B-unit plus M-units would lead to product ions corresponding the same mass-to-charge ratios of ions of double-linked procyanidins, or to cleavage of the glycosidic linkage, respectively. Additional complication is introduced by the presence of significant carbon isotope peaks for pentameric and higher procyanidins, which hamper unambiguous interpretation of such mass spectra. In fact, Le Roux et al. (1998) have proposed such polymeric proanthocyanidins in litchi pericarp built up of 1 to 6 A-type linkages as degree of polymerisation increases to 22 mainly by interpretation of mass spectra of single and multiple-charged ions in previously isolated procyanidin fractions.

In order to gather additional information on the presumably present A-type procyanidins (compounds II to III-g in figure 3.33), in this work, several CID-MS-MS and MS^3 experiments were carried out on the observed deprotonated ions. Figure 3.36 shows reconstructed MS-MS mass chromatograms and spectra of m/z 707 and m/z 737 corresponding to A-type procyanidin arabinoside and galactoside, respectively. In the absence of authentic standards or isolated compounds with unambiguously confirmed identity, a clear statement was not possible in the present work. Nevertheless, the product ion spectra indicate fragmentation pathways that appear to be in line with the observed fragmentation patterns in B-type procyanidins and quercetin glycosides, respectively (figure 3.36 bottom).

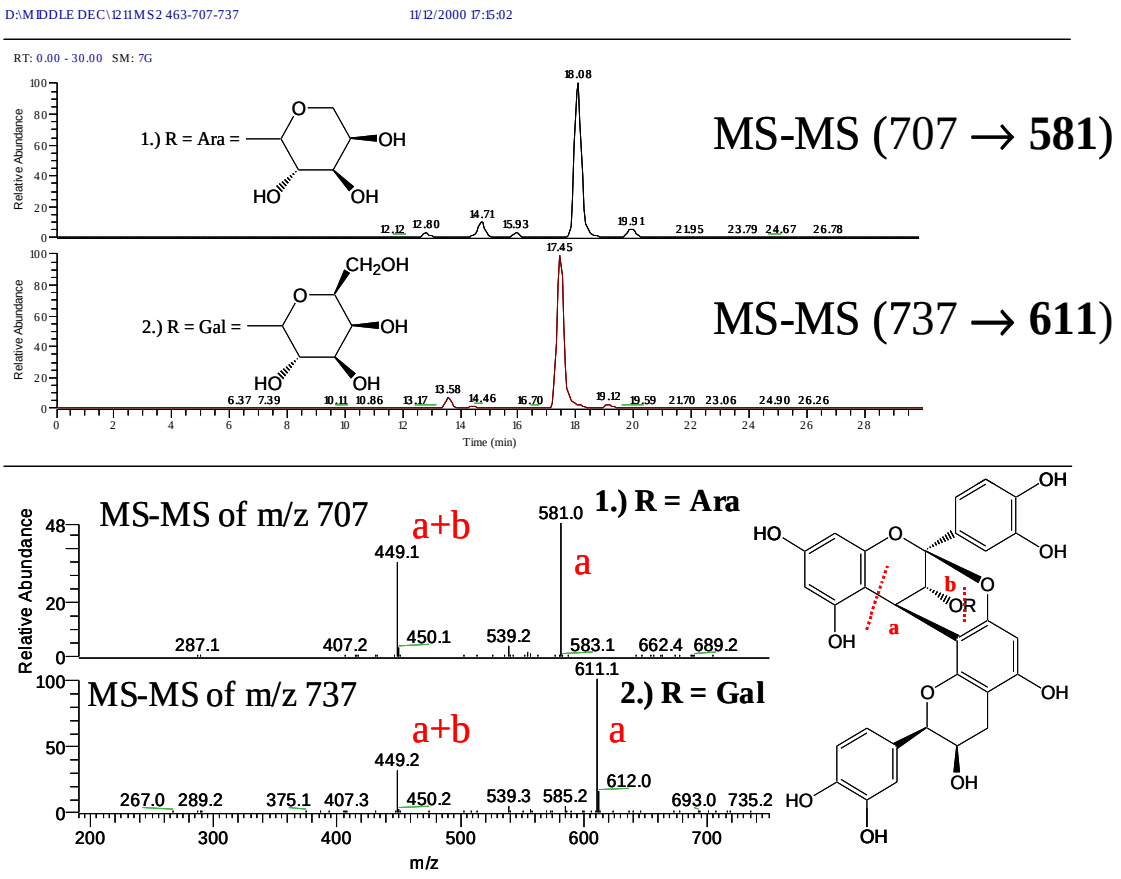


Figure 3.36 Reconstructed MS-MS mass chromatograms (top) of A-type procyanidin dimer-arabinoside (m/z 707 → 581) and -galactoside (m/z 737 → 611), respectively, as well as their respective CID-MS-MS spectra (bottom) - red dashed lines and letters indicate postulated fragmentation pathways.

Two fragmentation pathways may be postulated here from the obtained spectra in the CID-MS-MS experiments conducted. The loss of gallic acid (126 Da) leads to a product ion of m/z 581 in the case of the arabinoside and of m/z 611 in that of the galactoside corresponding well to the 30 Da mass difference between those two sugar moieties. Moreover, the second product ion m/z 449 can be observed in both spectra suggesting the loss of the sugar moiety together with gallic acid, as the mass difference to the former discussed product ions corresponds to the masses of the respective sugar moiety. In addition, fragmentation of the parent ion m/z 575, presumably being deprotonated A-type dimer aglykon, leads solely to m/z 449 as product ion. Minor product ions of all three compounds at m/z 289, m/z 287, and m/z 285 suggest interflavan cleavage as suggested for procyanidin B2. The same fragmentation pattern at lower ion intensities

can also be observed for the presumed trimeric compounds (III, III-a, and III-g). However, the absence of a product ion corresponding to the sole loss of the sugar moiety for the glycosides is surprising and is in contrast to the observations made for quercetin glycosides, where this loss was the dominating one requiring an additional CID experiment for structure elucidation.

Final confirmation must derive from isolation and unambiguous identification of these compounds. Attempts of isolating, purifying and stabilising these compounds were made as part of the present work. However, it was not possible to obtain sufficiently pure fractions by the applied techniques (described in chapter 3.2.3) in order to elucidate the structure of these compounds by means of NMR spectroscopy. Thus, in this work the identities of the A-type procyanidins were only tentatively assigned. Nevertheless, due to their putative quantitative relevance, these substances were monitored in the quantitative analyses (section 3.4), but values were considered only indicative in the absence of reference standard compounds.

3.2.3 Isolation, purification and characterisation of phenolic compounds from cocoa powder

As the presence of some of the compounds in cocoa liquor were only tentatively confirmed by the HPLC-MS experiments performed as part of this work (chapters 3.2.1 and 3.2.2) due to the absence of commercially available authentic standards it, was the objective to isolate and purify these substances from cocoa and elucidate their structure of by means of nuclear magnetic resonance (NMR) spectroscopy as well as HPLC-MS and hydrogen/deuterium exchange mass spectrometry.

Two procyanidins, a procyanidin dimer and a trimer, were isolated and purified from cocoa powder as described in chapter 2.2 by a combination of polyamide clean-up (flash chromatography), high-speed countercurrent chromatography (HSCCC) and semi-preparative HPLC. In addition, four hydroxycinnamic acid amides, namely caffeoyl-DOPA (clovamide), caffeoyltyrosine (monodeoxyclovamide isomer 1), p-coumaroyl-DOPA (monodeoxyclovamide isomer 2), and p-coumaroyltyrosine (dideoxyclovamide) were isolated and purified from cocoa powder by polyamide clean-up, followed by size-exclusion chromatography (SEC) using Sephadex LH-20 as porous

gel and final purification by repeated fractionation on semi-preparative HPLC (all described in chapter 2.2 and outlined in figure 2.1).

Both HPLC-DAD-ESI-MS and NMR were used on all extracts and fractions during isolation and purification of these six compounds in order to select optimal separation conditions and combine similar fractions for subsequent purification.

Crude cocoa polyphenols after polyamide clean-up contained all phenolic compounds as described in sections 3.2.1 and 3.2.2 with reduced amounts of procyanidin oligomers higher than tetramers as well as reduction of compounds appearing as unresolved "hump" in the UV-trace at 280 nm as confirmed by HPLC-DAD-ESI-MS analyses. In addition, this extract was virtually free of xanthine alkaloids (theobromine and caffeine) as well as on reversed-phase-HPLC early eluting hydrophilic matrix compounds.

Two polar two-phase solvent systems were used for HSCCC consisting of a) ethyl-acetate/ethanol/water (25/1/25 v/v/v) and b) ethyl-acetate/n-butanol/water (45/5/50 v/v/v) with the upper phase being used as stationary phase in both cases. In both systems hydrophilic higher oligomeric procyanidins eluted first and were combined as single fraction (IV), followed by well-resolved procyanidin trimers (fraction III) and dimeric procyanidins (fraction II). The latest eluting fraction was small in quantity and contained not identified compounds, whereas monomeric catechin and epicatechin remained in the Teflon tubes and were collected as "light phase" together with quercetin and clovamide compounds. HSCCC fractions III and II contained one major trimer and dimer, respectively, and the latter small amounts of another procyanidin dimer as revealed by HPLC-MS. The trimeric HSCCC fraction was almost pure while the major dimeric isomer was purified in an additional step by semi-preparative RP-HPLC.

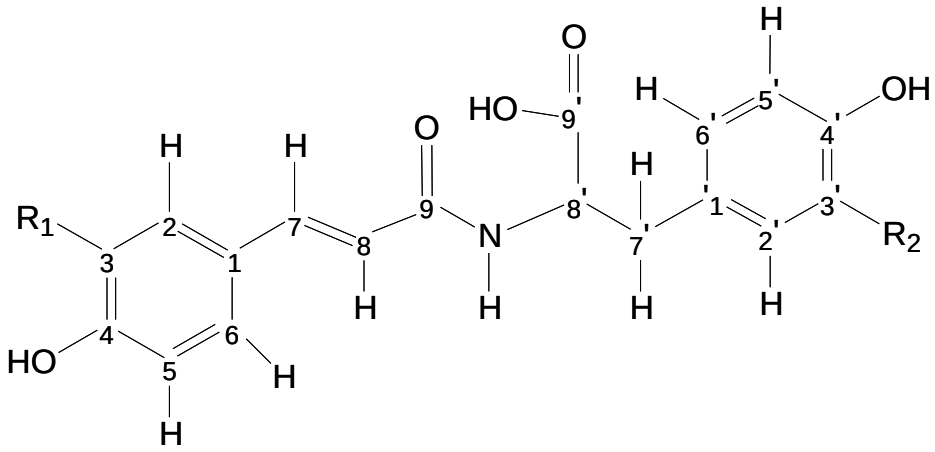
SEC on Sephadex LH-20 allowed separation of the early eluting clovamide derivatives from catechins and procyanidins, which eluted later in order of increasing degree of polymerisation although without sharp distribution between collection vials. Fractions containing the clovamides were reunified and single compounds were obtained by separation on semi-preparative HPLC since the clovamide and its monodeoxy- and dideoxy-analogues are well resolved in reversed-phase HPLC. Nevertheless, the two isomeric monodeoxyclovamides were not resolved and their identity had to be confirmed without separation.

3.2.3.1 Structure elucidation by nuclear magnetic resonance spectroscopy

The identity of four clovamides and two procyanidins previously tentatively described by HPLC-ESI-MS was confirmed applying ^{1}H and ^{13}C NMR analyses on the purified extracts. As monodeoxy derivates of clovamide (monodeoxyclovamides) have not been described in cocoa so far, additional two-dimensional NMR experiments have been carried out. Homonuclear (Corelated Spectroscopy, COSY) and heteronuclear (Heteronuclear Single Quantum Correlation, HSQC; Heternuclear Multiple Bond Correlations, HMBC) two dimensional spectra were acquired, confirming the presence of two monodeoxyclovamide isomers.

3.2.3.1.1 Hydroxycinnamic acid amides (clovamides)

Figure 3.37 shows the chemical structure of the four hydroxycinnamic acid amides including the numbering of the carbon atoms for signal assignment in NMR experiments.



R_1 = OH, R_2 = OH – Caffeoyl DOPA = clovamide
R_1 = OH, R_2 = H – Caffeoyltyrosine = monodeoxyclovamide 1
R_1 = H, R_2 = OH – p-Coumaroyl DOPA = monodeoxyclovamide 2
R_1 = OH, R_2 = OH – p-Coumaroyltyrosine = dideoxyclovamide

Figure 3.37 Chemical formulae of the 4 isomers of hydroxycinnamic acid amides (clovamides) identified in cocoa (DOPA = dihydroxyphenylalanine).

^{1}H NMR has been applied by Tebayashi et al. (2000) to all four clovamide derivatives isolated from red clover and obtained by chemical synthesis as well as ^{1}H and ^{13}C NMR to clovamide and dideoxyclovamide from cocoa by Sanbongi et al. (1998). These NMR data for were used as reference for NMR results obtained in this work.

Tables 3.4 to 3.6b summarise the comparisons between recorded NMR spectra in the present study and data from literature.

Table 3.4 ^{1}H spectra for clovamide (caffeoyl-dihydroxyphenylalanine)

C	**^{1}H in methanol-d_4 from cocoa (own data)**	**^{1}H in DMSO-d_6 from cocoa (Sanbongi et al., 1998)**	**^{1}H in DMSO-d_6 from red clover/ chemical synthesis (Tebayashi et al., 2000)**
2	6.94, 1H, d, J=1.8Hz	6.93, 1H, d, J=1.8Hz	6.96, 1H, J=1.8Hz
5	6.73, 1H, d, J=8.2Hz	6.74, 1H, d, J=8.1Hz	6.76, 1H, d, J=8.1Hz
6	6.82, 1H, dd, J=8.1, 1.8Hz	6.82, 1H, dd, J=8.0, 1.8Hz	6.86, 1H, dd, J=8.2, 1.8Hz
7	6.42, 1H, d, J=15.6Hz	6.41, 1H, d, J=15.7Hz	6.41, 1H, d, J=15.7Hz
8	7.15, 1H, d, J=15.7Hz	7.20, 1H, d, J=15.7Hz	7.21, 1H, d, J=15.7Hz
2’	6.59, 1H, d, J=1.2Hz	6.62, 1H, d, J=1.8Hz	6.63, 1H, s
5’	6.55, 1H, d, J=8.1Hz*	6.61, 1H, d, J=7.8Hz	6.62, 1H, d, J=8.2Hz
6’	6.55, 1H, d, J=8.1Hz*	6.57, 1H, dd, J=7.9, 1.8Hz	6.50, 1H, dd, J=8.1, 1.8Hz
7’	2.90, 1H, dd, J=13.6, 5.1Hz	2.90, 1H, dd, J=13.7, 4.9Hz	2.92, 1H, dd, J=13.9, 4.8Hz
	2.72, 1H, dd, J=13.5, 7.7Hz	2.72, 1H, dd, J=13.7, 9.1Hz	2.73, 1H, dd, J=13.9, 9.2Hz
8’	4.23, 1H, br	4.48, 1H, m	4.44, 1H, ddd, J=9.2, 8.0, 4.8Hz
NH	8.37, 1H, br	8.75, 1H, d, J=8.0Hz	8.21, 1H, d, J=7.2Hz
OH	8.4-9.2, 4H, br	8.62, 8.67, 9.07	8.7-9.4, br, 4H
		9.31, 4H	

* overlapped signals

Table 3.5 ^{13}C and ^{1}H spectra for dideoxyclovamide (p-coumaroyltyrosine)

C	^{13}C in DMSO-d_6 from cocoa (own data)	^{1}H in DMSO-d_6 from cocoa (own data)	^{13}C in DMSO-d_6 from cocoa (Sanbongi et al., 1998)	^{1}H in DMSO-d_6 from cocoa (Sanbongi et al., 1998)	^{1}H in DMSO-d_6 from red clover/chem. synthesis (Tebayashi et al., 2000)
1	127.8		127.7		
2	130.7	7.02, 1H, d, J=8.3Hz	130.7	7.02, 1H, d, J=8.6Hz	7.03, 1H, d, J=8.4Hz
3	116.8	6.66, 1H, d, J=8.4Hz	116.7	6.64, 1H, d, J=8.2Hz	6.65, 1H, d, J=8.4Hz
4	160.6		160.6		
5	116.8	6.66, 1H, d, J=8.4Hz	116.7	6.64, 1H, d, J=8.2Hz	6.65, 1H, d, J=8.4Hz
6	130.7	7.02, 1H, d, J=8.3Hz	130.7	7.02, 1H, d, J=8.6Hz	7.03, 1H, d, J=8.4Hz
7	142.2	6.57, 1H, d, J=15.6Hz	142.4	6.48, 1H, d, J=15.6Hz	6.48, 1H, d, J=15.7Hz
8	118.3	7.30, 1H, d, J=15.6Hz	117.9	7.27, 1H, d, J=15.6Hz	7.28, 1H, d, J=15.7Hz
9	168.8		169.0		
1’	129.6		129.1		
2’	131.4	7.44, 1H, d, J=8.6Hz	131.3	7.37, 1H, d, J=8.6Hz	7.38, 1H, d, J=8.6Hz
3’	116.2	6.83, 1H, d, J=8.6Hz	116.2	6.78, 1H, d, J=8.6Hz	6.79, 1H, d, J=8.6Hz
4’	157.2		157.3		
5’	116.2	6.83, 1H, d, J=8.6Hz	116.2	6.78, 1H, d, J=8.6Hz	6.79, 1H, d, J=8.6Hz
6’	131.4	7.44, 1H, d, J=8.6Hz	157.3	7.37, 1H, d, J=8.6Hz	7.38, 1H, d, J=8.6Hz
7’	38.1	3.05, 1H, dd, J=13.7, 4.7Hz	37.8	2.97, 1H, dd, J=13.7, 4.7Hz	2.98, 1H, dd, J=13.8, 4.8Hz
		2.87, 1H, dd, J=13.7, 7.4Hz		2.79, 1H, dd, J=13.7, 9.4Hz	2.80, 1H, dd, J=13.8, 9.2Hz
8’	56.4	4.30, 1H, m	55.5	4.46, 1H, m	4.47, 1H, ddd, J=9.2, 8.1, 4.8Hz
NH		7.81, 1H, d, J=8.2Hz		8.18, 1H, d, J=8.2Hz	8.19, 1H, d, J=8.1Hz
OH	176.3	8.7-9.3, 2H, br	174.9	9.19, 8.84, 2H, br	9.19, 8.82, 2H, br

The NMR results here are in good accordance with the literature (Sanbongi et al., 1998; Tebayashi et al., 2000) and the presence of clovamide and dideoxyclovamide in cocoa can be confirmed. For example, the proton NMR spectrum of dideoxyclovamide mainly exhibits four patterns of signals:

- a methylene signal split into two doublets of doublets at 2.87 and 3.05 ppm due to the position in α of the asymmetric carbon 8';
- a methyne signal at 4.30 ppm;
- two olefinic protons at 6.57 and 7.30 ppm exhibiting a coupling constant of 15.6 Hz which indicates their stereochemistry in trans;
- four aromatic doublets (6.66, 7.02, 6.83 and 7.44 ppm) corresponding to eight protons, which indicates the presence of an axial symmetry of the two aromatic cycles.

In addition, a ^{13}C NMR spectrum was acquired for dideoxyclovamide. These carbon-13 data are in good accordance with those published by Sanbongi et al. (1998) the variation of chemical shifts being inferior to 0.5 ppm for the majority of signals. A greater variation for the carbon 8´ in α position of the carboxylic and the amine groups and for the carboxylic carbon (C 9´) itself was observed. However, it is well established that such variations can occur for carbon directly linked or close to polar functional groups such as the hydroxyl or amine groups. Indeed, experimental conditions and particularly the nature of solvent and the analyte concentration can induce a shift of the resonance signals of such carbons. The use of deuterated dimethylsulfoxide in the present study instead of deuterated methanol employed by Sanbongi et al. (1998) could thus easily explain the observed variations. It should be mentioned that the chemical shift 131.4 ppm was simultaneously assigned to carbons 2' and 6' due to the symmetry of the corresponding ring contrary to the reported data 157.3 ppm for carbon 6' (Sanbongi et al., 1998).

The proton spectrum of the monodeoxyclovamide analogues fraction confirmed the presence of two isomers and revealed that caffeoyltyrosine was the predominant of the two isomers. A ratio of approximately 1.7:1 in favour of caffeoyltyrosine over p-coumaroyl-DOPA can be deduced from the integrals of the olefinic signals at 7.19 and 7.27 ppm (figure 3.38).

Whereas Sanbongi et al. (1998) present the assignment of ^{1}H NMR data to the respective protons for clovamide and dideoxyclovamide, such information is not given for the two monodeoxyclovamide isomers (Tebayashi et al., 2000). Thus, ^{1}H NMR data recorded here and published by Tebayashi et al. (2000) have been only tentatively assigned (tables 3.6a and b). In order to gain more confidence in the identity of these two hydroxycinnamic acid amides, additional multidimensional NMR experiments have been conducted including COSY, HSQC and HMBC spectra (figures 3.39 and 3.40a + b further below).

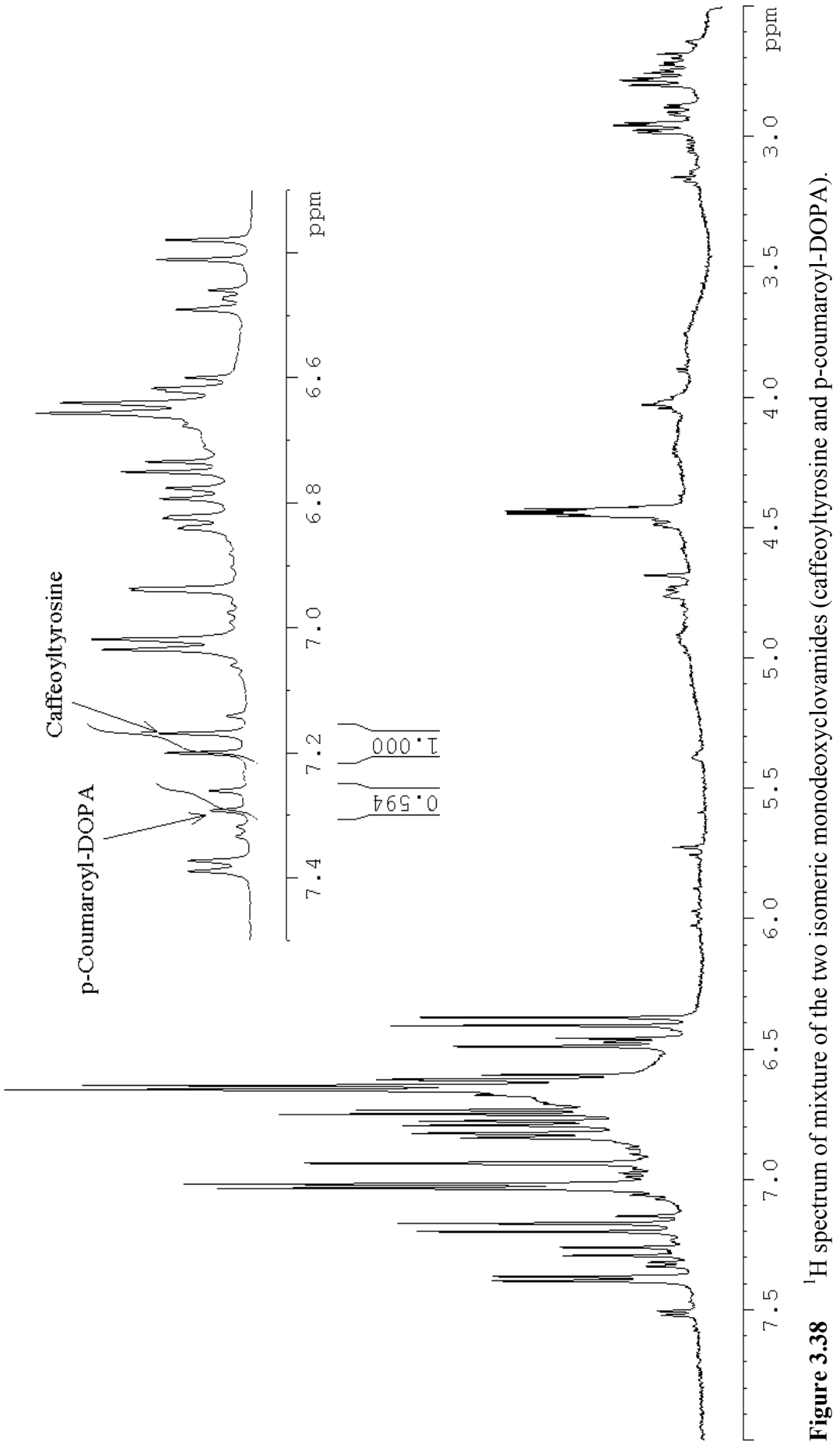


Figure 3.38 ^{1}H spectrum of mixture of the two isomeric monodeoxyclovamides (caffeoyltyrosine and p-coumaroyl-DOPA).

Table 3.6a ^{1}H spectra for caffeoyltyrosine (monodeoxyclovamide isomer1)*

C-No.	^{13}C	^{1}H in DMSO-d_6 from cocoa (own data)	^{1}H in DMSO-d_6 red clover/chem. synthesis (Tebayashi et al., 2000)
2	126.53	6.93, 1H, d, J=1.5Hz	6.94, 1H, s
5	115.65	6.74, 1H, d, J=8.2Hz	6.74, 1H, d, J=8.2Hz
6	120.68	6.83, 1H, dd, J=8.2, 1.5Hz	6.83, 1H, d, J=8.3Hz
7	118.00	6.39, 1H, d, J=15.7Hz	6.41, 1H, d, J=15.7Hz
8	139.84	7.19, 1H, d, J=15.7Hz	7.19, 1H, d, J=15.7Hz
2' & 6'	130.18	7.02, 2H, d, J=8.4Hz	7.03, 2H, d, J=8.2Hz
3' & 5'	115.10	6.64, 2H, d, J=8.4Hz	6.65, 2H, d, J=8.2Hz
7'	36.10	2.96, 1H, dd, J=14.0, 4.6Hz	2.98, 1H, dd, J=13.9, 4.8Hz
		2.76, 1H, dd, J=14.0, 9.6Hz	2.72, 1H, dd, J=13.8, 9.3Hz
8'	54.00	4.44, 1H, m	4.44, 1H, ddd, J=9.3, 8.2, 4.8Hz
NH		8.24, 1H, d, J=8.2Hz	8.21, 1H, d, J=8.2Hz
OH		8.6-9.9, 3H, br	9.06, 9.19, 9.36, 3H, br
1	126.53		
3	145.44		
4	147.43		
9	165.53		
1'	127.84		
4'	155.93		

*: Chemical shifts were referenced to DMSO resonances at 2.5 ppm (^{1}H) and 39.5 ppm (^{13}C). Carboxylic signal (^{13}C) could be observed in another optimised experiment (δC9': 173.33 ppm).

Table 3.6b ^{1}H spectra for p-coumaroyldihydroxyphenylalanine (monodeoxyclovamide isomer2)*

C-No.	**^{13}C**	**^{1}H in DMSO-d_6 from cocoa (own data)**	**^{1}H in DMSO-d_6 red clover/chem. synthesis (Tebayashi et al., 2000)**
2 & 6	129.44	7.38, 2H, d, J=8.5Hz	7.38, 2H, d, J=8.3Hz
3 & 5	115.72	6.78, 2H, d, J=8.5Hz	6.79, 2H, d, J=8.1Hz
7	118.12	6.47, 1H, d, J=15.8Hz	6.49, 1H, d, J=15.4Hz
8	139.84	7.27, 1H, d, J=15.8Hz	7.28, 1H, d, J=15.7Hz
2’	116.60	6.62, 1H, s	6.62, 1H, s
5’	115.1	6.60, 1H, d, J=8.5Hz	6.61, 1H, d, J=8.0Hz
6’	120.1	6.48, 1H, d, J=8.5Hz	6.48, 1H, d, J=8.1Hz
7’	36.36	2.89, 1H, dd, J=14.3, 5.0Hz	2.91, 1H, dd, J=13.8, 4.7Hz
		2.73, 1H, dd, J=14.3, 6.2Hz	2.73, 1H, dd, J=13.8, 9.2Hz
8’	54.00	4.44, 1H, m	4.44, 1H, ddd, J=9.2, 8.0, 4.7Hz
NH		8.19, 1H, d, J=7.9Hz	8.16, 1H, d, J=8.0Hz
OH		8.6-9.9, 3H, br	8.68,8.73, 9.83, 3H, br
1	125.99		
4	158.92		
9	165.44		
1’	128.35		
3’	143.72**		
4’	143.77**		

*: Chemical shifts were referenced to DMSO resonances at 2.5 ppm (^{1}H) and 39.5 ppm (^{13}C).
**: Assignements can be inverted. Carboxylic signal (^{1}H and ^{13}C) could not be observed.

Figure 3.39 shows the COSY spectrum of the monodeoxyclovamide mixture where the scalar couplings are displayed in red and blue for caffeoyltyrosine and p-coumaroyl-DOPA, respectively.

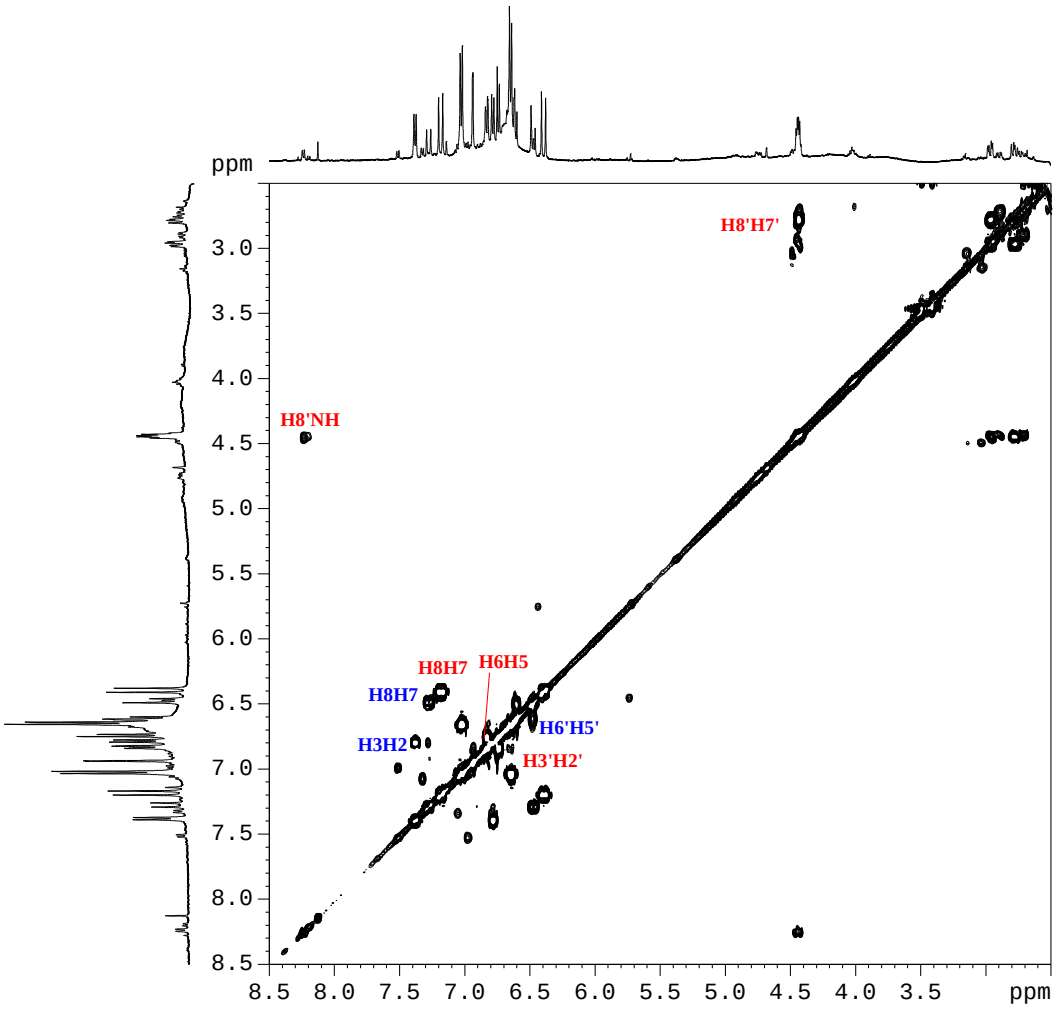


Figure 3.39 COSY spectrum of mixture of two isomeric monodeoxyclovamides.

Homonuclear correlations occur between:

- the proton corresponding to carbon 8’ (4.44 ppm) and the methylene protons (2.76 and 2.96 ppm; 2.73 and 2.89 ppm), correlations H8’H7’ of both isomers;
- the proton corresponding to carbon 8’ (4.44 ppm) and the amine proton (8.24 ppm), correlation H8’NH of caffeoyltyrosine;
- the olefinic protons, correlations H8H7 of both isomers;

- the aromatic protons corresponding to carbons 5, 6, 7, 8, 2’ and 3’, correlations H6H5, H8H7 and H3’H2’ of caffeoyltyrosine, and to carbons 2, 3, 7, 8, 5’ and 6’, correlations H3H2, H8H7 and H6’H5’ of p-coumaroyl-DOPA.

- scalar couplings in meta between protons corresponding to carbons 2 and 6 (caffeoyltyrosine) and to carbons 2’ and 6’ (p-coumaroyl-DOPA) could also be detected.

In addition, heteronuclear correlations spectra, HSQC and HMBC, were also acquired. HSQC gives rise to correlations of carbons and directly bonded hydrogens while HMBC allows the observation of long-range couplings between hydrogen and carbon distant by two or three chemical bonds. The assignment of both HSQC and HMBC spectra reported shown in figures 3.40a and b, respectively as well as in tables 3.6a and 3.6b, allowed unambiguous identification of the two monodeoxyclovamide analogues.

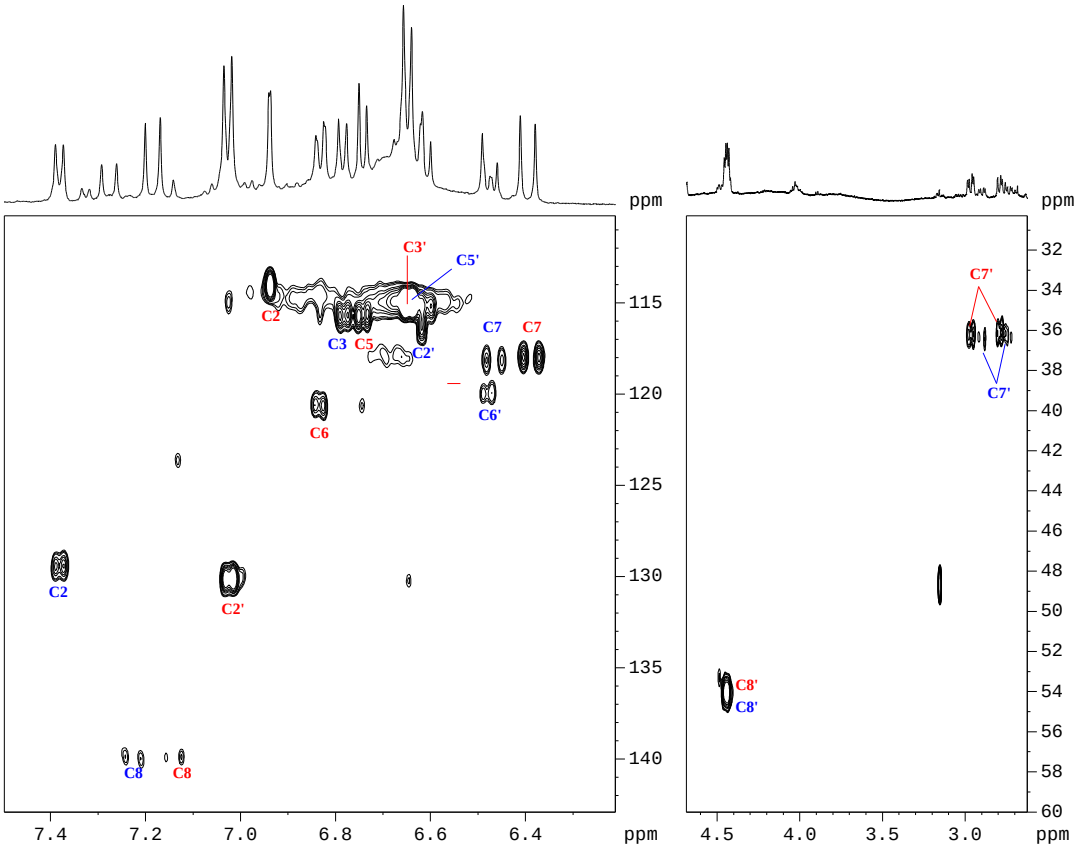


Figure 3.40a HSQC spectrum of mixture of caffeoyltyrosine (red) and p-coumaroyl-DOPA (blue).

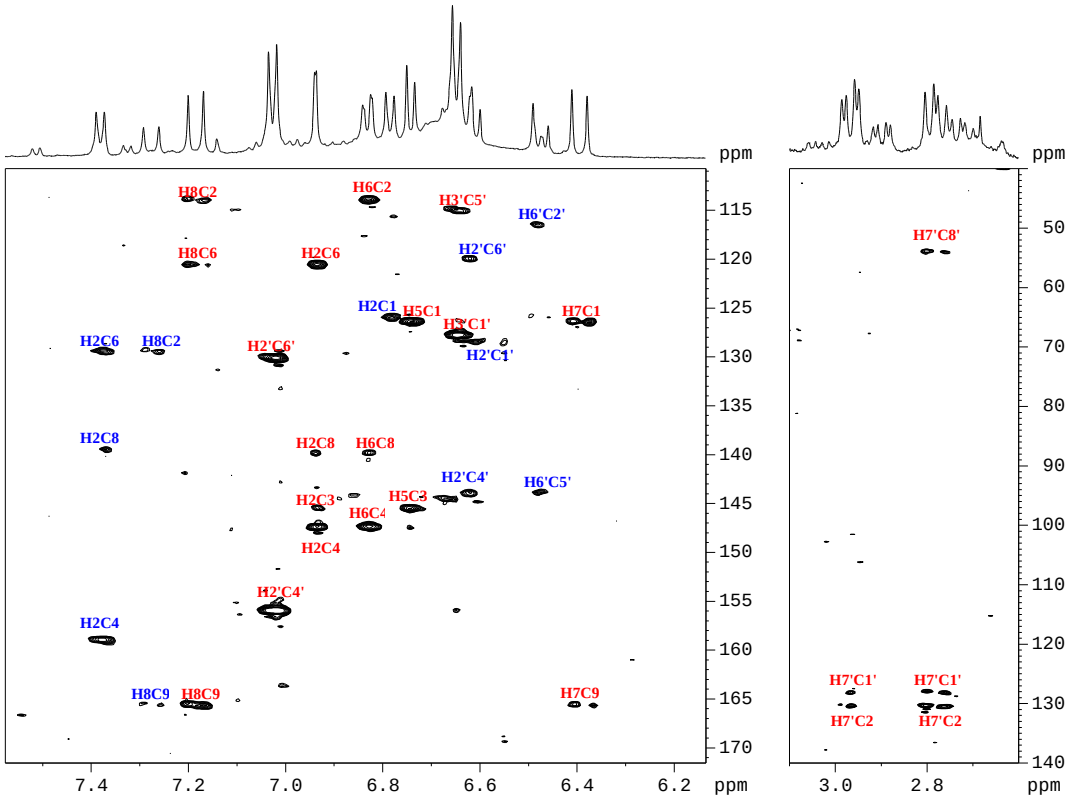


Figure 3.40b HMBC spectrum of mixture of caffeoyltyrosine (red) and p-coumaroyl-DOPA (blue).

3.2.3.1.2 Procyanidins

Procyanidins consist of two or more catechin and/or epicatechin units, which are linked together via 4β→8 or 4β→6 interflavan C-C bonds (Shoji et al., 2003). Nevertheless, the most predominant forms in the plant kingdom are epicatechin-based procyanidins with 4β→8 linkages (figure 3.41). The two procyanidin dimeric and trimeric compounds isolated here from cocoa powder by means of HSCCC and preparative RP-HPLC represent the overwhelming predominat dimeric and trimeric isomers in cocoa (Porter et al., 1991). Thus, the hypothetic structures of the isolated compounds to be confirmed by NMR. were epicatechin-4β→8-epicatechin (procyanidin B2) and epicatechin-4β→8-epicatechin-4β→8-epicatechin (procyanidin C1), respectively.

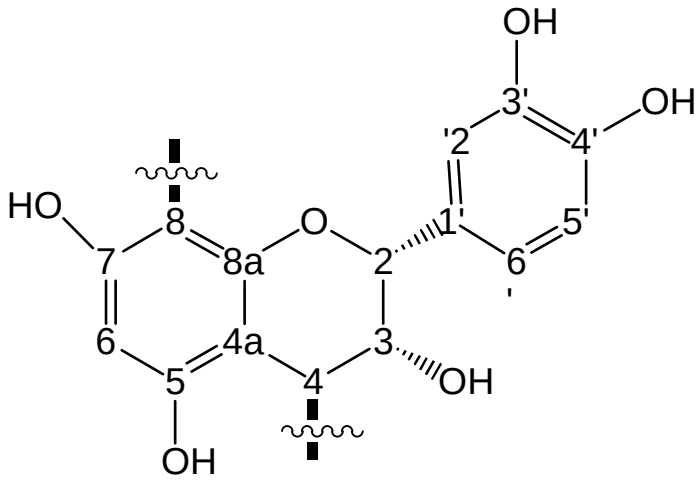


Figure 3.41 Basic epicatechin subunit of procyanidin dimer B2 and trimer C1, respectively, and carbon numbering.

Traditionally, the structure elucidation of procyanidins is based on a combination of spectroscopic data (NMR, optical rotation, circular dichroism (CD)) and chemical degradation studies (Rohr, 1999). NMR investigations have mostly required a previous derivatisation of procyanidins to their peracetate or methyl ether acetates (Shoji et al., 2003). However, degradation and derivatisation of procyanidins require substantial amounts of isolated compounds. In addition, isolated compounds cannot be used for further application as reference standard in qualitative and quantitative analyses or for physiological studies.

The difficulty in determining the structure of procyanidins by NMR derives from broadening of ^{1}H NMR signals due to atropisomerism, which results from steric interactions in the vicinity of the interflavan bond about which the flavanol units are free to rotate as well as conformational isomerism due to different geometries of the heterocyclic rings (Rohr, 1999; Shoji et al., 2003). Avoiding the derivatisation step of procyanidins for decreasing these broadening effects, Shoji et al. (2003) conducted experiments at low temperatures between –20 and –40°C (253-233K) for procyanidin dimers, trimers and a tetramer isolated from apple.

Preliminary experiments carried out as part of the present work recording ^{1}H NMR spectra of procyanidin B2 at low temperatures have shown promising improvements in the resolution of broad and overlapping signals (data not shown). Thus, ^{1}H and ^{13}C NMR spectra of isolated procyanidin dimer and trimer were recorded at 250 and 253K, respectively, and for structure elucidation ^{13}C NMR results compared with published data (Agrawal et al., 1989; Shoji et al., 2003). Tables 3.7 for procyanidin dimer B2 and table 3.8 for procyanidin trimer C1 summarise the comparisons.

Whereas chemical shifts in ^{13}C NMR of procyanidin B2 differ somewhat from the data in the literature, those for the trimer are in very good accordance, particularly to those reported by Shoji et al. (2003). This is likely due to the different solvent and temperature conditions used for the NMR experiments in the studies performed in this work being acetone-d_6 at 250K, and methanol-d_4 at 233K as well as acetone-d_6-water (1:1 v/v) in the references. It is well known that solvent composition affect chemical shifts in ^{13}C NMR of procyanidins, in particular that of C-4a (Agrawal et al., 1989). Nevertheless, since an authentic standard of procyanidin B2 has recently become commercially available, comparison of ^{1}H and ^{13}C NMR spectra as well as retention times and mass spectra in HPLC-MS analyses of isolated compound and standard have confirmed the identity making repeated measurements under comparable conditions unnecessary.

In contrast, ^{1}H and ^{13}C NMR spectra of the isolated trimer have been repeated under exactly the same conditions (methanol-d_4 at 253K) as reported by Shoji et al. (2003). Figure 3.42 shows the ^{13}C NMR spectrum of the isolated procyanidin trimer C1. All 45 C-atoms have been assigned in strong accordance with the ^{13}C NMR data reported by Shoji et al. (2003) confirming the identity of epicatechin-4β→8-epicatechin-4β→8-epicatechin (procyanidin C1).

Table 3.7 ^{13}C spectra of procyanidin dimer B2

C	**^{13}C in acetone-d_6 at 250K from cocoa (own data)**		**^{13}C in methanol-d_4 at 233K from apple (Shoji et al., 2003)**		**^{13}C in acetone-d_6–H_2O (1:1 v/v) at 303K from various plants(Agrawal et al., 1989)**	
	B-unit	T-unit	B-unit	T-unit	B-unit	T-unit
2	79.5	77.2	79.9	76.9	79.1	76.5
3	66.7	73.2	67.0	73.5	66.2	72.8
4	29.5	37.2	29.9	36.7	29.1	36.7
4a	97.5	100.9	99.6	103.9	100.6	102.2
5	156.3	157.9[a]	156.5	157.6[c]		
6	96.9	96.2	96.8	95.6	97.3	96.3
7	156.3	158.8[a]	156.6	157.8[c]		
8	107.2	96.2	107.2	95.5	107.6	95.9
8a	156.3	157.9[a]	154.5	157.7[c]		
1’	132.0	132.5	132.1	132.5		
2’	115.6[b]	115.3[b]	114.9	114.9		
3’	145.8	145.7	145.8	145.7		
4’	145.6	145.5	145.5	145.4		
5’	115.8	115.9	115.6	115.7		
6’	119.3	119.5	118.7	119.0		

[a-d] Assignments with the same letter are interchangeable

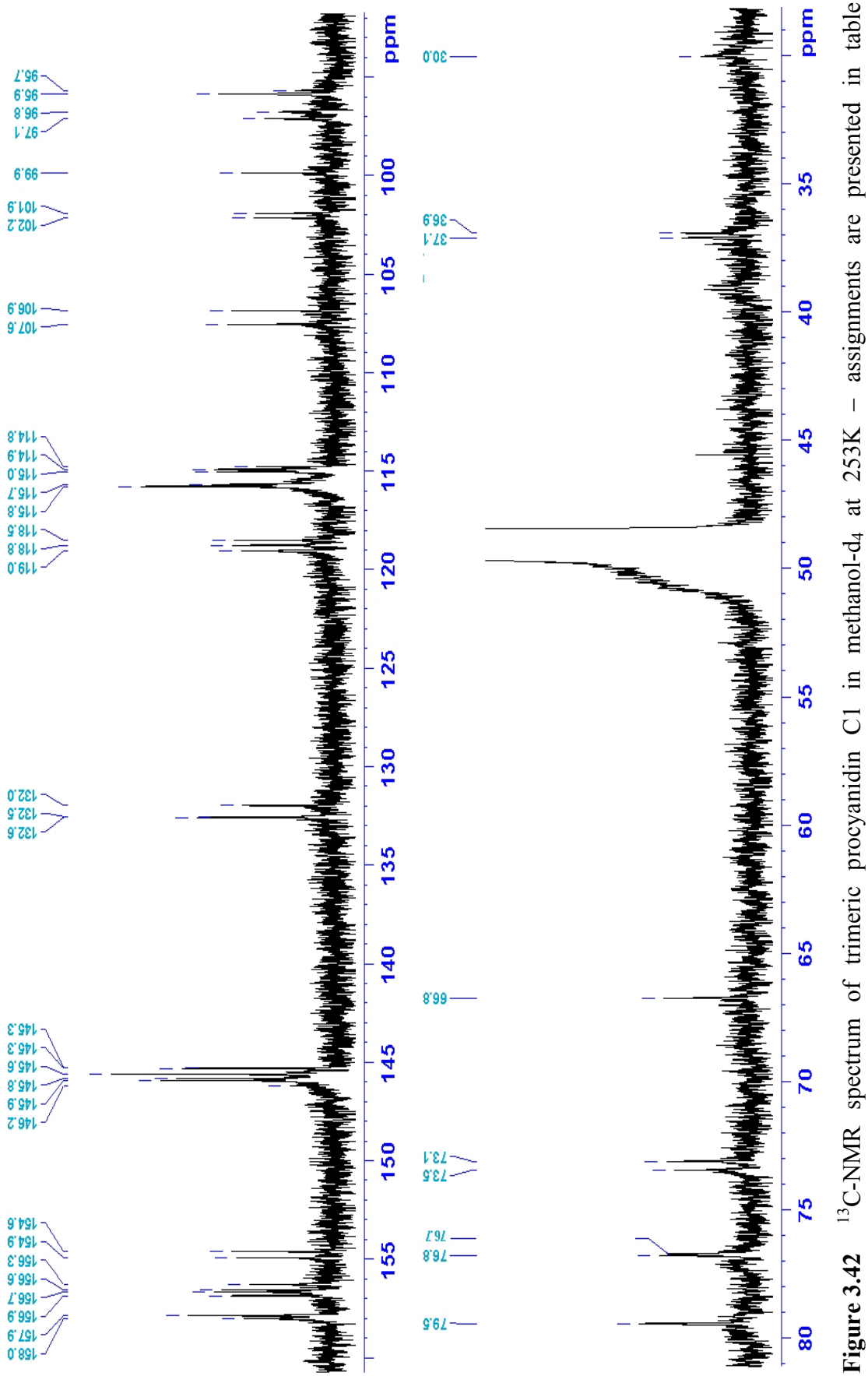


Figure 3.42 ^{13}C-NMR spectrum of trimeric procyanidin C1 in methanol-d_4 at 253K – assignments are presented in table 3.8.

Table 3.8 ^{13}C spectra of procyanidin trimer C1

C	^{13}C in methanol-d_4 at 253K from cocoa (own data)			^{13}C in methanol-d_4 at 253K from apple (Shoji et al., 2003)			^{13}C in acetone-d_6–H_2O (1:1 v/v) at 303K (Agrawal et al., 1989)		
	B-unit	M-unit	T-unit	B-unit	M-unit	T-unit	B-unit	M-unit	T-unit
2	79.5	76.7	76.8	79.4	76.6	76.7	78.9	76.5	76.5
3	66.8	73.1	73.5	66.7	72.9	73.4	66.2	71.9	73.0
4	30.0	37.1	36.9	30.0	37.1	36.9	29.2	36.8	36.8
4a	99.9	102.2	101.9	100.0	102.1	101.5	100.6	102.0	102.0
5	156.6	156.3	157.9	156.6	156.4	158.0			
6	97.1	96.8	95.7[a]	97.4	97.1	96.0[c]	97.5	97.1	96.4
7	156.6	156.7	158.0	156.6	156.9	158.2			
8	107.6	106.9	95.9[a]	107.4	106.8	96.1[c]	107.6	107.1	96.0
8a	154.6	154.9	156.9	154.4	154.7	157.7			
1’	132.0	132.5	132.6	131.8	132.3	132.4			
2’	115.0	114.8	114.9	115.0	114.9	115.0			
3’	146.2	145.9	145.6[b]	146.0	145.9	145.6			
4’	145.8[b]	145.3	145.3	145.6	145.4	145.4			
5’	115.7	115.8	115.7	115.7	115.8	115.8			
6’	118.5	118.8	119.0	118.7	118.9	119.0			

[a-c] Assignments with the same letter are interchangeable

3.2.3.2 Determination of labile protons by liquid phase hydrogen/deuterium exchange followed by mass spectrometry

The signals for hydrogen atoms of hydroxyl-groups are difficult to distinguish by ^{1}H NMR and usually appear as a broad peak (see tables 3.4 to 3.6b). In order to get additional confirmation on the number of OH-groups of the isolated hydroxycinnamic acid amides and procyanidin dimer and trimer, respectively, experiments applying hydrogen/deuterium exchange followed by mass spectrometry (H/D-MS) were carried out in this part of the work. In addition to the information on exchangeable protons of the OH-groups, additional structure information was gathered from H/D-MS experiments by extending the understanding of fragmentation pathways, which were postulated in chapters 3.2.1 and 3.2.2.

In fact, H/D exchange - both in gaseous and liquid phase - has been widely applied for studying structure, stability, folding dynamics, and intermolecular interactions in proteins (Lam and Ramanathan, 2002; Kamel et al., 2002) as well as identifying exchangeable protons, such as in OH, COOH, NH or SH (Wan et al., 2001). More recently, H/D exchange coupled to ESI-MS has shown its potential in the structure elucidation of drugs and their metabolites (Smith et al., 2000; Kamel et al., 2003) as well as of natural products (Bringmann et al., 2000).

The experiments were done in this work simply by dissolving the isolated compound in normal solvent (water-methanol 1:1 v/v) and separately in deuterated solvent (D_2O-methanol-d_4 1:1 v/v) at room temperature leading to the exchange of acidic protons of the isolated compound with protons (H^+) from the normal aqueous phase and deuterons (D^+) from the deuterated phase, respectively. Performing mass spectrometric analyses by direct infusion into the ion source of a mass analyser in aqueous solvent showed the normal compound spectrum of ions corresponding to $[M-H]^-$ in the negative ion mode and $[M+H]^+$ in the positive ion mode having a mass to charge ratio of minus one and plus one of the compound mass, respectively. In deuterated solvent ions correspond to $[M(D_x)-D]^-$ in the negative and $[M(D_x)+D]^+$ in the positive ion mode having a mass-to-charge ratio of minus **two** and plus **two** of the compound mass, which for its part has a molecular weight by the amount of exchangeable protons higher than the original one.

As an example of the performed studies within this work, the following discussion focuses at first on the mixture of the two monodeoxyclovamide isomers since these compounds have not been described in cocoa so far. Nevertheless, experiments were also carried out on the other two isolated hydroxycinnamic acid amides, namely clovamide and dideoxyclovamide, and provided both additional confirmation on the identity of these substances as well as helpful information for interpretation of fragmentation patterns of the isomeric monodeoxyclovamides. Figures 3.43a and figure 3.43b show the mass spectra of monodeoxyclovamides (MW 343 Da) in both ion modes as well as in aqueous and deuterated phases (MW 348 Da after H/D-exchange).

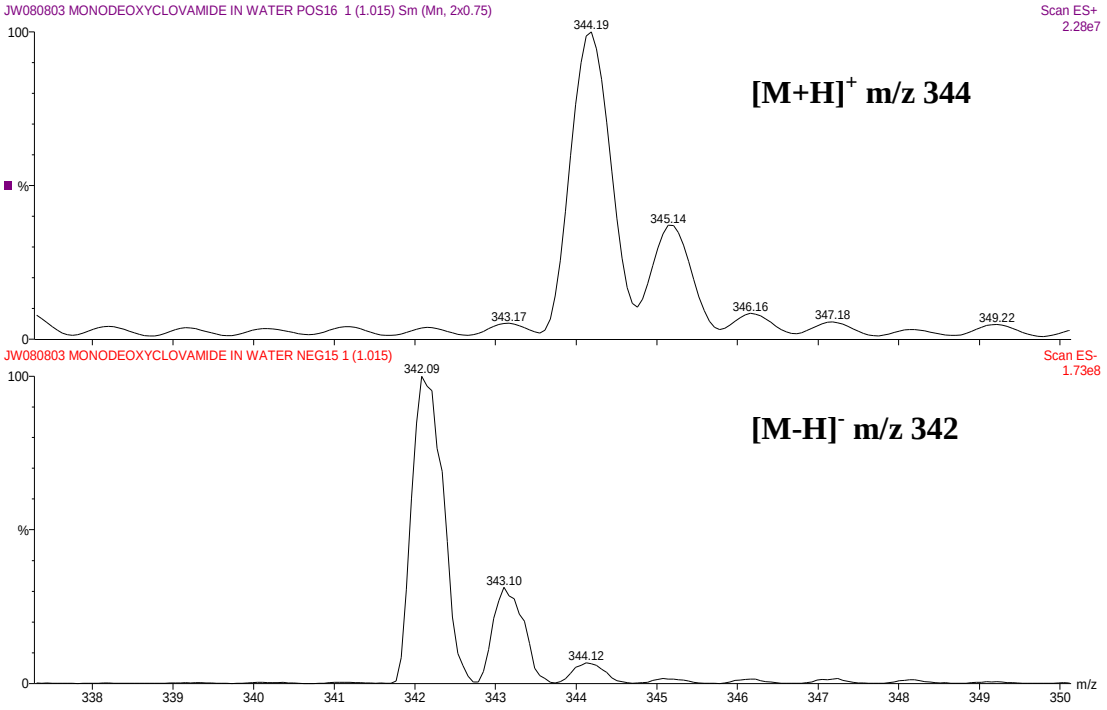


Figure 3.43a Mass spectra of monodeoxyclovamides (MW 343 Da) in water-methanol (1:1 v/v) in positive ion mode (top) and negative ion mode (bottom).

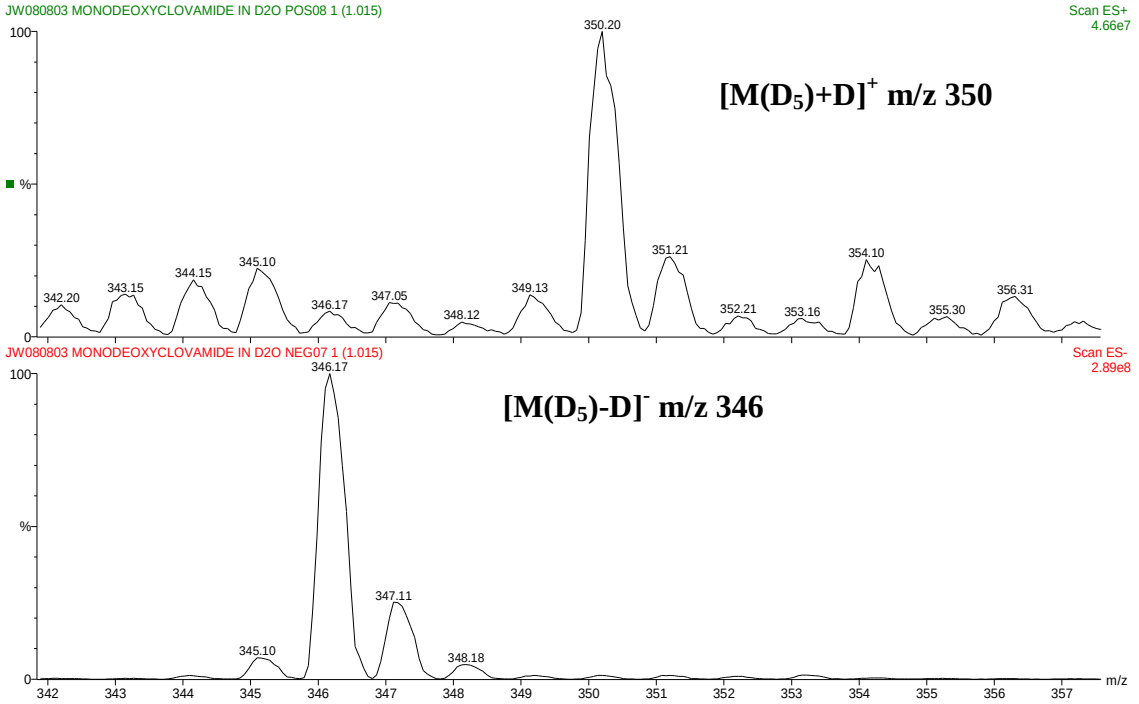


Figure 3.43b Mass spectra of monodeoxyclovamides after H/D exchange (MW 348 Da) in D_2O-methanol-d_4 (1:1 v/v) in positive ion mode (top) and negative ion mode (bottom).

These results suggest that monodeoxyclovamides contain five exchangeable protons being the three protons in the hydroxyl-groups (-OH) of the two aromatic rings, the proton of the carboxy-group (-COOH) and the proton of the amide bridge (-NH-). H/D-MS experiments performed on isolated cocoa clovamide and dideoxyclovamide showed six and four exchangeable protons, respectively, corresponding to the additional hydroxyl-group of the former and the lack of one hydroxyl-group in the latter (data not shown).

In order to get additional structural information, CID-MS-MS analyses on all isolated cocoa hydroxycinnamic acid amides were carried out. Figures 3.44a and 3.44b show CID-MS-MS spectra in the positive and negative ion mode, respectively, which were obtained for the isolated fraction presumably containing the mixture of two monodeoxyclovamide isomers (caffeoyltyrosine and p-coumaroyl-DOPA) resuspended in either aqueous phase or in deuterated solvent.

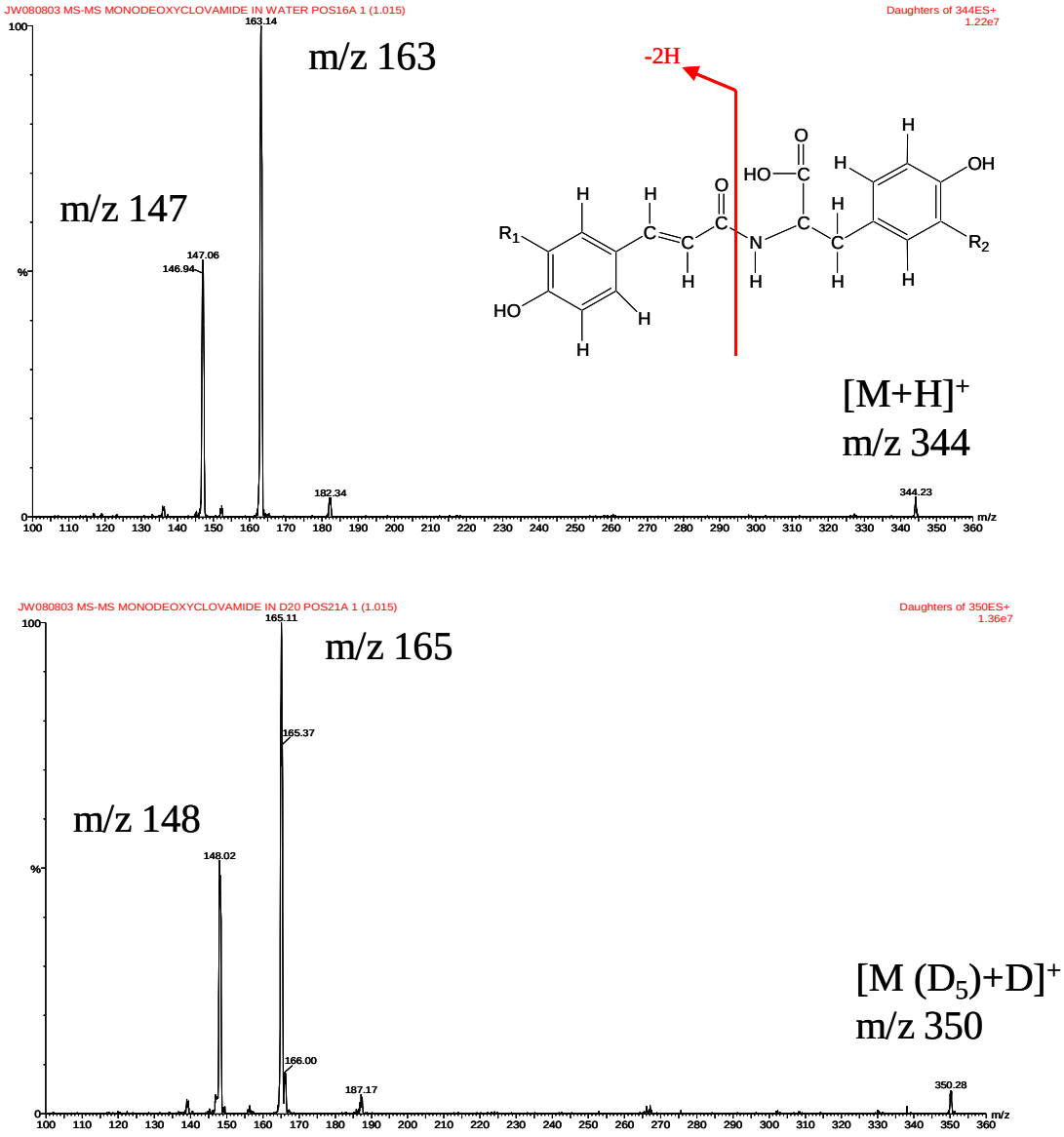


Figure 3.44a CID-MS-MS spectra in the positive ion mode of monodeoxyclovamides (MW 343 Da) in water-methanol (1:1 v/v) (top) and in D_2O-methanol-d_4 (1:1 v/v) (bottom).

The positive ion mode (figure 3.44a) shows very clearly a neutral loss of tyrosine or DOPA, respectively. In addition, from the fragmentation after H/D exchange, it can be deduced that the accompanied H rearrangement involves the movement of the proton from the hydroxyl group of the caffeoyl- or p-coumaroyl rest towards the aromatic amino acid residue. This can be seen from the neutral loss of tyrosine (181 Da) leading to m/z 163 in aqueous phase and tyrosine-d_4 (185 Da) leading to m/z 165 after H/D

exchange, as well as loss of DOPA (197 Da) for m/z 147 and DOPA-d_5 (202 Da) for m/z 148, respectively.

In contrast to the positive ion mode the negative ion mode shows a much more complex product ion spectra (figure 3.44b).

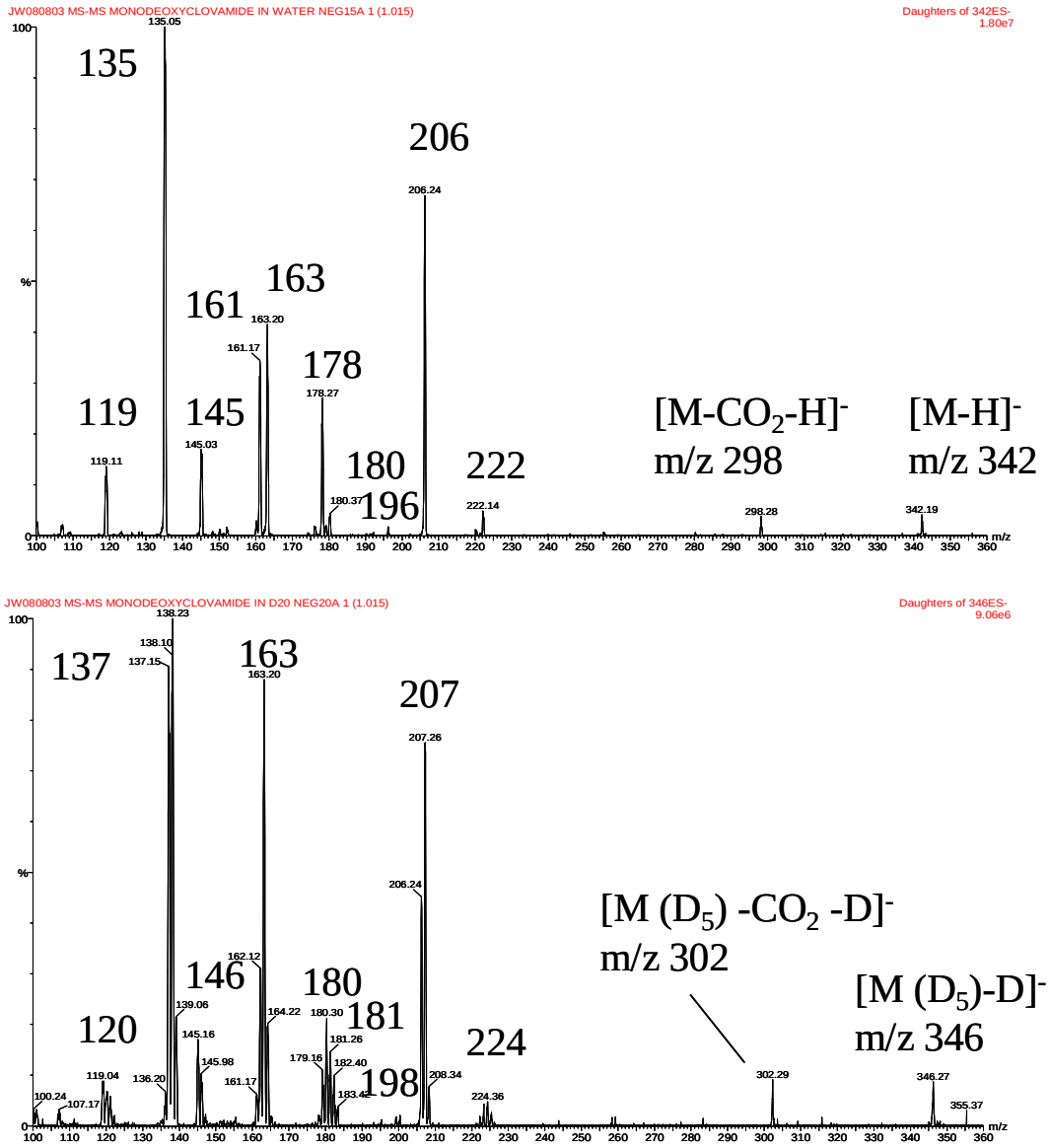


Figure 3.44b CID-MS-MS spectra in the negative ion mode of monodeoxyclovamides (MW 343 Da) in water-methanol (1:1 v/v) (top) and in D_2O-methanol-d_4 (1:1 v/v) (bottom).

The CID-MS-MS spectrum in the negative ion mode obtained in this part of the study differs somewhat from the one obtained in chapter 3.2.2.2 for the presumed monodeoxyclovamides in the HPLC-MS-MS analysis of cocoa liquor extract (figure 3.30b). In particular, the ion intensities of product ions with lower masses are higher in figure 3.44b and some more of such lower mass product ions appear. This is believed to be due to higher collision energy applied on the isolated monodeoxyclovamide fraction here resulting in fragmentation of more stable bonds as well as further multiple fragmentations of higher mass product ions before reaching the ion detector. In addition, experiments before and after H/D exchange were carried out on a triple quadrupole mass analyser due to the unavailability of the ion trap analyser used for the previously described studies (chapters 3.2.1 and 3.2.2).

Nevertheless, through H/D exchange on isolated cocoa monodeoxyclovamides, loss of CO_2 from the deprotonated (dedeuterated) ion as proposed earlier during HPLC-MS-MS of cocoa liquor extract (section 3.2.2.2 and figure 3.30b) can be easily confirmed. Moreover, the second postulated fragmentation mechanism leading to product ions of m/z 206 and m/z 135 for caffeoyltyrosine as well as m/z 222 and m/z 119 for p-coumaroyl-DOPA was also confirmed by the performed H/D-MS-MS experiments in this part of the work.

Fragmentation patterns in deuterated solvent suggest – analogous to the reflections made for the positive ion mode – a H rearrangement from the aromatic amino acid residue towards the phenolic acid part of the molecule involving a labile proton, probably being the proton from the amide bridge (-NH-).

An additional fragmentation pathway is proposed from the CID-MS-MS spectra in figure 3.44b leading to the product ions m/z 161 and m/z 180 for caffeoyltyrosine as well as m/z 145 and m/z 196 for p-coumaroyl-DOPA and is shown together with the aforementioned mechanisms in figure 3.45. This fragmentation is in principle analogous to the one identified for the positive ion mode, however, the H rearrangement appears to involve a move from the phenolic acid towards the amino acid side, whereas it was the opposite in the positive ion mode.

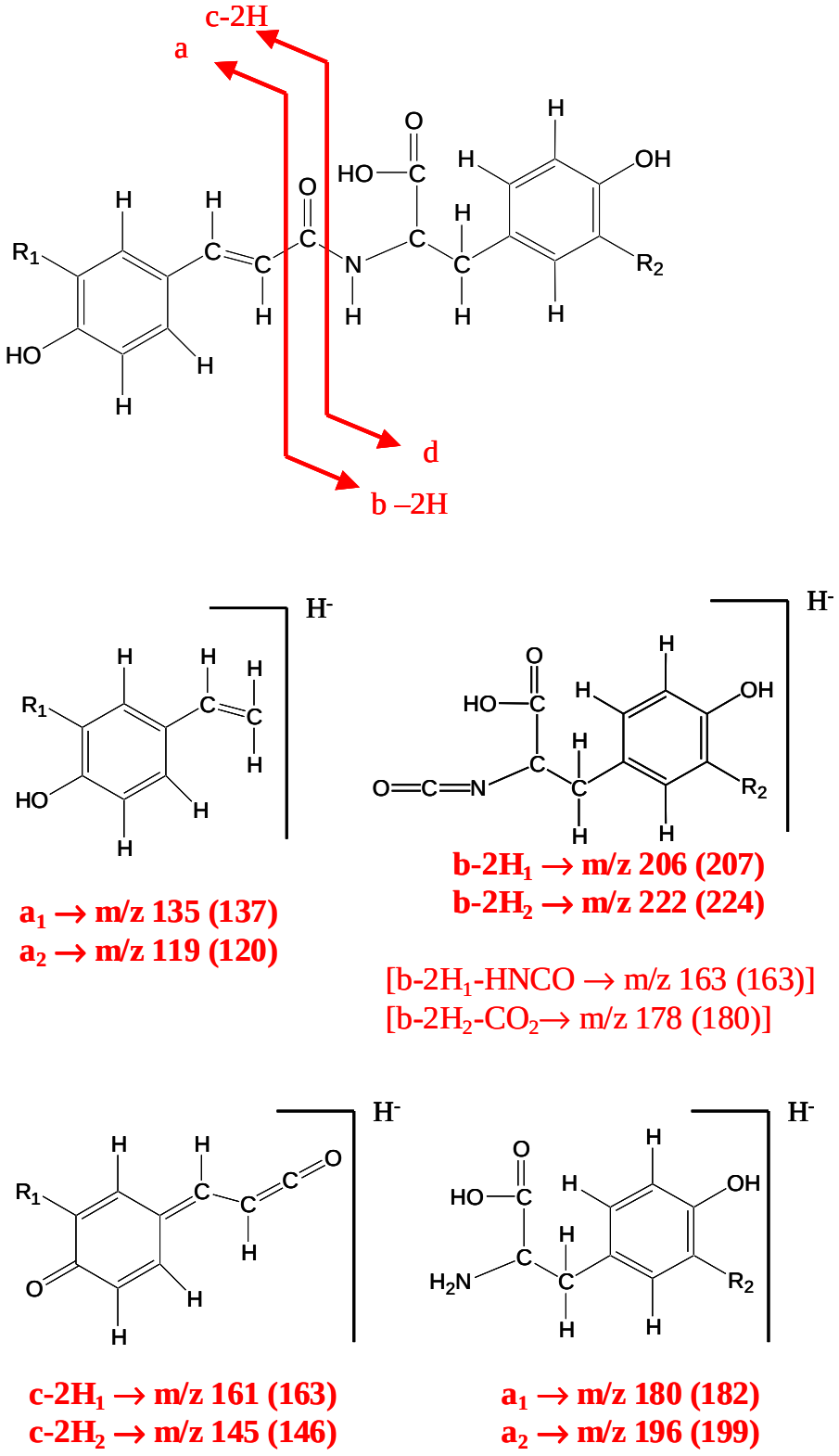


Figure 3.45 Proposed fragmentation pathways for monodeoxyclovamides (MW 343 Da) in CID-MS-MS experiments in the negative ion mode – m/z of product ions in deuterated solvent are given in brackets, subscript 1 corresponds to product ions of caffeoyltyrosine, subscript 2 of p-coumaroyl-DOPA.

Finally, there were two more significant product ions at m/z 163 and m/z 178 observed in the CID-MS-MS experiments carried out in the aqueous phase. Hypothetically, these ions can derive from various fragmentation pathways, both directly from the parent ion or by further decomposition of higher mass product ions. Further loss of HNCO of m/z 206 in aqueous phase (and DNCO of m/z 207 in deuterated solvent) could explain the increase in m/z 163 in deuterated solvent as it adds to the m/z 163 discussed above (m/z 161 in aqueous solvent). Further loss of CO_2 of m/z 222 would explain m/z 178 and additional evidence is gained after H/D exchange (m/z 224 $\rightarrow$ m/z 180). However, if these suggestions were true it would be surprising that CO_2 loss and loss of HNCO can be only observed in one of the related product ions and not in the respective other. More information from either multiple stages CID experiments in an ion trap analyser or exact mass determination of product ions and neutral losses by means of a Q-TOF hybrid instrument would be necessary to reveal the origin of these fragments.

Furthermore, the appearance of a product ion of m/z 138 in deuterated solvent cannot be explained from the proposed fragmentation mechanisms. If it corresponds to m/z 135 in aqueous solvent this product ion contains 4 deuterons after H/D exchange and subsequent fragmentation. Wan et al. (2001) observed movement of non-exchangeable hydrogen atoms in oligonucleotides upon fragmentation in a mass analyser if they are involved in rearrangements. This can also result in product ions with only partially H/D exchange as can be observed for several product ions in figure 3.44b including m/z 224, m/z 180, or m/z 163. Reed and Kass (2001) suggest that many H/D exchange experiments using mass spectrometry could be in error and thus particular care must be taken when interpreting CID-MS-MS spectra after H/D exchange.

H/D-MS and H/D-MS-MS experiments were further performed on the isolated cocoa procyanidin dimer and trimer fractions as described for the hydroxycinnamic acid amides. H/D-MS evidenced 11 exchangeable protons for the dimer (data not shown) and 17 for the trimer in each negative and positive mode (Figures 3.46a and 3.46b).

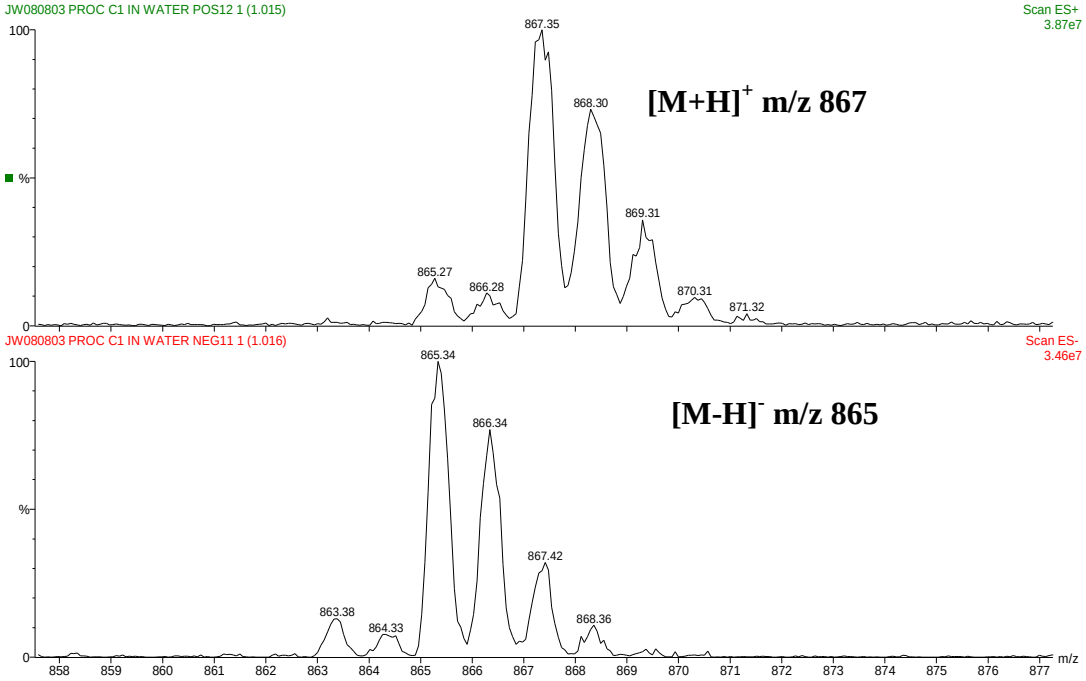


Figure 3.46a Mass spectra of procyanidin trimer C1 (MW 866 Da) in water-methanol (1:1 v/v) in positive ion mode (top) and negative ion mode (bottom).

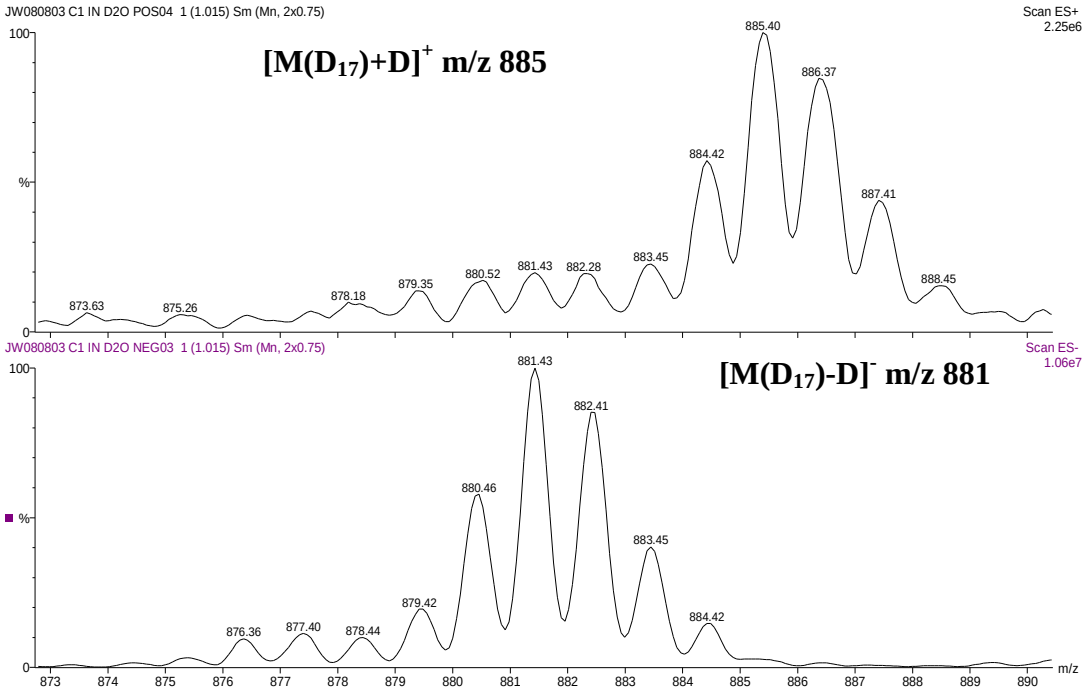


Figure 3.46b Mass spectra of procyanidin trimer C1 after back-exchange (MW 883 Da) in D_2O-methanol-d_4 (1:1 v/v) in positive ion mode (top) and negative ion mode (bottom).

These results were surprising as the dimer contains 10 and the trimer 15 hydroxyl-groups. Hence, the dimer showed H/D exchange of one and the trimer of two additional protons at sites normally considered non-labile, which therefore was not anticipated. Nevertheless, H/D exchange of the methyl groups on a pyrimidine ring of sulfonamides has been reported before (Smith et al., 2000) as well as H/D exchange at non-labile sites of aromatic and aliphatic carbons (Reed and Kass, 2001).

In order to extend on these observations H/D-MS experiments were performed on a commercially available authentic standard of monomeric catechin. As expected for this substance, the experiments evidenced five exchangeable protons, which correspond to the five hydroxyl-groups of the molecule, but no additional exchangeable proton.

Apparently, these data suggest that the proton on the C4-atom becomes sufficiently labile for H/D exchange through the interflavan bondage to the lower C8-atom of an aromatic ring with two hydroxyl-groups.

CID-MS-MS experiments on catechin, procyanidin dimer B2 and trimer C1 both in aqueous as well as deuterated solvent have been carried out in order to further investigate on this hypothesis. The postulated fragmentation mechanisms from HPLC-MS of cocoa liquor extract as discussed in section 3.2.1 were confirmed by the H/D-MS-MS experiments carried out in this part of the work. However, from these data no information could be extracted for the understanding of the location of the one or two additionally exchangable protons.

In contrast to the simplicity in gathering additional structure information on labile protons by H/D-MS for hydroxycinnamic acid amides, such facile determination of number of hydroxyl-groups was not possible for procyanidins. Nevertheless, the additional incorporation of deuterium into procyanidins could - when controlled - offer new strategies to study structure-specific H/D exchange, e.g., between 4β→8 and 4β→6 bound compounds, or between single and doubly linked procyanidins as well as better understanding of fragmentation mechanisms in mass spectrometry.

3.2.4 Phenolic compounds suggested in literature but not confirmed

Very recently, Hatano et al. (2002) and Sanchez-Rabaneda et al. (2003) have suggested the presence of additional polyphenols in cocoa liquor and cocoa powder, including free naringenin, luteolin, apigenin and these flavonoids linked to glycosides as well as epicatechin-8-C-β-D-galactoside.

Since the presence of the abovementioned substances in cocoa were proposed at a time, where the HPLC-MS instrumentation was no longer available for performing additional experiments, *a posteriori* analyses of the recorded HPLC-MS data were performed in this stage of the work. These records contain full scan mass spectra over the whole time of the chromatogram for ions with mass-to-charge ratios of 250-2200, and thus, mass chromatograms were reconstructed from these spectra for ions corresponding to deprotonated or protonated ions of the proposed phenolic compounds in the same way as for the cocoa polyphenols descroibed in chapters 3.2.1 and 3.2.2.

In fact, exploring on mass chromatograms for ions corresponding to deprotonated ions of the three flavonoids (naringenin, luteolin, apigenin) as well as their respective galactosides and arabinosides revealed the possible occurrence of some of these substances in cocoa liquor extract. However, ion intensities are rather low compared to the quercetin compounds described in section 3.2.2.1 suggesting the occurrence of only trace amounts of the observed substances. Moreover, chromatographic conditions used in the present work (described in chapter 2.1.6) differed significantly from the conditions applied by Sanchez-Rabaneda et al. (2003) making a comparison of retention times for gathering additional evidence for the presence of these substances in the cocoa liquor extract used within this work impossible. Consequently, it was not possible by these *a posteriori* analyses performed here to judge with sufficient confidence on the presence of the suggested compounds in cocoa.

In contrast, experimental HPLC-MS studies were carried out during the exploratory studies in this work on deprotonated ions having mass-to-charge ratios of 451 and 421 corresponding to (epi)catechin-galactoside and (epi)catechin arabinoside, respectively, since these ions were observed at intensities suggesting significant amounts of the corresponding compounds in cocoa liquor extract. Analogous to the studies on quercetin-glycosides and A-type procyanidin glycosides (chapters 3.2.2.1 and 3.2.2.3),

CID-MS-MS experiments were performed on those ions searching for a product ion corresponding to deprotonated (epi)catechin after neutral loss of the sugar residue. In fact, both m/z 289 corresponding to deprotonated (epi)catechin and m/z 245, a major fragment ion of deprotonated (epi)catechin (section 3.2.1), were observed for both parent ions (figure 3.47).

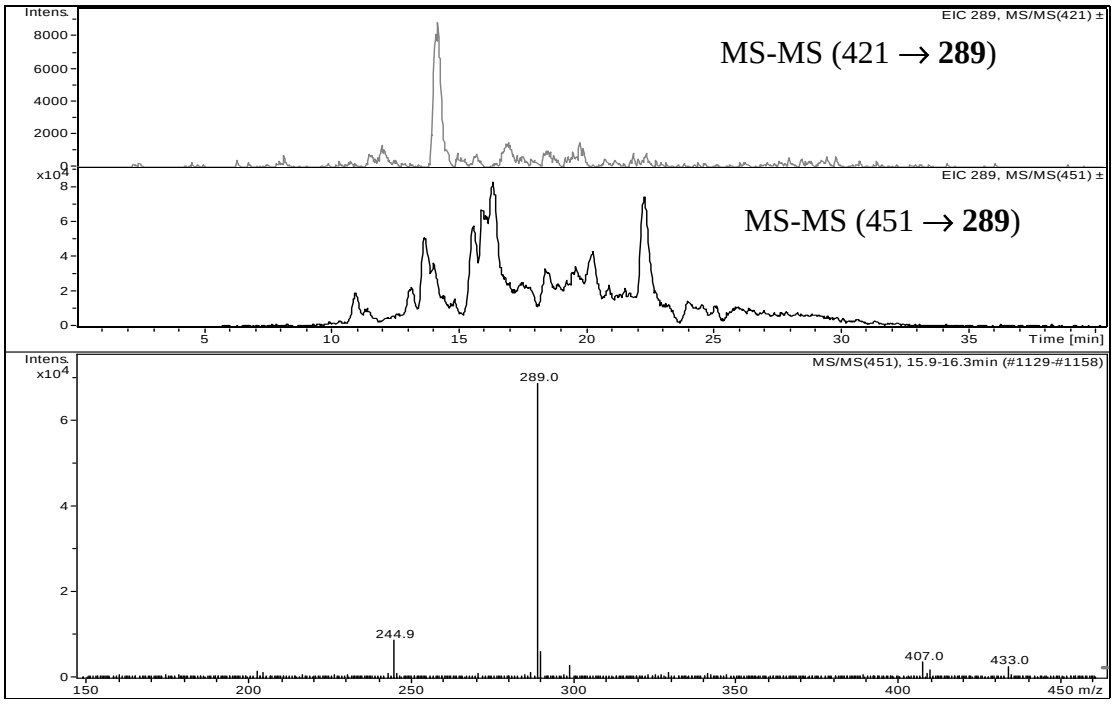


Figure 3.47 Reconstructed MS-MS mass chromatograms (top) of presumable (epi)catechin-arabinoside (m/z 421 → 289) and -galactoside (m/z 451 → 289), respectively, as well as the CID-MS-MS spectrum (bottom).

The ion intensity of m/z 421 was approximately 10 times lower than those of m/z 451, however, the presence of several peaks over the main chromatographic region even for the selective detection of MS-MS (m/z 451 → 289) is very surprising (figure 3.47). Repeated CID of the product ion m/z 289 revealed the typical MS-MS spectrum of epicatechin, strongly suggesting that this daughter ion is deprotonated epicatechin deriving from the parent(s) m/z 451. Nevertheless, the same mass chromatogram result in MS-MS and MS^3 (m/z 451 → 289→ 245) showing several compounds with identical MS and even MS^n properties (figure 3.48).

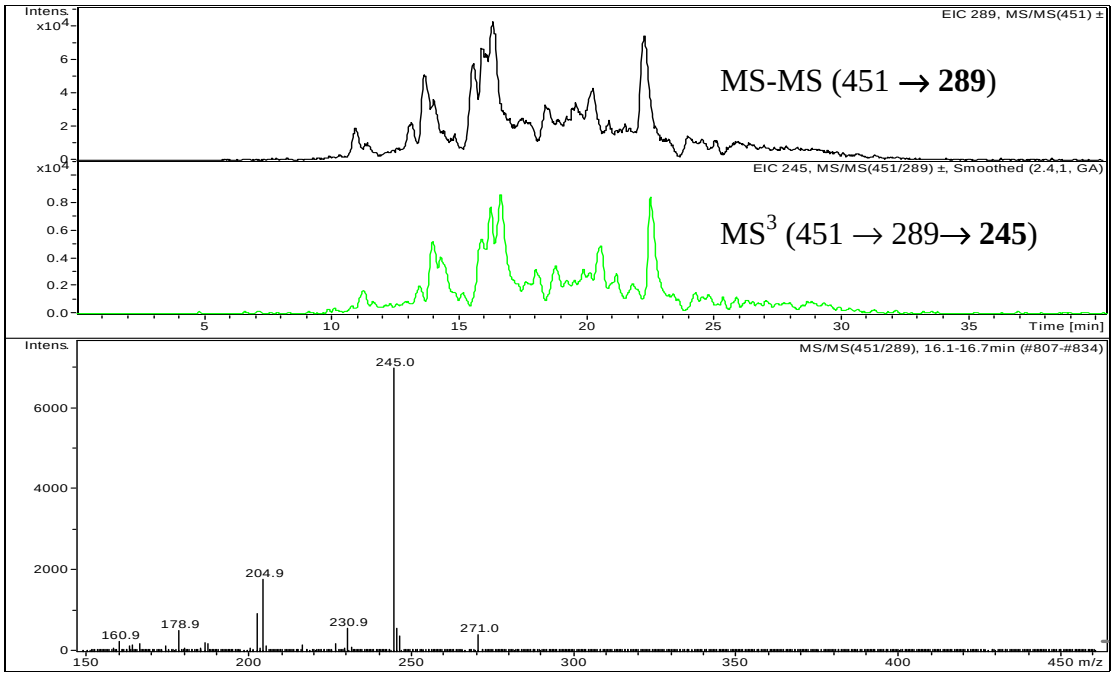


Figure 3.48 Reconstructed mass chromatograms (top) of presumable (epi)catechin-galactoside after CID-MS-MS (m/z 451 → 289) and CID-MS^3 (m/z 451 → 289 → 245), respectively, as well as the CID-MS^3 spectrum of m/z 451 → 289 →) (bottom).

The presence of 10 or more (epi)catechin galactosides or glucosides at significant amounts in cocoa must be seen as very unlikely. Nevertheless, the observed CID fragment of m/z 451→ 289 is very likely to be deprotonated (epi)catechin because of the repeated CID mass spectrum being identical to that of an authentic standard.

A hypothesis was that the parent ion of m/z 451 has its origins in the ion source of the mass spectrometer rather than resulting from deprotonation of several compounds with molecular masses of 452 Da eluting from the column. Therefore, it was explored for a series of substances, which have m/z 451 as common product ion in the negative ion mode and elute at the relevant time of 11 to 23 minutes from the HPLC column.

Re-evaluation of CID-MS-MS spectra of procyanidin dimers and higher oligomers revealed a significant product ion m/z 451 resulting from loss of gallic acid of deprotonated procyanidin dimer (figures 3.15 and 3.16). Higher procyanidin oligomers also show this product ion after CID-MS-MS experiments. Hence, repeated CID experiments of the product ion m/z 577 → 451 of deprotonated procyanidin dimer B2

were carried out in order to investigate for further fragmentation of this ion. In effect, CID-MS^3 spectra showed m/z 577 → 451 → **289** corresponding to deprotonated epicatechin, the identity of which was further evidenced performing CID-MS^4 resulting in 577 → 451 → 289→ (**245 + 205 + 179**), identical to the observed fragmentation patterns of m/z 451 in figures 3.47 and 3.48.

For this reason it was concluded that fragmentation inside the ion source ("in-source CID") of procyanidin oligomers, resulting in several product ions including m/z 451, were the underlying group of compounds leading to the tentative suggestion of the presence of (epi)catechin galactosides or –glucosides in cocoa liquor. In order to prove in-source CID, HPLC-MS analyses under the conditions described in chapter 2.1.6 used throughout the work described in chapter 3.2.1 and 3.2.2 were carried out on a commercially available authentic standard of procyanidin dimer B2, and a full-scan mass spectrum was obtained for the chromatographic region corresponding to the retention time of this compound. Figure 3.49a highlights the relevant part of this mass spectrum showing not only the deprotonated parent ion m/z 577 but also the product ions observed for this ion (m/z 451, 425, 407, 289, and 287) in "*ion-trap-CID*" experiments involving isolation of the parent ion prior to fragmentation, thus indicating *in-source CID* of m/z 577. In contrast, applying "softer" conditions within the electrospray ionisation source, i.e. selecting a smaller accelerating field, avoided in-source CID of deprotonated procyanidin dimer B2 standard in the intermediate pressure region between source and mass analyser and led thus to a mass spectrum consisting almost exclusively of m/z 577, namely deprotonated procyanidin B2 (figure 3.49b).

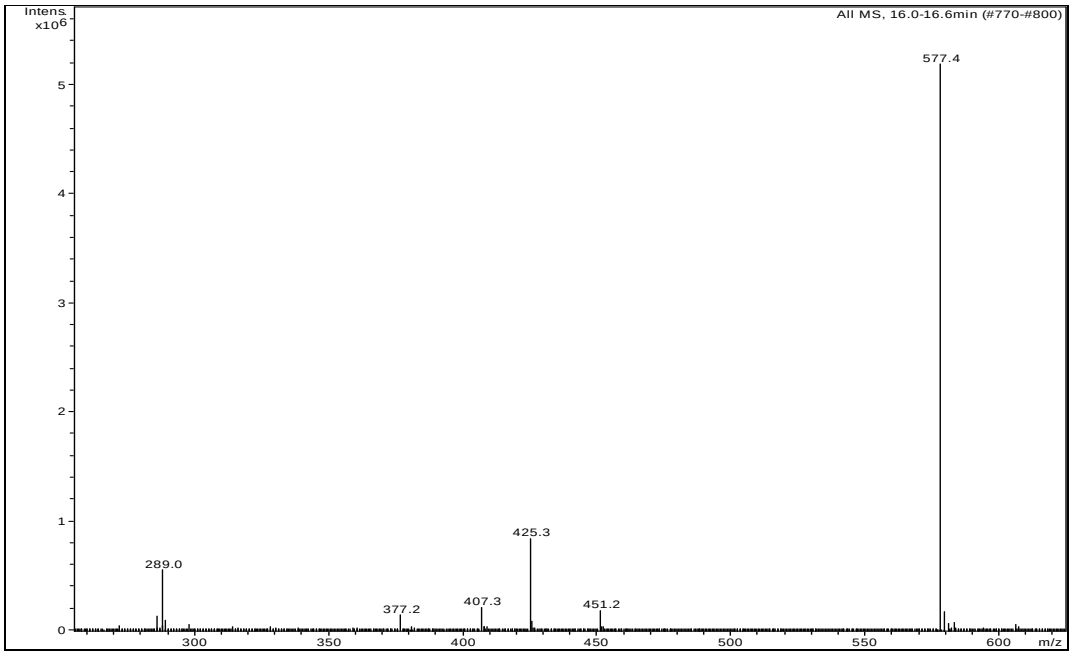


Figure 3.49a Mass spectrum of procyanidin B2 under ion source conditions resulting in in-source CID of deprotonated ion m/z 577**.**

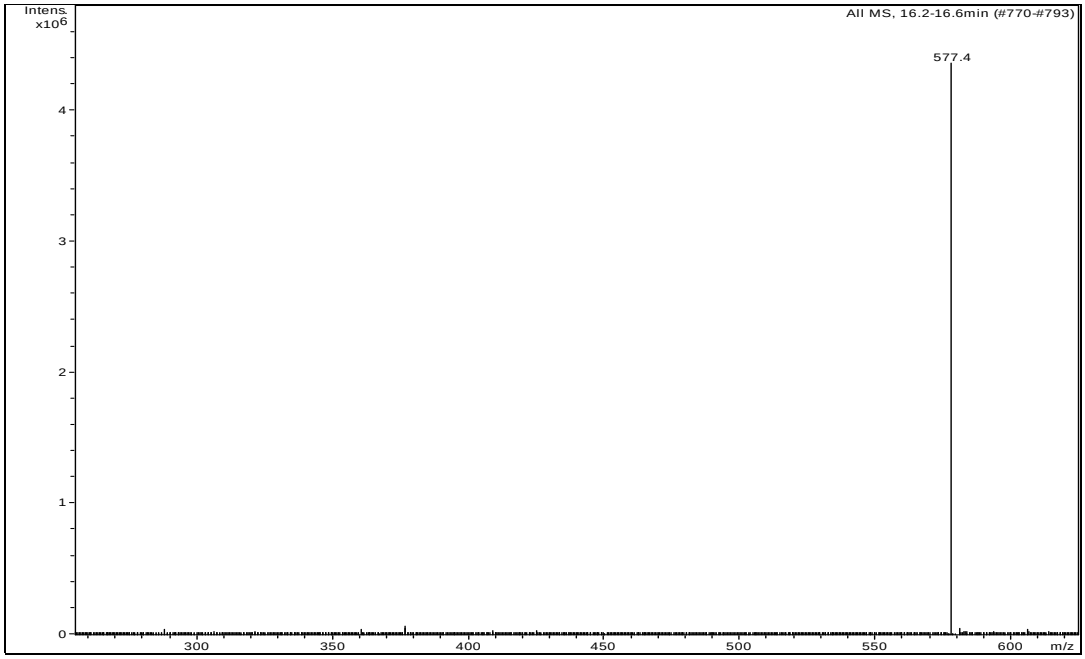


Figure 3.49b Mass spectrum showing the deprotonated ion m/z 577 of procyanidin B2 without fragmentation under “soft” ion source conditions avoiding in-source CID.

Repeating HPLC-MS analysis of cocoa liquor extract, but applying the soft ionisation setting for obviating in-source CID, no ions or only very low intensities of ions with m/z 451 were observed. These low intensities may still more likely derive from remaining but minimal in-source CID of procyanidins rather than from an (epi)catechin galactoside or glucoside. Moreover, obviating in-source CID also resulted in the disappearance of ions with m/z 421 corresponding to putative (epi)catechin arabinoside. Nevertheless, m/z 421 is not seen in the product ion spectra of any procyanidin, and consequently the origin of this ion remains unresolved within this work.

In conclusion, the presence of flavan-glycosides in relevant amounts in cocoa is not suggested, and flavonoid glycosides other than derived from quercetin are considered of quantitative negligible importance.

3.2.5 Summary of identified phenolic compounds in cocoa

26 phenolic compounds including catechins, procyanidins, flavonols, and hydroxycinnamic acid amides have been tentatively identified by HPLC-DAD-ESI-MS. This technique offers the ability to detect and characterise compounds present in small quantities in difficult matrices combining the information on retention time and molecular weight easily deduced from mass-to-charge ratios of protonated and deprotonated ions. Additional structure information can be obtained from CID-MS^n experiments on those ions followed by the interpretation of MS^n mass spectra through logical fragmentation pathways. Nevertheless, interpretation of such spectra must be done carefully, particularly in cases where many structurally very similar or related compounds are present as in the case of cocoa polyphenols in order to avoid premature conclusions

Only 9 of these 26 tentatively identified polyphenols are commercially available for final structure confirmation as well as for use as quantitative reference. Five additional compounds have been successfully isolated, purified and characterised by NMR. Two isomeric monodeoxyclovamides are described **for the first time** in cocoa to the author's knowledge. However, procyanidin tetramers to heptamers as well as A-type procyanidin compounds have not been possible to isolate as pure reference standards within the frame of this work. Table 3.9 summarises the work described in section 3.2.

Table 3.9 Overview on phenolic compounds in chocolate and chocolate raw products identified and/or confirmed in this study.

Name	**Means of identification**			
	Tentatively by means of LC-MS^n (see 3.2.1)	Comparison with authentic standard	Isolated from cocoa & identified by NMR (3.2.3)	Reference for compound described in cocoa previously
Catechin	YES	YES		Jalal and Collin, 1977
Epicatechin	YES	YES		Jalal and Collin, 1977
Gallocatechin	YES	YES		Forsyth, 1955
Epigallocatechin	YES	YES		Forsyth, 1955
Procyanidin B2	YES	YES	YES	Porter et al., 1991;
Procyanidin B5	YES			According to retention on RP-HPLC columns (Rohr, 1999); Porter et al., 1991
Procyanidin C1	YES		YES	Porter et al., 1991;
Procyanidin tetramer to heptamer	YES			Hammerstone et al., 1999;
A-type procyanidin dimers (aglykon, arabinoside, galactoside)	YES			Porter et al., 1991;
A-type procyanidin trimers (aglykon, arabinoside, galactoside)	YES			Hatano et al., 2002
Quercetin	YES	YES		Andres-Lacueva et al. (2000)
Quercetin-arabinoside	YES	YES		Porter et al., 1991;
Quercetin-galactoside	YES	YES		Porter et al., 1991;
Quercetin-glucuronide	YES			Andres-Lacueva et al. (2000)
Clovamide	YES		YES	Sanbonghi et al., 1998
2 monodeoxy-clovamide isomers	YES		YES	NOT described in cocoa so far
Dideoxy-clovamide	YES		YES	Sanbonghi et al., 1998
Chlorogenic acid	YES	YES		Jalal and Collin, 1977

Compounds in bold are of quantitative relevance and have been considered for quantitative studies in chapter 3.4.

3.3 Set-up and validation of methodologies for quantitative analysis

As the objectives in this work included not only identification of polyphenols in cocoa but also determination of their concentration in chocolate and chocolate raw products used in chocolate making, including fermented cocoa beans, cocoa nibs, and cocoa liquor, it was the objective in this part to set up the HPLC-MS based methodology used in chapters 3.2.1 and 3.2.2 for quantification of cocoa polyphenols. This included in-house validation comprising the examination of repeatability, recovery, linearity and range of application of the method as well as the participation in a proficiency testing exercise for the determination of catechin and epicatechin in baking chocolate. The Folin-Ciocalteu assay (chapter 3.1.1) was applied as fast and simple estimation of total polyphenols for optimising the extraction solvent and procedure, respectively.

3.3.1 Sample extraction and sample preparation

Prior to the instrumental analysis of polyphenols these compounds had to be extracted from the cocoa matrix. The first step in analysis of cocoa beans, cocoa nibs, cocoa liquor, and chocolate was to grind the raw material in order to allow a better contact of the extracting solvent with the sample and to ensure that the extracted portion is representative for the entire sample. As heat is developed during grinding due to shear forces, liquid nitrogen was used for keeping the temperature low avoiding the liquefying of cocoa butter and preserving polyphenols from risk of degradation. Subsequently, cocoa butter was extracted twice with hexane in an ultrasonic bath followed by centrifugation resulting in virtually fat-free cocoa solid residues.

Generally, the extraction of polyphenols from cocoa and other food or plant matrices is achieved using either solely water, aqueous methanol, ethanol or acetone, respectively, with up to 95% organic solvent as well as pure methanol with some mechanical treatment, such as ultrasonication, maceration or other means of homogenisation with the extraction solvent (Wollgast and Anklam, 2000a).

Thus, the objective in this part of the work was to compare and choose the extraction solvent system in order to maximise the extraction of polyphenols from the various cocoa matrices. In addition, for the strongest solvent system, the extraction procedure

was to be optimised in terms of ratio of solvent to cocoa sample and extraction frequency, respectively.

Aqueous acetone, methanol, and ethanol at 3 different amounts of organic solvent as well as the addition of 1% acetic acid for improving polyphenol solubility and stability have been systematically studied. Extraction for each of the 12 conditions was carried out in triplicate on defatted cocoa liquor and the polyphenol content in the extract was determined in duplicate by the Folin-Ciocalteu assay. In addition, extracts were assayed qualitatively HPLC-ESI-MS as described in chapters 3.2.1 and 3.2.2.

Results were subjected to analysis of variance (ANOVA) and multiple comparisons of means (post hoc tests). However, data could not be used untransformed for statistical analyses due to the violation of the assumption of variance homogeneity as can be demonstrated by plotting the observed standard deviations against the mean concentration showing a proportional increase of variance (or standard deviation) with increased concentration (figure 3.50a).

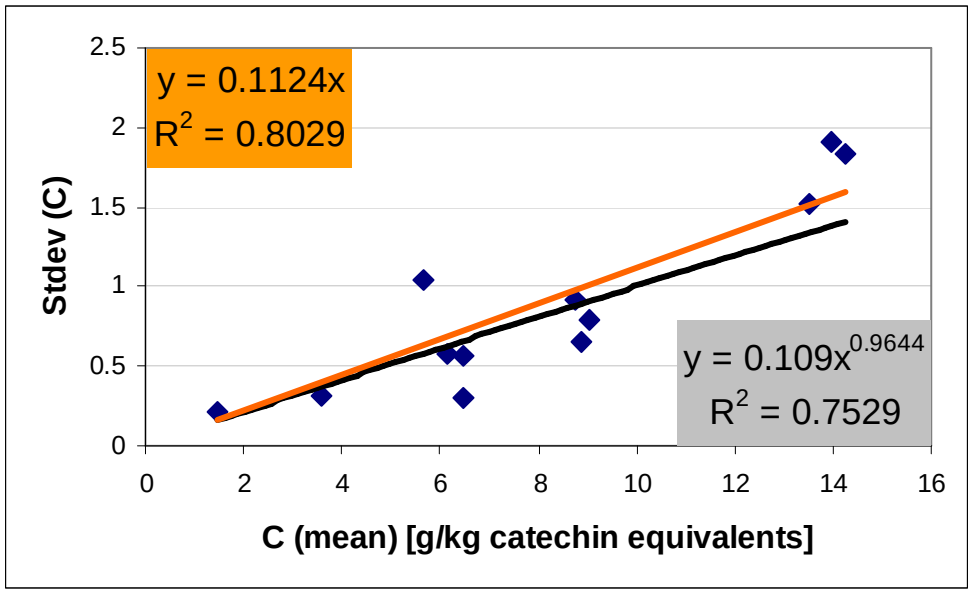


Figure 3.50a Mean total cocoa polyphenol concentration against standard deviation of six replicate determinations at different concentrations ranging from 1.5 to 14.2 g/kg – method precision clearly shows concentration dependence (black line represents power-, orange line linear-curve fitting, respectively).

This phenomenon is widely recognised in analytical chemistry, and the empirically derived Horwitz function ($\sigma H = 0.02c^{0.85}$) describes this phenomenon for the compound concentration dependence of the reproducibility standard deviation in intercollaoborative trials for a wide concentration range from 0.138 to $1.2*10^{-7}$ expressed in dimensionless mass ratios (Thompson, 2000).

However, in practice for concentration ranges over one or two magnitudes (or at concentration below 120 ppb), the relationship is often close to linear instead of an exponential relationship, i.e. the power is close to 1, here being 0.9644 (figure 3.50a). Consequently, the relation between standard deviation and mean concentration becomes linear (s = a*c) and the relative standard deviation (RSD) becomes unaffected by different concentrations, i.e. constant (RSD = s/c = a). If the original data can be transformed into a new variable in such a way that the standard deviation of the new variable is equivalent to the RSD of the original data and thus, a constant, more powerful parametric statistical analyses, such as ANOVA and multiple comparisons of means would be possible to apply. This criterion of transformation is fulfilled by natural logarithm of original data: $c_{new} = \ln(c)$. The standard deviation of c_{new} is calculated from the variance of c by substituting this expression into the law of error propagation receiving the following equation:

$$s_{cnew} = \sqrt{s_c^2 \cdot (d\ln(c)/dc)^2}$$

As the derivative of ln(c) is 1/c this equation can be simplified to

$$s_{cnew} = \sqrt{s_c^2 \cdot (1/c)^2}$$

which is equivalent to

$$s_{cnew} = s_c / c$$

which is equivalent to RSD of the original mean concentration as required.

Figure 3.50b demonstrates graphically the independence of the standard deviation of the new variable ($s_{c(new)}$) from the new variable itself (c_{new}).

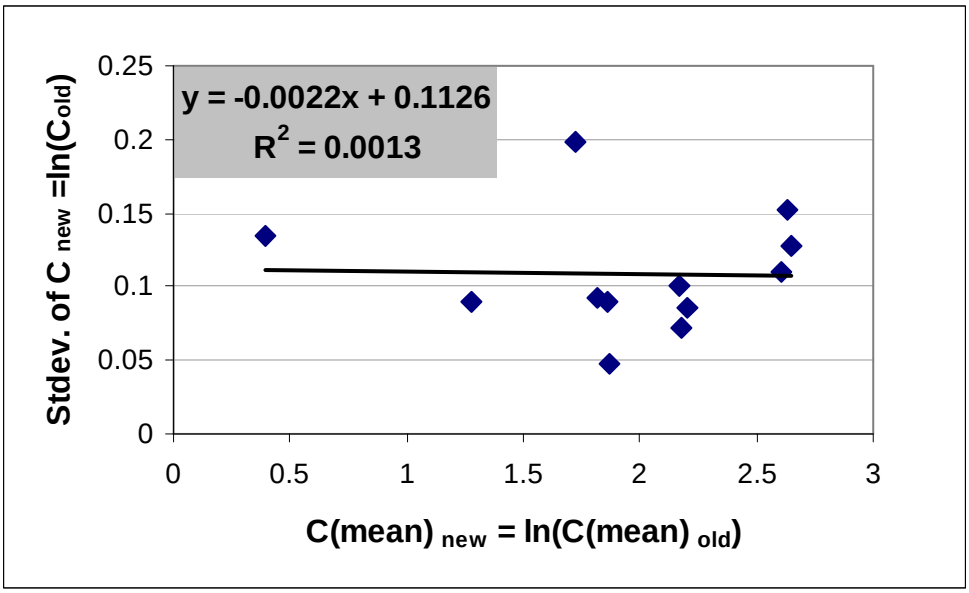


Figure 3.50b Mean against standard deviation - situation after data transformation (C_{new} = ln(C_{old}): standard deviation is independent and constant over the concentration range fulfilling the assumption of variance homogeneity for commonly used statistical analyses.

Therefore, prior to statistical data analysis in this part of the study as well as in subsequently described statistical data analysis of this work, original data of mean concentrations were transformed through natural logarithm.

A convenient side effect of this transformation is that the precision expressed as RSD of a method can be directly derived from the square root of the "mean of squares" of the error (MS_{error}) in an ANOVA analysis of transformed data. In fact, the precision of the Folin-Ciocalteu assay determined in this way for the performed analyses of 36 extracts in duplicate was approximately 11%, which is in good agreement with the estimation of precision for this assay in cocoa liquor samples in section 3.1.1 (10%).

Figure 3.51 shows the result of total polyphenols concentration as determined by the Folin-Ciocalteu method in cocoa liquor extracted with 12 different solvent systems.

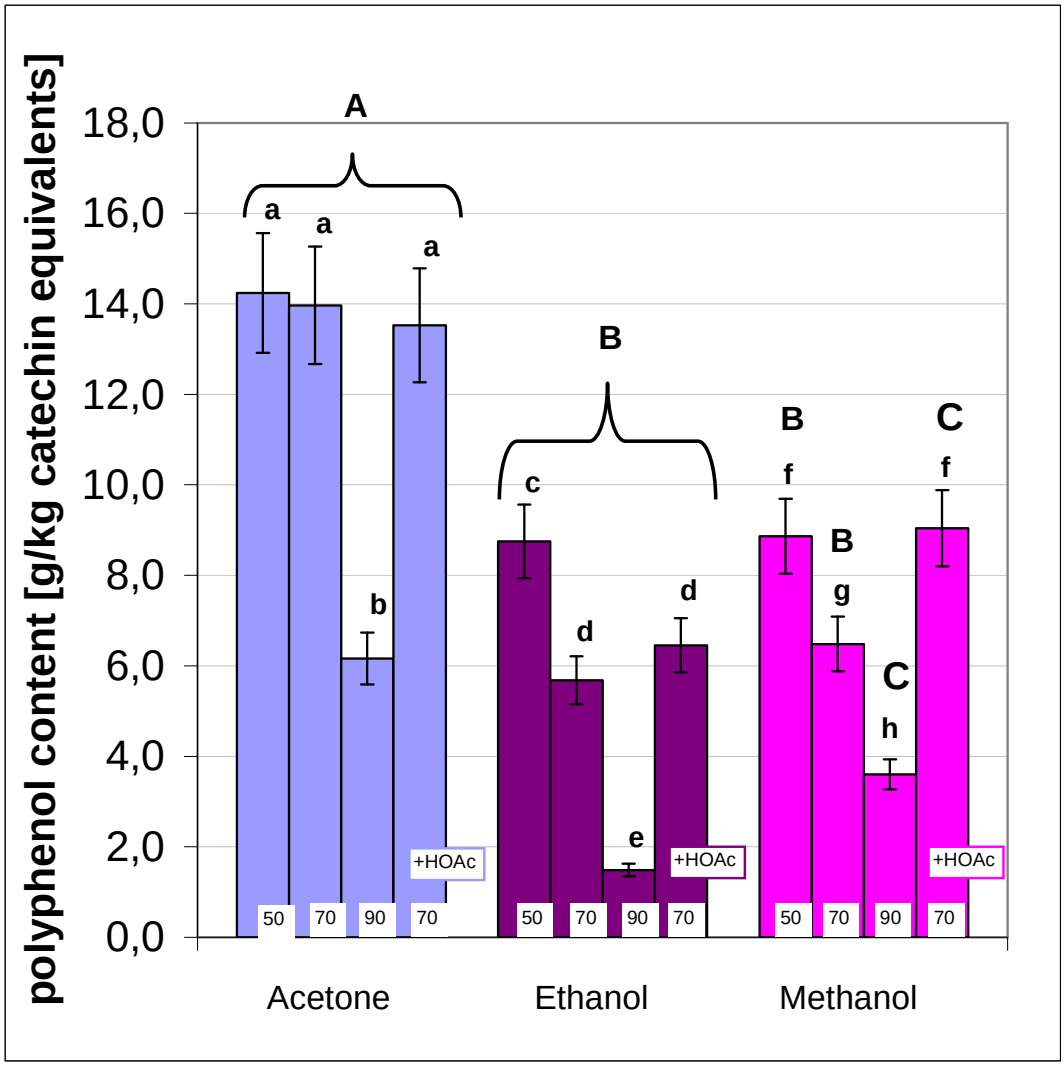


Figure 3.51 Comparison of extraction solvents for the extraction of polyphenols from cocoa liquor as determined using the Folin-Ciocalteu method – concentrations are means of 6 values, error bars represent the expanded uncertainty of the mean values with a coverage factor of 2 corresponding to a confidence level of 95%. Concentrations having different letters are significantly different ($p < 0.05$), small letters compare levels of organic solvent within on type and capital letters compare different organic solvents.

Aqueous acetone in concentrations between 50 and 70% organic solvent in the presence or absence of 1% acetic acid was shown to extract the highest amount of polyphenols as determined by the Folin-Ciocalteu assay and monitored by HPLC-ESI-MS, which confirmed the highest amount of oligomeric procyanidins with this extraction solvent

(figure 3.51). At slightly acidic conditions polyphenols are more stable and there is little risk of precipitation of higher oligomers from the extraction solution (Rohr, 1999). Thus, aqueous acetone at 70% with 1% acetic acid was considered the best solvent systems for extraction in subsequent studies.

In the second evaluation step, the minimum amount of solvent per gram of cocoa liquor was to be determined for efficient extraction without unnecessary wastage of solvent. Additionally, it was evaluated whether a single extraction step would suffice for efficient extraction of polyphenols from the cocoa matrix in order to keep solvent use and sample extraction time as low as possible. Extraction time in the ultrasonic bath was kept constant at 10 minutes as preliminary trials have shown no further improvement with longer extraction times.

Six combinations of ratios of solvent volume to sample mass (5, 10, and 20 ml per gram defatted cocoa liquor) as well as single or duplicate extraction were examined and the results are presented in figure 3.52.

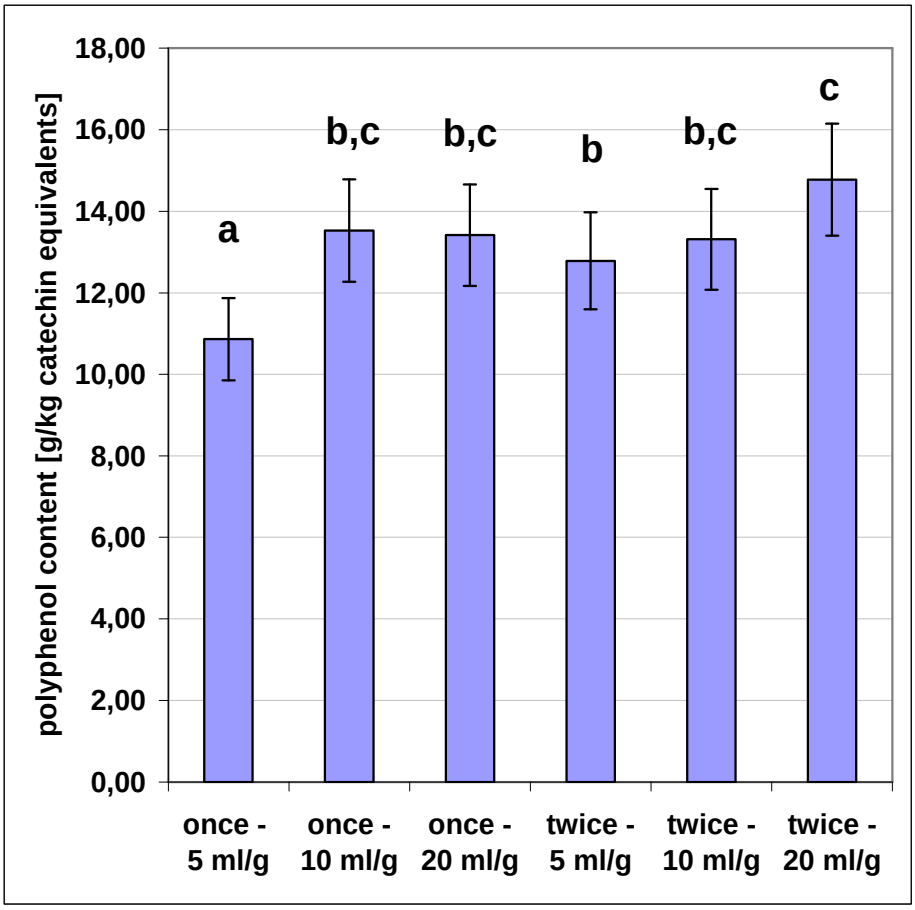


Figure 3.52 Comparison of extraction efficiency of single or double extraction at different sample-extraction solvent ratios for the extraction of polyphenols from cocoa liquor by acidified aqueous acetone (acetone-water–acetic acid 69.5:29.5:1 v/v/v) as determined using the Folin-Ciocalteu method – concentrations are means of 6 values, error bars represent expanded uncertainty of the mean values with a coverage factor of 2 corresponding to a confidence level of 95%. Concentrations having different letters are significantly different ($p < 0.05$).

A single-step extraction of 1 gram of defatted cocoa liquor with 10 ml of extraction solvent (acetone-water–acetic acid 69.5:29.5:1 v/v/v) has shown no inferior efficiency compared to higher amounts of solvent or a second extraction step, respectively (figure 3.52). Thus, for further method validation as well as quantitative studies a sample extraction procedure including grinding, defatting with hexane (twice) and a single extraction of polyphenols from defatted cocoa by acidified aqueous acetone was exerted as described in chapter 2.3.2.

3.3.2 High-performance liquid chromatography coupled to electrospray ionisation-mass spectrometry as quantitative method of analysis for polyphenols in chocolate and chocolate raw products

The objective here was to further develop the HPLC-ESI-MS method used for qualitative analysis (chapter 3.2) as a tool for quantitative analysis of polyphenols in chocolate and chocolate raw products. 22 in section 3.2 identified as quantitatively relevant polyphenols have been selected with the aim to determine all of their concentrations with a single method of sample extraction and preparation as well as a single chromatographic run and quantitation by mass spectrometric detection.

After having demonstrated specificity of HPLC-MS for each of the cocoa polyphenols through separation and unambiguous detection (described in section 3.2), the method was applied under identical chromatographic and optimised mass spectrometric detection conditions (negative ion mode, soft ionisation conditions) as described in chapter 2.3.3. This method was then validated in an in-house procedure covering linearity, recovery, range of application, and repeatability. These four parameters present the core characteristics that at least have to be examined before a quantitative analytical method should be applied to a study of real samples (Green, 1996; Bressolle et al., 1996; Causon, 1997).

3.3.2.1 Selection of best suited mass spectrometric detection technique

For mass spectrometric detection two MS-systems were available: a single quadrupole mass analyser equipped with an ESI-source with the spray oriented horizontally towards the sampling orfice as common in earlier LC-MS systems (described in section 2.3.3.1) as well as an ion trap mass analyser equipped with an orthogonal spray ESI-source, which better supports high liquid flow rates and coeluting not ionised matrix compounds (described in section 2.3.3.2).

The former analyser may be used in scan mode, however selective ion monitoring (SIM) is generally applied with quadrupole MS systems for better sensitivity because, very few ions compared to a scan over a wider mass range have to be isolated and detected one after the other. In contrast, the ion trap mass spectrometer in the scan mode gives almost no loss of sensitivity compared to the SIM mode due to the simultaneous

collection of ions in the ion trap prior to much quicker selective ejection of ions for detection. Thus, an ion trap analyser retains all information on compounds that are ionised for *a posteriori* control of unexpected chromatographic events or coeluting compounds without compromising much method sensitivity.

Alternatively, mass spectrometric detection with an ion trap system may be performed in the more selective MS-MS mode. The use of mass chromatograms of unique fragment ions subsequent to CID-MS-MS of the parent ions of eluting analytes significantly reduces background noise due to coeluting matrix compounds. Comparable to the SIM mode of a quadrupole analyser (or the multiple reaction mode MRM of a triple quadrupole system), this enhances sensitivity of the analysis for the cost of lost information of unknown or unpredicted events.

In order to select the appropriate mass spectrometric detection for the objectives of the present work including the quantitative analyses of polyphenols in chocolate and chocolate raw products, SIM with the single quadrupole analyser as well as full-scan MS analysis and MS-MS detection of compound specific product ions were compared in a pre-validation by evaluation of recovery of the detection of an internal standard (taxifolin) and precision of 3 selected polyphenols (epicatechin, catechin, and procyanidin B2) in each 12 repeated analyses of aliquots of a beforehand extracted cocoa liquor sample.

For recovery, a taxifolin standard was injected five times in order to create a reference value and recovery of taxifolin for each of the 12 spiked samples has been calculated. The single quadrupole analyser in the SIM mode gave recoveries of only 76±7% suggesting ion suppression due to cocoa matrix compounds. Both MS full-scan and MS-MS detection with the ion trap analyser showed recoveries of 103±3% and 99±2%, respectively. Statistical analysis comparing the five areas under the curve ion counts of taxifolin standard with those 12 of the spiked cocoa extract using the Student’s t-test revealed no significant differences for the full-scan MS and MS-MS detection, respectively, suggesting the absence of any interferences from matrix compounds in both detection modes.

The reason for this difference between the mass analysers is believed to be due to the orientation of the ESI-source spraying directly onto the orfice of the older quadrupole

mass spectrometer compared to the newer development of orthogonal or Z-oriented spraying systems allowing constant ionisation of compounds in the presence or absence of coeluting matrix rather than due to the differences in ion isolation and detection between the two systems.

Precision for the determination of the three selected cocoa polyphenols were 6-10% for the quadrupole SIM MS detection, 3-5% for the full-scan MS detection as well as 2-7% for MS-MS detection with the ion trap system.

Thus, the ion trap mass spectrometer operating in the negative ion mode recording full-scan MS spectra is selected for further method validation and quantitative analysis, because of the maximum amount of available information together with equal or superior detector accuracy and precision.

3.3.2.2 Validation of the high-performance liquid chromatography-mass spectrometry method for the quantitative analysis of polyphenols in chocolate and chocolate raw products

Method validation is the process of proving that an analytical method is acceptable for its intended purpose (Green, 1996). Generally, validation must include studies on specificity, linearity, trueness (recovery), precision (repeatability), and range of application (Bressolle et al., 1996; Causon, 1997). Further criteria may include limit of detection (LOD), limit of quantitation (LOQ), and robustness of a method.

3.3.2.2.1 Linearity

A linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to concentrations. Preliminary HPLC-MS analyses carried out for polyphenols in cocoa beans, cocoa liquor, dark chocolate and milk chocolate performed in this study showed that polyphenol concentrations range between 1 and 3000 mg/kg depending on the type of cocoa product and the polyphenol under consideration, respectively. With the developed procedure of sample extraction and preparation (described in section 2.3.2) this concentration range corresponded to 0.2 to 600 ng injected “on column”. In order to cover this range safely, a range of 0.1 to 1000 ng polyphenol on column was selected for linearity studies.

A stock solution of available standard compounds including epicatechin, catechin, procyanidin B2, procyanidin C1, clovamide, monodeoxyclovamides, dideoxyclovamide, quercetin, quercetin-galactoside, and quercetin-arabinoside was prepared and diluted in a way to cover the predicted concentration range. Taxifolin, a synthetic polyphenol not present in cocoa, was used as internal standard in every calibration solution at a constant concentration of 10 µg/ml. Peak area ratios of polyphenol standard and internal standards were calculated for every concentration and a linear regression carried out. A linear relationship between concentration and peak area ratio was to be confirmed from the slope of the linear calibration curve being statistically different from zero ($p<0.05$) and the intercept not statistically different from zero ($p<0.05$). Additionally, acceptability of linearity was to be judged from a correlation coefficient (R^2) being greater than 0.99.

However, when performing common linear regression analysis for the results obtained from HPLC-MS analyses of the calibration solutions, the intercept did in fact show statistically difference from zero disproving the linearity hypothesis despite satisfying the other two conditions. This is believed to be due to the common increase in variance with increase in concentration (heteroscedasticity) over a wide concentration range (here four orders of magnitude) and is best managed by use of a weighted regression such as $1/y$ or $1/y^2$ (Causon, 1997). Both types of weighted regressions led to results satisfying all three criteria for good linearity with weighting by $1/y$ generally giving stronger statistic significance as well as higher values of R^2. These linear regression lines are presented for three selected polyphenols, epicatechin, procyanidin B2, and quercetin-galactoside on the right hand side of figure 3.53. In order to create more evidence for method linearity, plots of relative response factors versus concentration show that response factors remain within 10% of the mean response factor over the whole concentration range for all compounds (figure 3.53 right) indicating good linearity of the method.

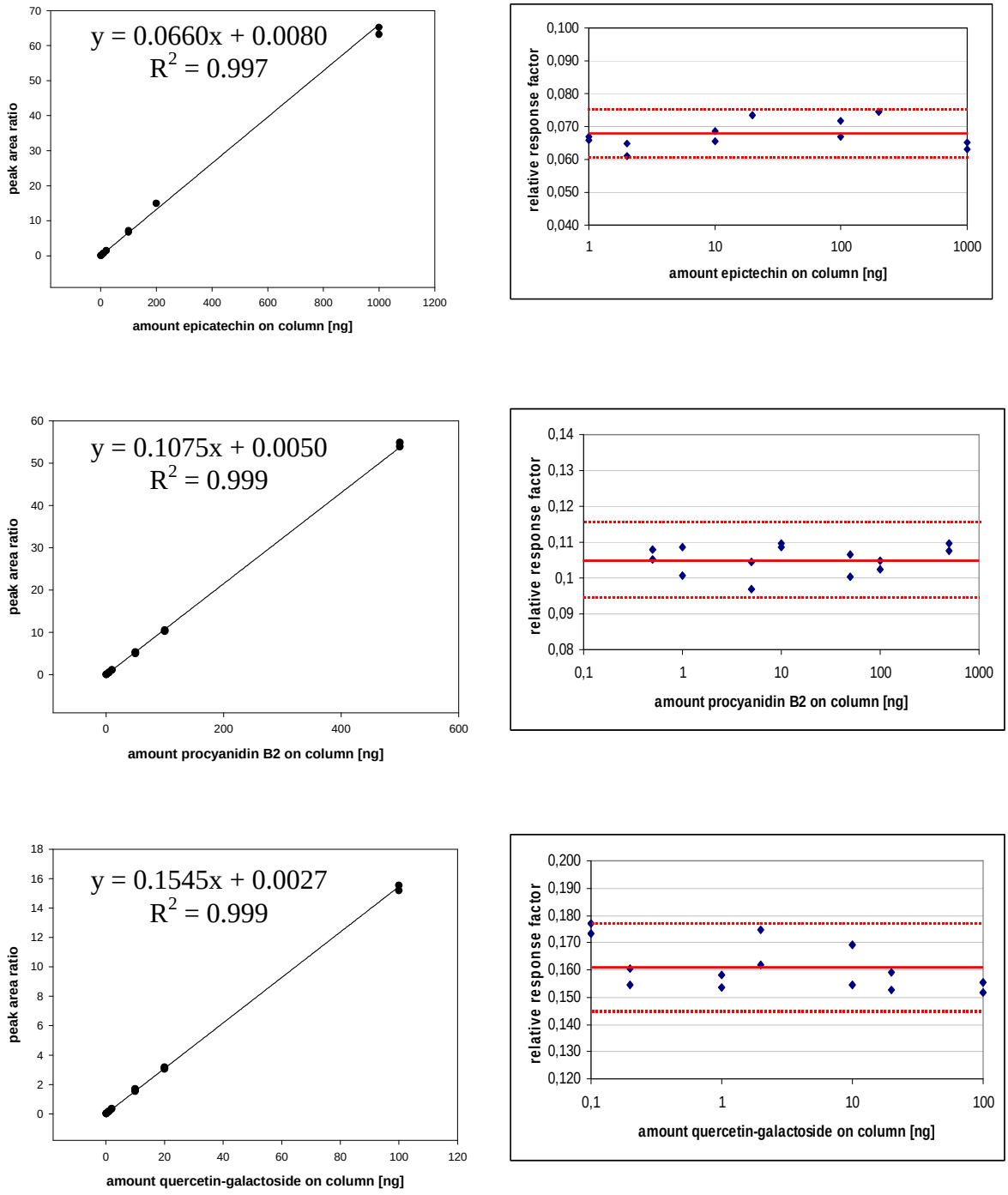


Figure 3.53 Peak area ratios (left) and relative response factors (right) versus concentration for epicatechin (top), procyanidin B2 (middle), and quercetin-galactoside (bottom) – left: resulting functions of 1/y weighted linear regression analyses and correlation coefficients; right: red line represents mean relative response factor, dashed lines ± 10% limits.

3.3.2.2.2 Trueness, precision and range of application

The trueness of a method is the closeness of the measured value to the true value for the sample (Green, 1996). Trueness can be assessed by analysing a sample of known concentration (reference material) and comparing the measured value to the true value. Alternatively, results obtained with a new method can be compared to those measured with an existing method that is known to be accurate. Neither a reference material with known polyphenol concentration nor an accurate measurement method for cocoa polyphenols are available to date. Thus, trueness assessment is based on recovery studies in the present work. As a blank cocoa matrix containing no polyphenols cannot be obtained, the standard addition technique was employed.

First, a sample of cocoa liquor was analysed in 12 separate complete analyses in order to get a solid reference for the non-spiked sample. For practical reasons the data for all polyphenols from theses analyses were also used to estimate the method precision (repeatability), as precision of a method means the amount of scatter in the results obtained from multiple analyses of a homogeneous sample (Green 1996). Subsequently, recoveries for epicatechin, catechin, procyanidin B2, quercetin, and quercetin-galactoside used for the studies of trueness here, were determined by adding approximately 25%, 50%, 75%, and 100% of the amount determined in the non-spiked sample. For each spiking level analyses were performed in triplicate and the recovery calculated as follows (adopted from Horwitz, 1995):

$$\%\,\mathrm{Recovery} = \frac{C_f - C_u}{C_a} \cdot 100\% \text{ with}$$

C_f = concentration of analyte found in fortified (spiked) sample

C_u = concentration of analyte found in unfortified sample

C_a = concentration of added amount of analyte

Precision for all 17 cocoa polyphenols investigated here ranged from 6% for epicatechin to approximately 15% for procyanidin heptamer with an overall method precision of 10% for all compounds at different concentration levels. The relatively low precision in analysis of procyanidin heptamer is likely to be due to the low concentration (ca. 4 ng

on column) and a broad peak shape due to chromatographic difficulties under the conditions applied here for large molecules (MW 2018 Da). Nevertheless, the observation of increasing relative standard deviation for concentrations approaching the detection limit of a method was expected (Green, 1996) and can thus be used to determine the lower limit of the application range, which can also be considered as the limit of quantitation (LOQ) of this method. The term lower limit of the range of application is preferred here, as the application range of an analytical method is defined as the concentration interval over which acceptable linearity, trueness, and precision are obtained (Green, 1996), hence, the range for which the method has been validated. Commonly in literature, quantitation limit and detection limit are simply determined by defining them as concentrations producing a signal 10 times (LOQ) and 3 times (LOD) the noise level of the system. Thereafter, the system LOD here was between 5 and 20 pg on column depending on the phenolic compound corresponding to 25 to 100 µg/kg polyphenol in cocoa products, and the LOQ would be at 15 to 60 pg on column or 75 to 300 µg/kg polyphenol in cocoa, respectively. However, at below 100 pg on column or 500 µg/kg polyphenol in cocoa the precision increases to levels above 15-20% and thus, the lower limit of application of this method corresponding to the lowest concentration that can be determined with sufficient accuracy was set to 0.5 mg/kg polyphenol in cocoa. The upper limit of the application range is somewhat above the highest concentration measured in screening studies and is defined by the highest concentration used for calibration (1000 ng on column) corresponding to a polyphenol concentration in cocoa of 5000 mg/kg.

Recoveries resulting from the studies of added analyte as described above ranged between 95% and 102% and showed no statistically significant difference from 100% (one-sample Student’s t-test) suggesting complete recovery. This result was expected since sample extraction and preparation did not include a clean-up or concentration step where usually analyte losses have to be anticipated.

However, whereas complete extraction and recovery of low molecular weight compounds can be assumed, it has to be kept in mind that extraction of higher oligomeric procyanidins is unlikely to be exhaustive (Rohr, 1999). This can be also seen from acid hydrolysis (proanthocyanidin assay, section 3.1.5) of the residue after repeated extraction with different solvents yielding red cyanidin indicating the presence

of procyanidins. Hence, true recovery of higher oligomeric and polymeric procyanidins must be considered to be significantly below 100% especially for higher molecular weight compounds. Nevertheless, as this value cannot be determined from spiking with procyanidin standards, the quantity of procyanidin in the residue was estimated within this work by the proanthocyanidin assay. Moreover, as procyanidin levels in chocolates and chocolate raw materials were determined using the same method of extraction and analysis in the same laboratory comparison of relative amounts of procyanidins can still be done accurately under the premise of good method precision.

During the course of this work the HPLC-ESI-MS method was applied for the measurement of catechin and epicatechin in a proficiency testing exercise organised by the National Institute of Standards and Technology (NIST), Gaithersburg, Maryland, USA. This allowed the comparison of the measured value with the assigned true value of this newly issued standard reference material (SRM) baking chocolate (NIST code: SRM 2384) as determined by NIST through the average of all reported results from the participants in this proficiency study (Sharpless et al., 2002). Results for catechin and epicatechin obtained here through quadruplicate analyses of the SRM were 83±5% and 92±7% of the assigned true value for this reference material, respectively. These values can be used as a measure of trueness for the method applied here and suggests a recovery below 100%. However, the assigned value for SRM 2384 baking chocolate itself has an expanded uncertainty (coverage factor 2 corresponding to 95% confidence) of 21% and 20% for catechin and epicatechin, respectively. Thus, the values obtained in this laboratory fell within the borders of the uncertainty of the values for the SRM. Additionally, a student's t-test assuming unequal variances showed no significant differences between measured and assigned values ($p<0.05$). Consequently, these results do not disprove the demonstration of complete recovery as demonstrated in the added analyte approach described above.

In summary, the method is specific for every phenolic analyte and was demonstrated to be linear over the entire application range from 0.5 to 5000 mg/kg polyphenol in cocoa with complete recovery and an overall precision of 10%. Thus, the method has been shown valid for the intended purpose of analysing 22 phenolic compounds in chocolate and chocolate raw products by a single step polyphenol extraction procedure and multicompound quantitative HPLC-ESI-MS analysis.

3.4 Polyphenol content in chocolate & chocolate raw products – Identification of important process parameters

In this part of the study the objective was to determine the concentrations of 22 polyphenols in dark chocolate and milk chocolate as well as in chocolate raw products including fermented cocoa beans from various origins, cocoa nibs, and cocoa liquor in order to study the influence of raw material selection and process parameters in chocolate making on contents and profile of phenolic compounds in the final products.

For this, samples were taken from chocolate manufacturing plants in order to study procedures that are common practise in user countries rather than laboratory scale experiments. The procedures studied include cocoa liquor and dark chocolate manufacturing processes, a milk chocolate making procedure, as well as a process of combined roasting and alkalising of cocoa nibs. The process steps of these procedures as well as the relevant information regarding temperature and time of the treatment and also the sampling points together with sample type and sample codes are all described in chapter 2.4 including schematic outlines (figures 2.2 to 2.5).

Since the raw material for cocoa processing in user countries are fermented cocoa beans, ten available samples of fermented cocoa beans from different origins were collected in one manufacturing plant for quantitative determination of polyphenol concentrations as well, in order to obtain a first indicative screening on the variation in polyphenol content among deliveries from countries of origin, where the fermentation and drying of cocoa beans is carried out. Sample origins include the countries of Ivory Coast (5 samples), Ecuador, Nigeria, Venezuela, Papa New Guinea, and Jamaica.

Finally and in order to have a suggestive view on the variations in the final products, polyphenol contents were determined in several dark chocolates and milk chocolates, respectively. Since a large study on polyphenol patterns and contents in chocolates available on local, national or international markets was beyond the scope of the present work, samples were collected from three local supermarkets in a way to obtain some indication on variations in products from the same producer as well as such variations in products between a few manufacturers. This was done by purchasing several products of the same manufacturer, which have been produced on different days, as deduced

from the expiry dates and production codes, as well as some additional chocolates from different chocolate makers. All samples had the amount of “cocoa solids” stated on the label, which was considered important for product comparison.

Concentrations of 22 phenolic compounds were determined using the validated multi-compound method based on HPLC-ESI-MS. Additionally, the Folin-Ciocalteu assay was employed in parallel to study the potential of this simple assay for studying the influence of processing on total polyphenol content in cocoa. In order to get an estimate on non-extractable polyphenols (NEPP) the modified proanthocyanidin assay as described in section 3.1.5 was applied on the residue of the aqueous acetone extraction of the multi-compound HPLC-ESI-MS method.

3.4.1 Sampling

The outcome in the form of quantitative measures of the cocoa polyphenol content as well as the conclusions drawn from these in this part of the work not only depend on the accuracy of the analytical methodology applied but also significantly on the representativeness of the sampling. The overriding criterion is “fitness for purpose” being the universally accepted principle among analytical scientists as the correct approach to obtaining data of appropriate quality (Thompson and Fearn, 1996). Obviously, however carefully analyses are carried out, the result will be of limited value unless the portion of the sample taken for analysis is truly representative of the whole population of interest. Hence, for inhomogeneous products, where the sampling error is large and usually exceeds by far the measurement error, there is little value in trying to improve the analytical method. Better results will be obtained by the analysis of a larger number of samples. Sampling and analysis are inextricably linked and thus, both sampling and analysis, must be carried out in such a way that the final data obtained enable correct and sensible conclusions and decisions to be taken (Crosby and Patel, 1995).

The measurement error is caused by the measured value failing to accurately represent the true value of the polyphenol concentration within a sample, and this error was already studied, described and quantified as *measurement uncertainty* through the validation procedure of the analytical method used within the present work (section 3.3.2). Therefore, the objective in this part of the work was to assure that sampling was

carried out in such a way to draw sufficiently representative analytical samples, subject to quantitative analyses of polyphenols in the studied cocoa products, for identifying influences of chocolate making process parameters as well as polyphenols variations in cocoa beans and chocolates, respectively.

Sampling is the procedure used to draw or constitute a sample from an entire population of interest, so that characteristics from the sample can be used to draw conclusions or make interferences about the entire population of interest (Codex Alimentarius, 2002). Sampling is also associated with an error ("*sampling error*") caused by the sample failing to accurately represent the population from which it was collected. Parameters that contribute to the sampling error include the heterogeneity of the inspected characteristics, the random nature of sampling, as well as the known and acceptable characteristics of the sampling procedure.

A representative sample is a sample in which the characteristics of the lot from which it is drawn are maintained. It is in particular the case of a simple random sample where each of the items or increments of the lot has been given the same probability of entering the sample (Codex Alimentarius, 2002). Consequently, representativeness is the ability to generalise from a sample to the entire lot or population of interest. Obviously, the sample strategy has to adapt to the heterogeneity of the inspected characteristics. Finally, a lot is homogeneous (*heterogeneous*) relative to a given characteristic, if the characteristic is (*not*) uniformly distributed according to a given probability law (commonly the normal distribution) throughout the lot.

In the present work the representativeness of sampling was examined experimentally through the repeated analyses of at least ten samples taken in the same manner from the same lot.

For cocoa liquor and chocolate mass 10 samples of approximately 100 grams were taken from the container in the manufacturing plants and each sample was analysed for polyphenol content by the HPLC-ESI-MS method in duplicate using ca 1 gram for extraction. For chocolates in bars 10 bars were taken and duplicate analytical samples of ca 1 gram were analysed for their polyphenol content in the same way. For dark and milk chocolate samples purchased at the local supermarket it was not the objective to have a representative subset of samples for chocolates on the regional, national or even

European market, but to have some indicative values for chocolates of the same producer - but produced at different times - compared to those of other manufacturers. Representativeness was to be examined for chocolate bars produced and filled into bars on the same day. Results from statistical analysis of variance (ANOVA) revealed no significant difference ($p<0.05$) between the within sample error (measurement error) and the between sample error (measurement error + sampling error). In other words, the sampling error is small compared to the measurement error and thus, good representativeness of this sampling procedure has been proved. This result was expected since both cocoa liquor and chocolate mass remain in liquid state and are continuously stirred in big containers before the latter is filled hot into cold forms of chocolate bars, where it suddenly becomes solid during the process of cocoa liquor and chocolate manufacturing. Thus, theses samples are surely homogeneous for polyphenol content and the randomly taken samples should assure good representativeness.

However, this is not the case for cocoa nibs and in particular for cocoa beans after having been fermented under greatly varying conditions (discussed in chapter 1.3) before delivery in big shipments from the countries of origin to the user countries. In fact, when examining fermented cocoa beans of a 20 kg jute sack, it was evident from the occasional occurrence of a violet colour that not all beans were completely fermented. Additionally, the shell of some of from their brown colour apparently well-fermented beans could be removed only with some difficulty, presenting another indication of a lower degree of fermentation. Screening analyses of several single cocoa beans using the Folin-Ciocalteu assay showed extremely high total polyphenol contents for violet beans and significantly lower levels for brown beans showing a relative similar content for beans that can be easily deshelled. This strongly suggests heterogeneity in the polyphenol content of fermented cocoa beans even from the same origin. As it was not possible to control for the representativeness of shipments of cocoa beans from the countries of origin nor for the representativeness of keeping two subsamples of 20 kg of such shipments by the cocoa liquor manufacturer for the reason of quality assurance, a lot for the purpose of this study was considered, as two 20 kg jute sacks of cocoa beans, which polyphenol content should be best possible described by the sampling procedure followed by quantitative analysis of the analytical sample taken.

For practical reasons of transport and analysis, which starts with the deshelling of cocoa beans, 100 g sub-samples corresponding to 80 to 100 cocoa beans were taken from the 20 kg sack to the laboratory. The demonstration of acceptable representativeness of this sub-sampling strategy was to be examined on sixteen 100 g containers, 8 from each two jute sacks of a selected cocoa bean batch, followed by duplicate analyses as described for cocoa liquor and chocolate mass or bars, respectively.

However, the heterogeneity of polyphenol content in theses sub-samples still had to be accounted for. This has been achieved by making use of the practical consequences of the central limit theorem, a fundamental result of the theory of probability. In its simplest form this theorem states that the sum (or the average) of a large number of independent observations from the same distribution has an approximate normal distribution. Or in other words, the distribution of the phenomenon under study does not have to be normal because its sum (average) will be. A useful side effect of the normal distribution of the sum (average) is the applicability of more powerful parametric statistics, such as analysis of variance, student's t-test, principal component analysis etc. Applied to the problem here this means that with a large number, usually considered greater than 30 observations, of independent analyses of polyphenol content in small portions of cocoa beans, which originally have a heterogeneous polyphenol content distribution, the sum (average) becomes approximately a normal variable and can be thus considered homogeneous in the sub-sample. Practically, this has been done by grinding the complete 80 to 100 cocoa beans of the sub-sample followed by taking approximately 35 portions of 25 to 30mg each with a micro-spatula. As for the normality of the distribution of the sum of > 30 portions it does not matter, whether the portions are analysed separately or after reunification to a composite sample, the latter approach has been applied for reasons of practicability.

In order to study representativeness of the practise of taking 100 g subsamples from the 20 kg jute sacks, all sixteen 100 g sub-samples were analysed in duplicate in this mode and results subjected to analysis of variance. There was no significant difference between the within sample error and the between sample error ($p<0.05$) and thus good representativeness of the 100 g sub-sampling procedure from the 20 kg jute sacks was proved for fermented cocoa beans. Moreover, representativeness of taking 100 g subsamples of cocoa nibs from the mixer and toaster of the manufacturing plants was

demonstrated in the same manner. The same procedure of grinding and collecting a composite sample of 35 to 40 portions for analysis was applied to cocoa nibs as well in order to assure normal distribution.

3.4.2 Results from the monitoring of the chocolate manufacturing processes

In order to study the influence of process parameters in the chocolate manufacturing procedures on cocoa polyphenol content 16 samples were taken from basically three different process types: a cocoa liquor production with two different time-temperature regimes during roasting (outlined in figure 2.2 in chapter 2.4) followed by the separate manufacture of dark chocolate (figure 2.3) starting with a cocoa liquor from a procedure like the one outlined in figure 2.2, a milk chocolate manufacturing procedure applying a petzomat, a thin-layer roaster applying pressurised water vapour at over 100 °C being a rather gentle roasting procedure with a very short residence time of the cocoa liquor (figure 2.4), as well as a combined roasting-alkalising procedure for cocoa nibs (figure 2.5). All 16 samples were analysed in duplicate for 22 phenolic compounds by the validated HPLC-ESI-MS method thus making up a data table with a total of 704 data points.

With the aim of getting a first overview on the influence of cocoa processing and the difference of the three processes studied, the sum of the 22 measured polyphenols has been calculated and duplicate data analysed by ANOVA followed by multiple comparisons of means (Tukey's post-hoc test). Figure 3.54 presents a visual extract of these data analyses showing highly significant ($p < 0.001$) decreases in the summed polyphenol content through all cocoa processing procedures. As anticipated, the milk chocolate manufacture shows the best preservation of the overall polyphenol content likely due to the gentlest roasting conditions, whereas alkalising appears to add an additional threat to cocoa polyphenols resulting in major losses.

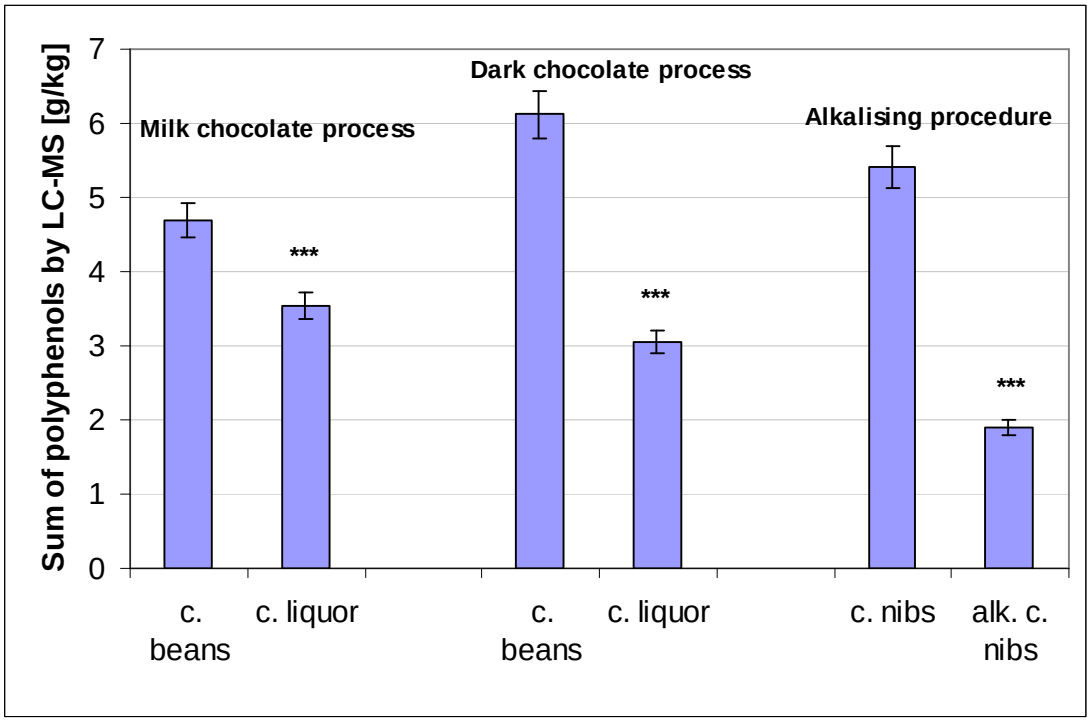


Figure 3.54 Polyphenol losses during three different types of cocoa processing. Values are expressed as means of duplicate analyses; error bars represent expanded measurement uncertainty with a coverage factor of 2 corresponding to a confidence level of 95%.
***: differences are significant at $p < 0.001$.

While a sum parameter, such as the total content of 22 polyphenols calculated here, gives a quick view on major changes during cocoa processing and may represent a measure of remaining overall antioxidative potential of the marketed product, a lot more specific information is available studying alterations of every single quantified phenolic compound. Depending on the respective measurement uncertainty for each analyte as well as its sensitivity to process induced changes statistically significant ($p < 0.05$), variations or only likely trends were observed from multivariate statistical analyses. However, deriving conclusions from multiple comparisons of more than 320 results showed to be too complex, and thus there was a need for grouping the results or variables both for simpler visualisation and interpretation of the multivariate data. In a first attempt to identify key phenolic compounds or compounds that may be grouped, relative polyphenol distribution in fermented cocoa beans as starting material as well as in both manufactured dark and milk chocolate were compared (figure 3.55).

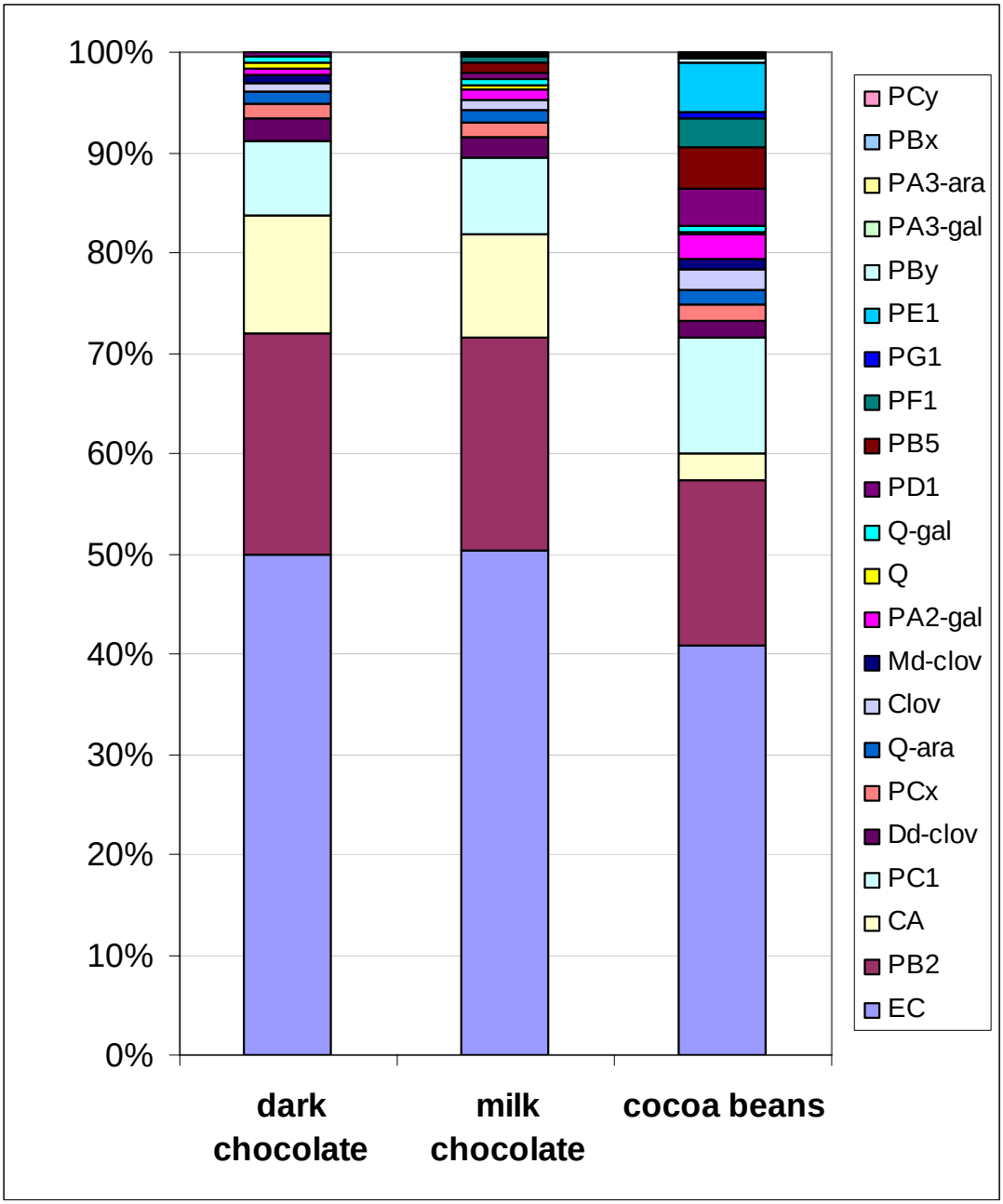


Figure 3.55 Relative polyphenol distribution in fermented cocoa beans as well as in processed dark and milk chocolate, respectively.
EC = epicatechin, CA = catechin, PB2, PB5, PBx, Pby = procyanidin dimers B2, B5 and two unknown isomers, PC1, PCx, Pcy = procyanidin trimer C1 and two unknown isomers, PD1, PE1, PF1, PG1 = procyanidin tetramer to heptamer, PA2-gal, PA3-ara, PA3-gal = doubly linked (A-type) procyanidin dimer and trimers linked to galactose and arabinose, respectively, Q, Q-ara, Q-gal = quercetin and quercetin linked to galactose and arabinose, respectively, Clov, Md-clov, Dd-clov = hydroxycinnamic acid amides (clovamide and its monodeoxy and dideoxy analogues).

At first, it is striking that epicatechin is the major phenolic compound in cocoa beans (40%) as well as in dark and milk chocolate (50%). Moreover, only four polyphenols, epicatechin, procyanidin dimer B2, catechin and procyanidin trimer C1 account for 90% of all phenolics in dark and milk chocolate whereas the remaining 18 substances make up only 10%. In contrast, the former four compounds describe 70% of all polyphenols in cocoa beans and the major substances contributing to the remaining 30% include the higher procyanidin oligomers. It is tempting to conclude from this that focussing on the four major cocoa polyphenols identified here is sufficient to characterise chocolates and chocolate raw products.

Nevertheless, large data tables usually contain a large amount of information, which may be partly hidden because the data are too complex to be easily interpreted. Principal Component Analysis (PCA) is a projection method that helps to visualise all the information contained in a data table. The concept behind PCA is to describe the variance (information) in a set of multivariate data in terms of a set of underlying orthogonal variables (principal components) (Summer et al., 2003). The original variables (here: polyphenol contents) may be expressed as a particular linear combination of the principal components (PCs). PCA is a linear additive model, in the sense that each PC accounts for a portion of the total variance of the data set. They are computed iteratively in such a way that the first PC is the one that carries most information (or in statistical terms: most explained variance). The second PC will then carry the maximum share of the residual information (i.e. not taken into account by the previous PC) and so on. Usually, only the first PCs contain genuine information, while the later PCs most likely describe noise. Therefore, it is useful to study the first PCs only instead of the whole raw data table: Not only is it less complex, but it also ensures that noise is not mistaken for information. Plotting the data in the space defined by the two to three largest PCs provides a rapid means of visualising similarities or differences in the data set (Summer et al., 2003).

The PCs are displayed as a set of ‘scores’, which highlight clustering or outliers, and a set of ‘loadings’, which highlight the influence of input variables on the scores set (Brindle et al., 2002). Figure 3.56 shows the scores plot of all samples taken during the various cocoa processes and figure 3.57 presents the loadings plot of the polyphenols analysed quantitatively in those samples.

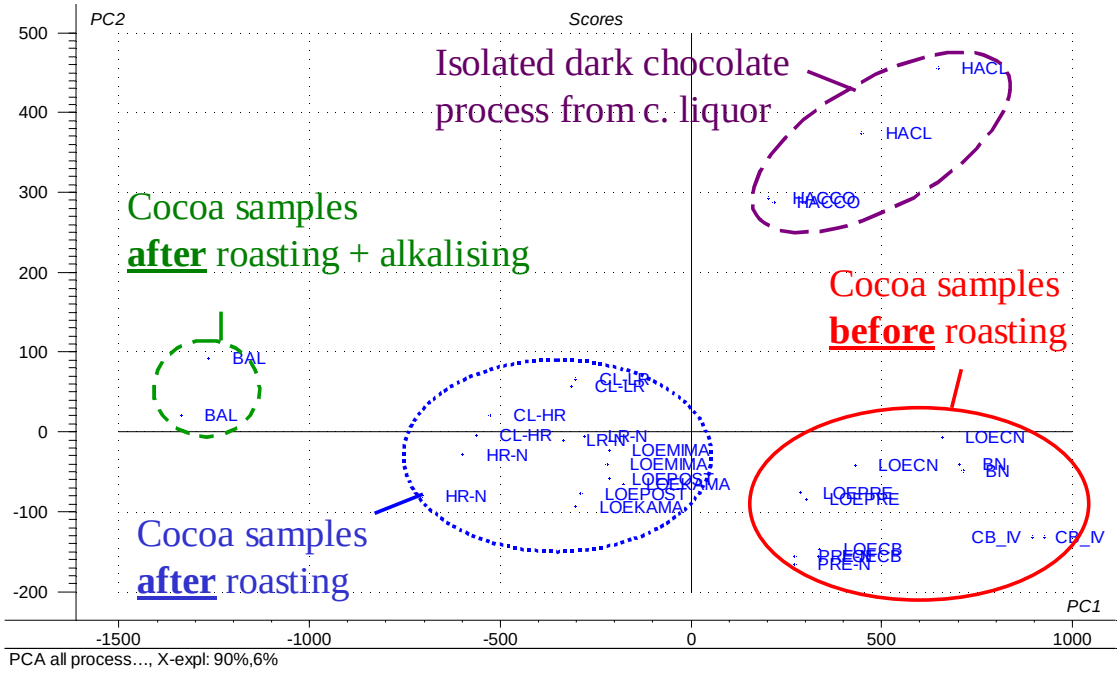


Figure 3.56 Result of principal component analysis – scores plot of all 16 samples analysed in duplicate taken during cocoa processes (figures 2.2 to 2.5 in chapter 2.4) – PC1 explains 90% and PC2 6% of the total variance.

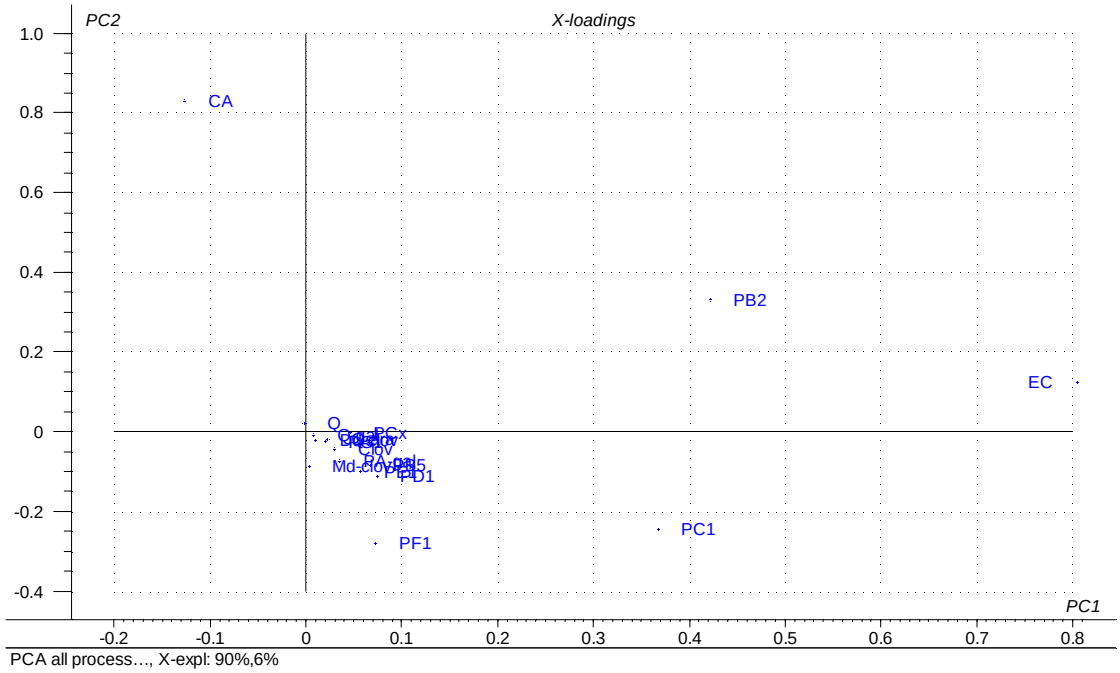


Figure 3.57 Result of principal component analysis – loadings plot of 22 polyphenols (variables) quantified in the 16 samples analysed in duplicate taken during cocoa processes (figures 2.2 to 2.5 in chapter 2.4) – codes for polyphenols are the same as in figure 3.55.

The scores plot allows the identification of four clusters in the samples highlighted as ellipses in figure 3.56. The sample clusters inside the red (continued line) and blue (dotted line) ellipses describe cocoa samples before the roasting and after the roasting processes, respectively, whereas the additional alkalising step in combination with the roasting leads to a clearly different polyphenol pattern in those samples (green short-dashed ellipse).

Generally, samples from further down the process of chocolate making are more towards the left in the plot, cocoa products in the early process stages and in particular starting material are located more to the right. Furthermore, the softer the time-temperature conditions of a process are, especially during roasting, the smaller is the left-right distance in the scores plot between those samples taken before and after the respective process step. The two samples (in duplicate) inside the violet (long-dashed) ellipse were taken from the dark chocolate manufacture process that starts with delivered cocoa liquor in a detached process. The location in the upper right quarter of the scores plot is unexpected, as the cocoa liquor used for dark chocolate making is principally like the one produced and studied here from the cocoa liquor manufacturing and thus, the location of the violet (long-dashed) ellipse cluster should have been close to the blue (dotted) ellipse cluster in the lower left quarter. Consequently, this cocoa liquor and the subsequently produced dark chocolate mass in the violet (long-dashed) ellipse differ significantly in their polyphenol composition from the two cocoa liquors that were produced in another manufacturing plant (blue dotted ellipse). The reasons for this remain unknown and could be either due to an unexpected strong variation in the polyphenol pattern of those cocoa liquors or due to a voluntary, but from the dark chocolate producer not reported variation in the recipe, such as the addition of fat free cocoa powder or other ingredients.

In order to give a more concrete sense to the location of samples in the scores plot in terms of polyphenol concentrations and/or patterns the meaning of the PCs has to be elucidated. This can be quickly achieved by looking at the loadings plot (figure 3.57), in which the loading reflects how much one variable contributed to a PC, how well that PC takes into account the variation of that variable over the data points, and finally, how much the variables correlate with each other.

PC1, which alone explains 90% of the total variance, has high positive loadings for epicatechin, procyanidin dimer B2 and trimer C1 contents, a negative loading for catechin concentration and rather small loadings for all other polyphenols (figure 3.57). If the score of a sample and the loading of a variable on PC1 have the same sign, the sample has higher than average value for that variable and vice-versa. The larger the scores and loadings are, the stronger is that relation.

The biggest contributor to PC2 is catechin concentration, however, PC2 accounts for only 6% of total variance, which is rather small compared to PC1 and overall variation in the data. Furthermore, from the loadings plot it can be deduced that epicatechin, procyanidin B2 and C1 quantities in cocoa samples are positively correlated with each other (and PC1) and all are negatively correlated with catechin content. Moreover, absolute differences in these four variables between samples explain most of the observed variance explained by PC1, which in turn explains 90% of total variance.

This finding from PCA is in accordance with the assumption that with the sole variance of epicatechin, catechin, procyanidin B2 and C1 contents samples and process influences might be characterised well tentatively deduced from the observation in figure 3.55 that these four polyphenols make up 90% of total polyphenol content in the final product (dark and milk chocolate). While this probably reflects the real major quantitative influence of these polyphenols on the selected samples, this does not exclude that also the variations of the content of minor phenolic compounds in the samples can describe equally well or better the influence of processing on cocoa polyphenols. I.e., these variables might be highly correlated with one or the other PC and thus, their variance would be well explained by such a PC and in turn those variables might give a good characterisation of the differences in the samples set.

Correlation loadings for every variable can be calculated in order to take into account the amount of explained variance in the interpretation. The correlation loadings plot helps to discover the structure in the data more clearly. Figure 3.58 shows the correlation loadings plot for the same PCA as in figure 3.57.

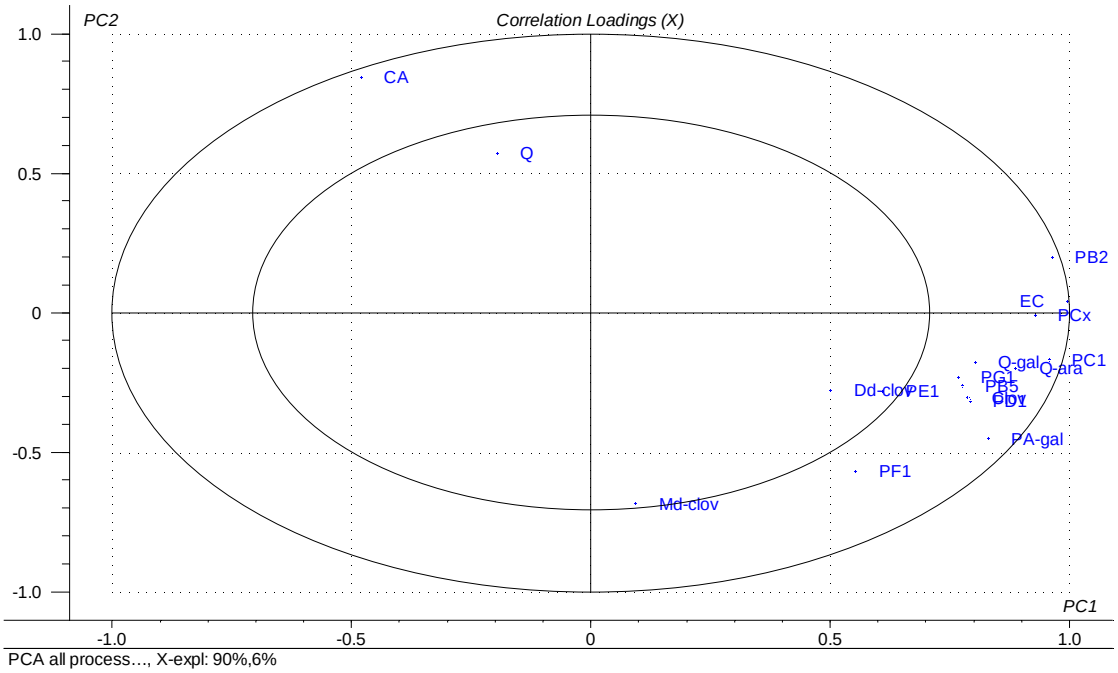


Figure 3.58 Result of principal component analysis as in figures 3.56 and 3.57 – correlation loadings plot of 22 polyphenols (variables) - the outer ellipse indicates 100% and the inner ellipse indicates 50% of explained variance, respectively – codes for polyphenols are the same as in figure 3.55.

In the correlation loadings plot the importance of individual variables is visualised more clearly compared to the ordinary loadings plot. In fact, the importance of the other oligomeric procyanidins as well as quercetin glycosides and clovamide becomes much more obvious when the correlation loadings ellipses are shown.

It appears as if in the PCA plots in figures 3.56 and 3.57 strong variations of variables might be overseen, if quantitative relevance on total polyphenol content is low. That is because PCA is a projection method, which is based on finding directions of maximum variation. Thus, it depends on the absolute variance of the variables in the sample set. However, if one wishes to explore on variables that are sensitive to process changes despite their contribution to total polyphenol content, variables can be weighted so that only the relative differences among the variance of the variables influence the model. The weighting 1/standard deviation is used to give all the variables the same variance for the studied sample set (i.e., a variance of one). In doing so, all the variables are given the same chance to influence the estimation of the PCs.

Figure 3.59 shows the ordinary loadings plot for a PCA with 1/standard deviation weighting of polyphenol concentrations.

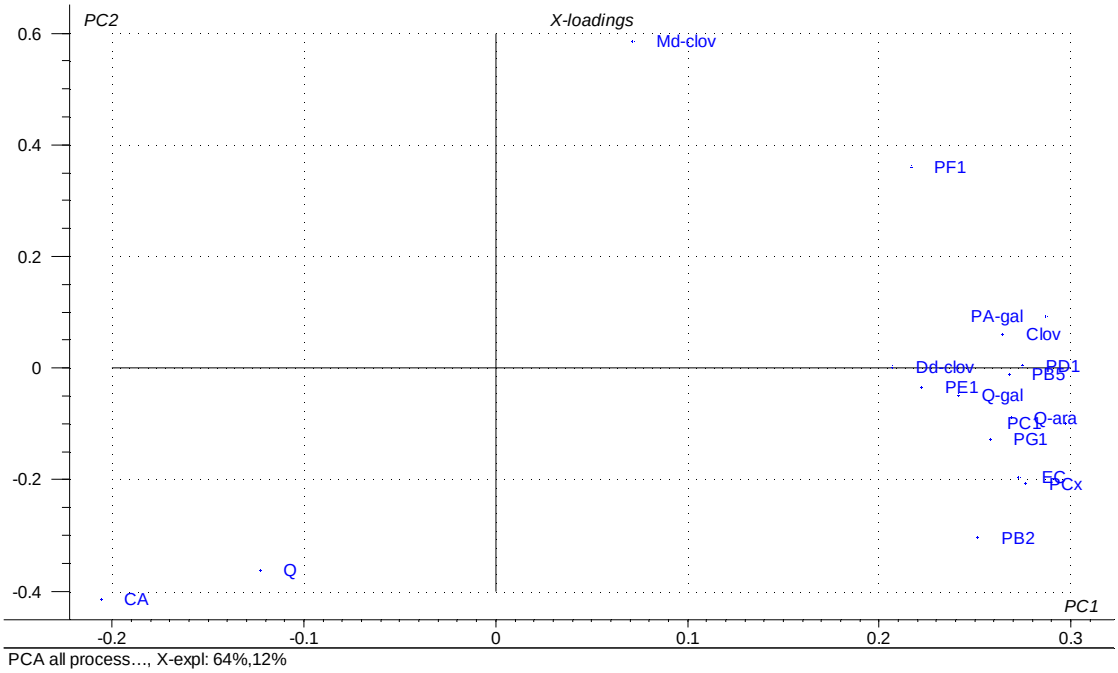


Figure 3.59 Result of principal component analysis with 1/standard deviation weighting of polyphenol concentrations (variables) – loadings plot of 22 polyphenols – codes for polyphenols are the same as in figure 3.55.

In this visualisation it is also clearly seen that relative variations in polyphenol concentrations of epicatechin, procyanidins and quercetin glycosides are highly positively correlated, whereas catechin and quercetin contents are negatively correlated with the former variables. In the scores plot of this PCA the orientation along PC1 of the samples is very similar as in figure 3.56 and the same clusters of samples can also still be observed (plot not shown) indicating that both approaches can be used complementary for studying the influence of cocoa processing on polyphenol content.

In conclusion, PCA has led to the following main findings that were used for further description and interpretation of process mediated changes in cocoa polyphenols within this study:

- There is a significant loss in the concentration of most cocoa polyphenols during chocolate manufacturing, particularly during roasting and combined roasting/alkalising with losses for epicatechin, and procyanidins B2 and C1 being quantitatively most relevant. In contrast, concentrations of catechin and quercetin increase during the processing, which is likely to be due to predominant epimerisation of epicatechin towards its non-epi structure catechin as reported for green tea catechins (Wang and Helliwell, 2000) and for epigallocatechin in banana fruits (Tanaka et al., 2000a) as well as the deglycosation of quercetin glycosides to quercetin aglykon, respectively.

- Losses of minor phenolic compounds including oligomeric procyanidins and quercetin glycosides correlate well with losses in major cocoa phenolics and thus can be used for describing cocoa process induced changes as well. Moreover, some compounds that are highly correlated such as procyanidins deserve further consideration as they themselves or calculated new parameters such as sums or ratios of original variables could be highly sensitive parameters describing also more subtle influences of other process parameters than roasting.

Following these intermediate conclusions from PCA several new parameters have been calculated including: (i) sum of oligomeric procyanidins of tetramers through heptamers (abbreviation used in graphical presentations: **Sum P>4**) representing a group of related compounds that show high correlations between each other, (ii) sum of hydroxycinnamic acid amides (clovamides, **Sum Clov**) a somewhat separate class of phenolic compounds in cocoa, (iii) both a sum of all quercetin compounds (aglykone and glycosides, **Sum Q**) and a ratio of quercetin glycosides to quercetin aglykone (**(Q-Ara+Q-Gal)/Q**)since it appears that part of the losses of the glycosides is due to deglycosylation leading to increases in the aglykone concentration, which might be of interest for bioavailability and potential bioactivity of these flavanols, (iv) both the sum of epicatechin and catechin (**EC+CA**)as well as the ratio of epicatechin to catechin (**EC:CA**) to study losses of epicatechin due to epimerisation to catechin, which might preserve the bioactivity potential of monomeric cocoa catechins despite the decrease of epicatechin as major compound, and losses due to other degradation reactions of one or both catechins leading to a loss of potential phenolic bioactivity of these two flavan-3-ols.

Figures 3.60a and 3.60b show relative changes of the four major cocoa polyphenols epicatechin, catechin, procyanidins B2 and C1 as well as the above described and through calculation derived parameters during milk chocolate manufacturing, production of cocoa liquor that may serve further for dark chocolate fabrication, and the combined roasting-alkalising procedure, respectively.

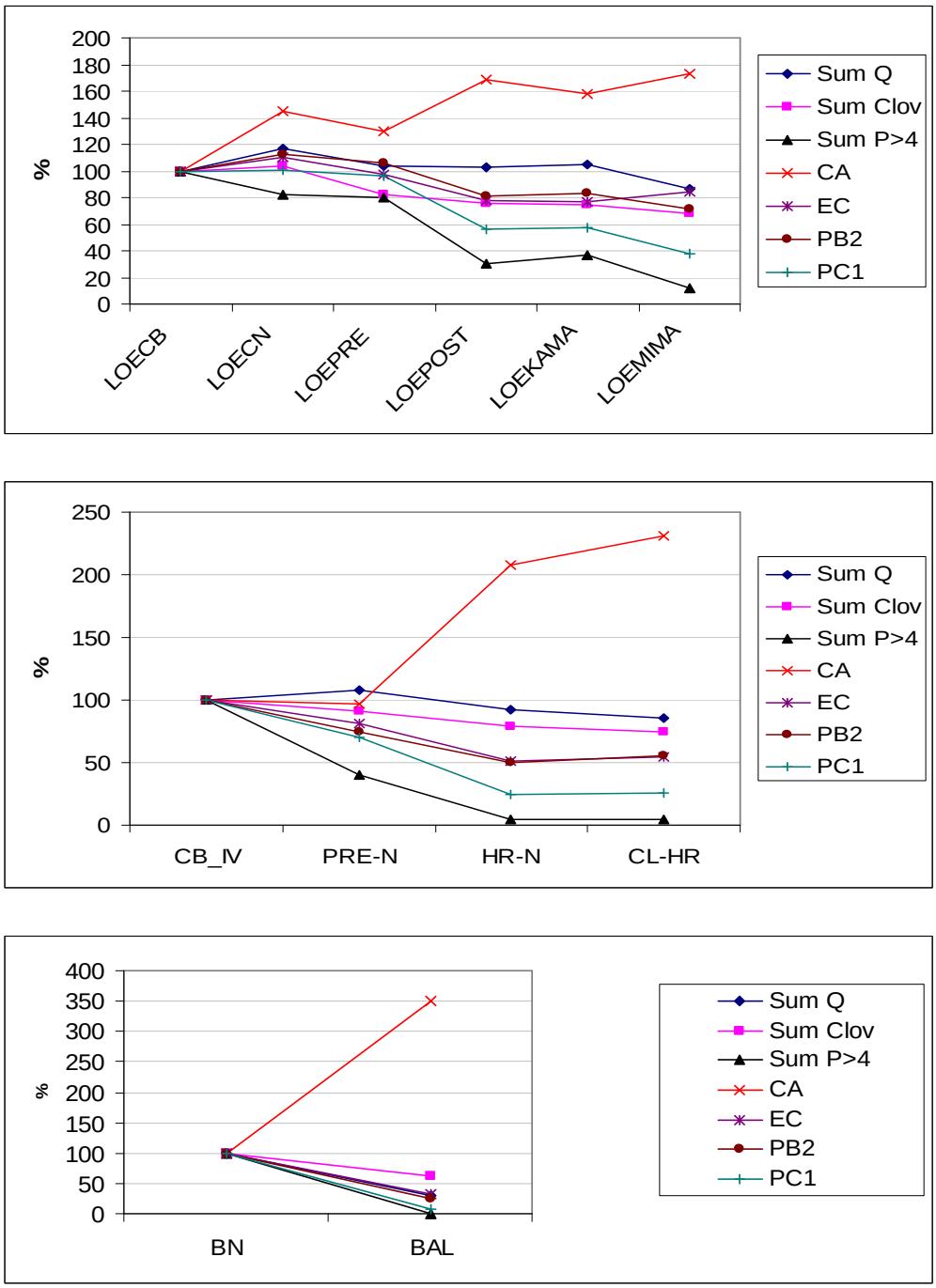


Figure 3.60a Relative changes of polyphenol contents during milk chocolate manufacturing (top), cocoa liquor making (middle), and combined roasting-alkalising (bottom).
Sum Q = sum of quercetin and quercetin glycosides, Sum Clov = sum of clovamide and mono- and dideoxy analogues, Sum P>4 = sum of procyanidin tetramers, pentamers, hexamers and heptamers, CA = catechin, EC = epicatechin, PB2 = procyanidin dimer B2, PC1 = procyanidin trimer C1 – error bars representing expanded measurement uncertainty as well as signs referring to statistical significant changes have been omitted for retaining clarity.

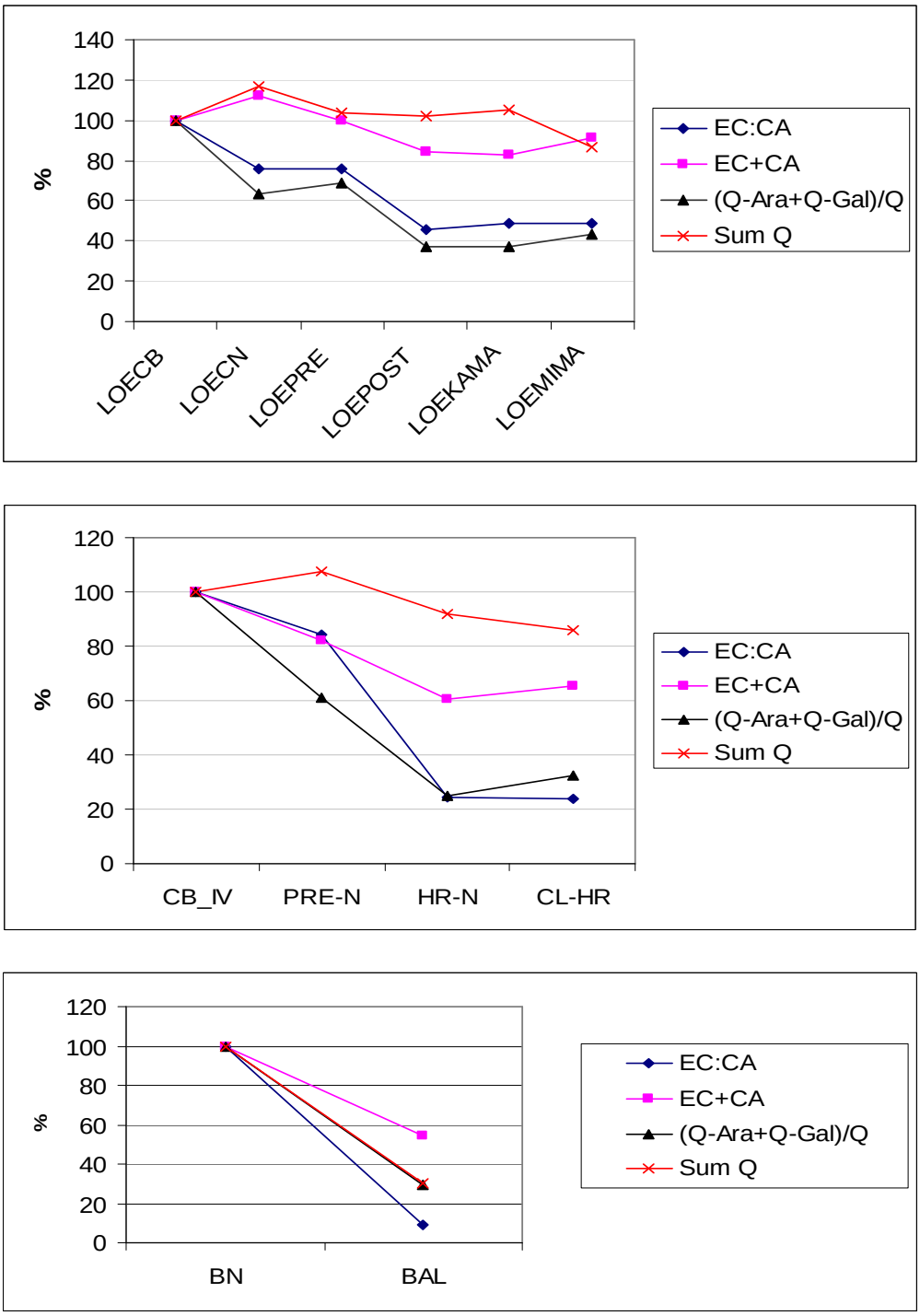


Figure 3.60b Relative changes of polyphenol contents during milk chocolate manufacturing (top), cocoa liquor making (middle), and combined roasting-alkalising (bottom)
EC:CA = epicatechin to catechin ratio, EC+CA = sum of epicatechin and catechin, (Q-Ara+Q-Gal)/Q = ratio of the sum of quercetin arabinoside and quercetin galactoside to quercetin aglykone, Sum Q = sum of quercetin and quercetin glycosides - error bars representing expanded measurement uncertainty as well as signs referring to statistical significant changes have been omitted for retaining clarity.

As expected from PCA relative changes in the four major cocoa polyphenols show positive correlations between epicatechin, procyanidin B2 and C1 with the former two having almost identical relative concentration decreases. The procyanidin trimer C1 is the compound that is most sensitive as demonstrated by the highest loss during processing. Changes in epicatechin as well as procyanidins B2 and C1 concentrations are negatively correlated with changes in catechin content, which increase through the process lines (figure 3.60a).

Relative decreases in minor polyphenols grouped in compound classes as procyanidins, quercetin and quercetin glycosides as well as clovamides show principally the same course as epicatechin, procyanidin B2 and C1 as predicted from PCA correlation loadings (figure 3.58) and PCA with variable variance standardisation (figure 3.59), respectively. Generally, procyanidins are the most sensitive and clovamides the most robust among the studied substances during cocoa processing (figure 3.60a).

When comparing the three different process types that mainly differ in the cocoa bean deshelling and roasting technology characterised by different time-temperature profiles as well as the presence or absence of a simultaneous alkalising procedure, it is striking that the harsher the process conditions are the more evident are also the described polyphenol alterations. Small changes induced by process steps that have mild conditions - as most steps of the milk chocolate process line - were statistically significant ($p<0.05$) in multiple comparisons of means only for the most sensitive phenolic compounds being the procyanidin oligomers and the trimer C1, but not in epicatechin that makes up half of the total polyphenol content of cocoa samples. This was also evident in the visualisation of the changes in polyphenol concentrations (figure 3.60a). In contrast, the combined roasting-alkalising process leads to a drastic decrease in all polyphenols even for the most robust clovamides and with the striking exception of augmenting catechin content likely due to epimerisation of epicatechin.

With the relative change of the parameters shown in figure 3.60b being the sums and ratios of epicatechin and catechin as well as quercetin glycosides and quercetin, respectively, both the influence of cocoa processing can be described and more interestingly, some information of the mechanism of changes in these compounds under varying process conditions can be deduced. For instance, the mild process of milk

chocolate manufacturing appeared to lead to only negligible degradation of epicatechin and catechin as well as quercetin glycosides and aglykone, as observed in close to inexistent changes in the sum parameters. However, epimerisation reactions between the former compounds with a predominant epi- to non-epiform direction as well as deglycosylation of the quercetin glycosides to free quercetin occured apparently, which was deduced from the strong alteration in the ratio variables.

On the contrary, degradation reactions for catechins became evident for the harsher process conditions in the cocoa liquor factory and for all the five compounds in the combined roasting-alkalising step. It is well known that polyphenols are unstable in alkaline conditions and oxidative degradation is likely to occur (Zhu et al., 2003). Thus, it is probable that the observations for the roasting-alkalising step in figure 3.60b (bottom) can be explained through degradation reactions that dominate over deglycosylation and epimerisation, although the latter is still an important chemical change shown by the 3 ½ fold increase in catechin content (figure 3.60a).

In parallel to the quantitative analysis of 22 single polyphenols by HPLC-ESI-MS all samples were analysed as well for total polyphenol content by the Folin-Ciocalteu assay. Figure 3.61 presents the relative changes during milk chocolate manufacturing, cocoa liquor making as well as combined roasting-alkalising for total polyphenols expressed as catechin equivalents measured by Folin-Ciocalteu and also for the sum of polyphenols determined by LC-MS.

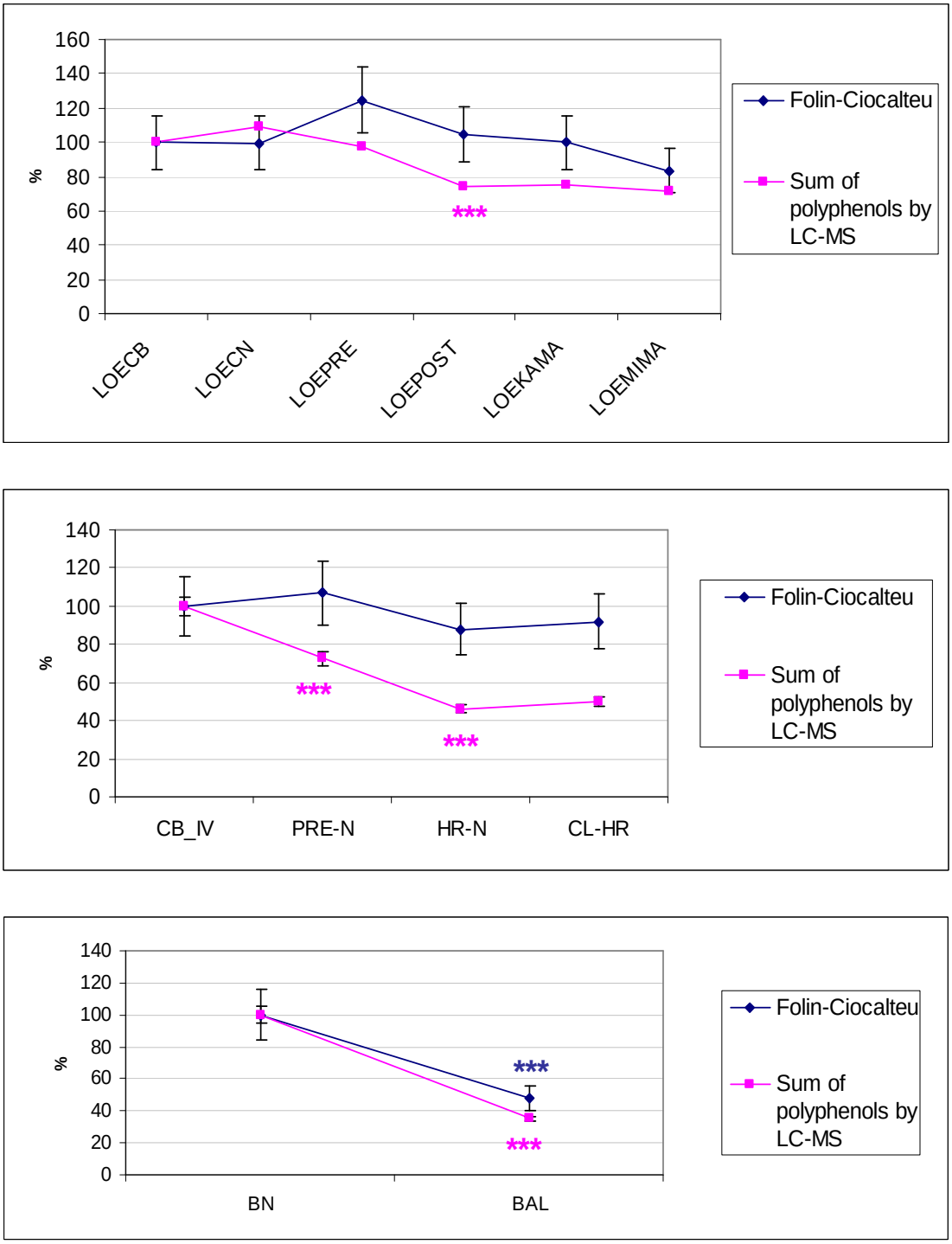


Figure 3.61 Relative changes of total polyphenol contents determined applying the Folin-Ciocalteu assay and by summing up single polyphenols measured by HPLC-ESI-MS during milk chocolate manufacturing (top), cocoa liquor making (middle), and combined roasting-alkalising (bottom) - error bars represent expanded measurement uncertainty with a coverage factor of 2 corresponding to 95% confidence, *** refers to a statistical significant change ($p<0.001$) of a processed sample to the sample before the respective processing.

It is striking that the total polyphenol content measured by the Folin-Ciocalteu assay did not change significantly ($p < 0.05$) during cocoa processing with the exception of the combined roasting-alkalising procedure, whereas the sum of polyphenols calculated from LC-MS analyses did. This may in part be accounted for by the 3-fold higher measurement uncertainty of the Folin-Ciocalteu method compared to the LC-MS sum parameter. However, from the mean values presented in figure 3.61 it appears that the lack of decrease in the parameter measured by the colorimetric assay would be real. Two possible hypotheses might explain this phenomenon: (i) only a small part of the measured colour development in the Folin-Ciocalteu assay was due to the cocoa polyphenols as identified within this work, whereas other matrix compounds varying little during processing are major contributors, such as proteins, polysaccharides, and fibre and (ii) new compounds were formed during cocoa processing having sufficient reducing potential to cause the colour formation in this assay, such as Maillard reaction products formation or conversion of known cocoa polyphenols in unknown compounds that retain all or most of the original reducing potential as likely through epimerisation reactions.

In conclusion, the approach chosen here for studying the influence of cocoa processing on cocoa polyphenols during chocolate manufacturing in user countries by measuring a range of phenolic compounds by application of a multi-parameter analytical method in combination with multivariate statistical data analysis such as PCA allowed for a deeper understanding of the alterations in single polyphenols as well as the phenolic pattern of cocoa products. In contrast to the focussing on a single major compound, such as epicatechin or the use of rapid measures of total phenols as the Folin-Ciocalteu assay, hypotheses can be created for chemical changes caused by process conditions, which in turn, could be the basis for possible future process optimisation. The possible discovery of preserved reducing potential of cocoa products during conventional processing without an alkalisation step would be particularly interesting regarding a possible preservation of desired bioactivity of cocoa. However, the latter would have to be proved through physiological studies. Selection of polyphenol-rich starting material as well as mild conditions during chocolate making might be promising strategies to produce final products with enhanced contents of putatively health beneficial polyphenols.

3.4.3 Polyphenol content of fermented cocoa beans of various origins

The starting material for the manufacturing of both dark and milk chocolate are the fermented cocoa beans as they arrive from the countries of origin to the user countries. The place of origins of these cocoa beans include countries in Asia, Africa, and Latin America and it is also well known that fermentation and drying are far from being standardised procedures (Kim and Keeney, 1984). Thus, quite different amounts as well as patterns of phenolic compounds composition were to be expected among cocoa beans from different origins. To get a rough idea of this variation, samples of cocoa beans, that were available from quality control samples preserved in one cocoa processing plant, were analysed for their polyphenol content by the multi-parameter HPLC-ESI-MS method as well as the colorimetric assays.

The origins of the fermented cocoa beans studied in the present work can be deduced from the sample codes used for the following graphical presentations. Five samples from Ivory Coast were coded **IVC1** to **IVC5,** the other samples were shipped from Ecuador (**ECU**), Jamaica (**JAM**), Venezuela (**VEN**), Nigeria (**NIG**), and Papa New Guinea (**PNG**), respectively.

As described above, both PCA and multiple comparisons of mean values were carried out on the obtained quantitative results. The results of PCA for 10 samples of cocoa beans that derive from 6 diverse origins and 5 samples from Ivory Coast of different shipments analysed in duplicate are presented in the scores plot (figure 3.62).

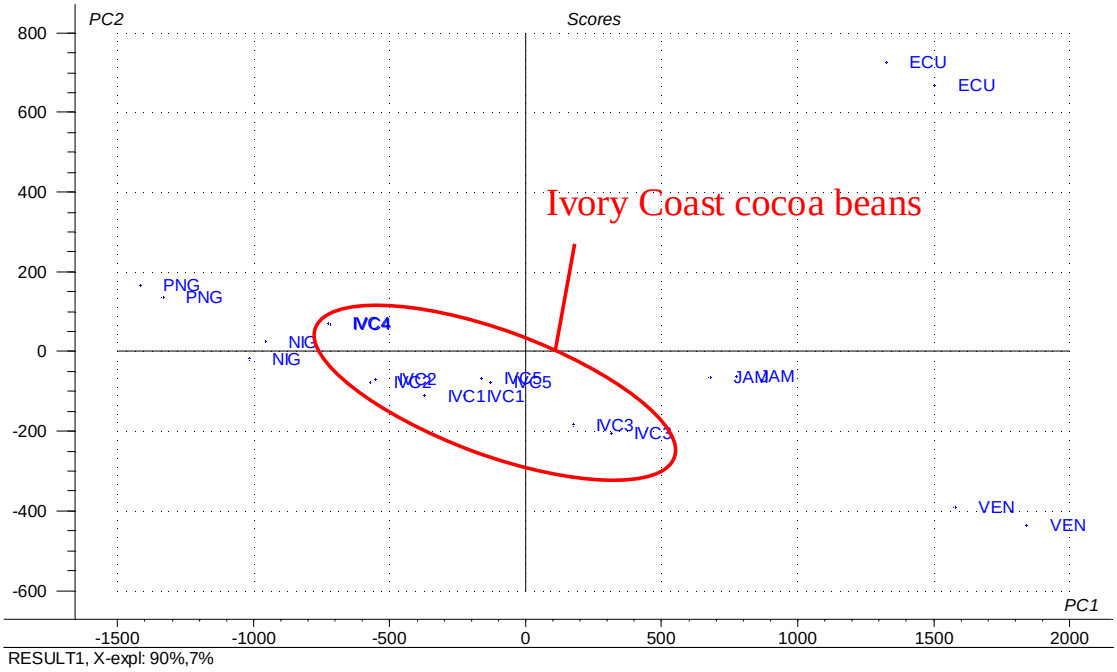


Figure 3.62 Result of principal component analysis – scores plot of all 10 samples of fermented cocoa beans analysed in duplicate – PC1 explains 90% and PC2 7% of the total variance – cocoa bean samples came from: Ivory Coast (IVC1 to IVC5), Ecuador (ECU), Jamaica (JAM), Venezuela (VEN), Nigeria (NIG), and Papa New Guinea (PNG), respectively.

In the PCA scores plot it is obvious that the five samples from Ivory Coast have a relatively similar polyphenol composition, whereas cocoa beans from other origins, in particular those from Venezuela and Ecuador, differ strongly from the Ivory Coast samples. The loadings plot of this PCA (not shown) is similar to the PCA of samples from cocoa processing, i.e., samples to the right have significantly higher values in most cocoa phenolics, such as epicatechin and procyanidin dimers to heptamers. In addition, the major variable contributing to PC2 was identified as catechin. Thus, cocoa beans from Ecuador should have significantly higher catechin contents than cocoa beans from other origins.

Figure 3.63 shows the total amount of polyphenols (top) calculated from the sum of single polyphenol concentrations in HPLC-MS analyses and the relative polyphenol composition (bottom) of the cocoa bean samples studied in this work.

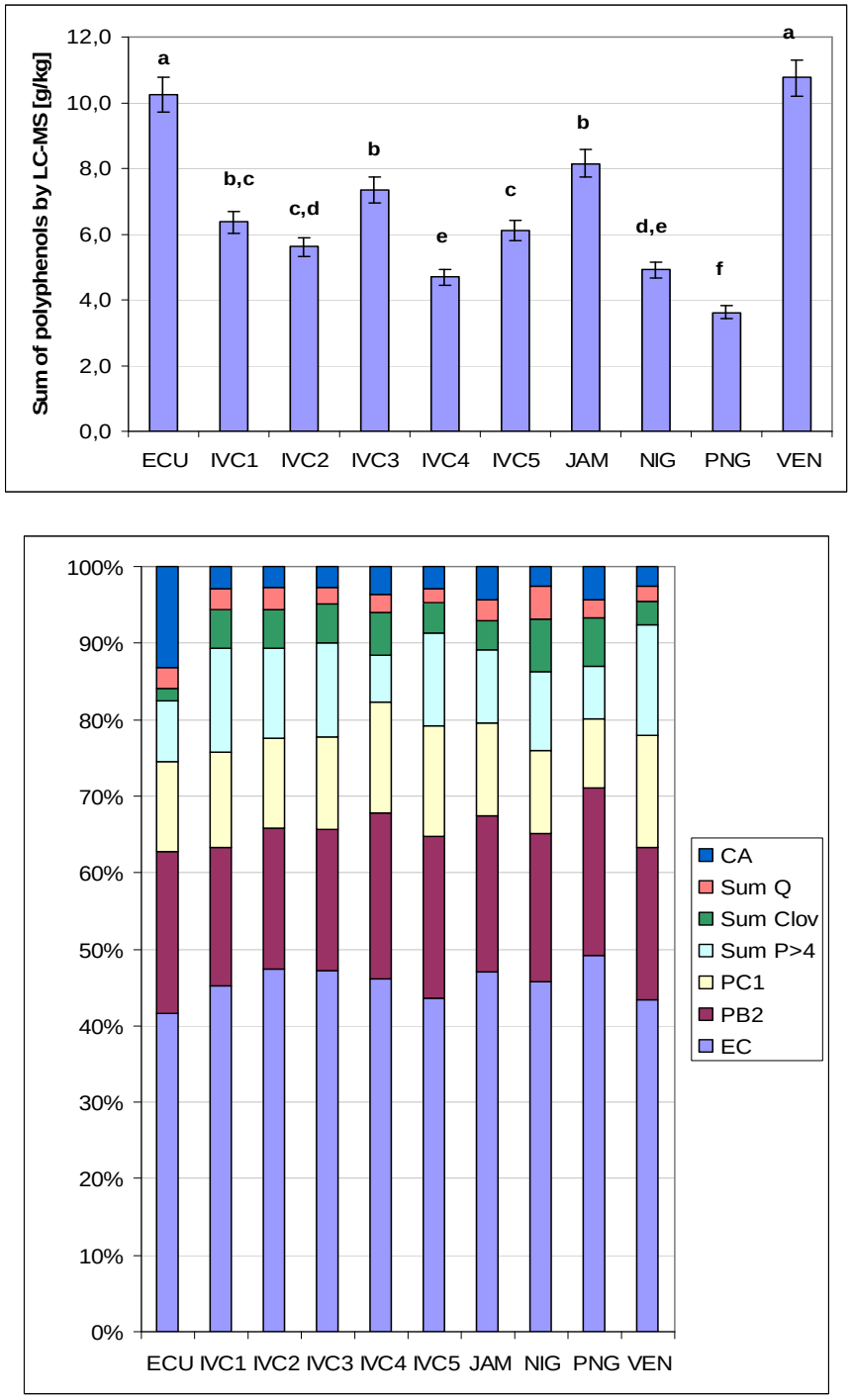


Figure 3.63 Polyphenol contents (top) and composition (bottom) in fermented cocoa beans of various origins (Ivory Coast (IVC1 to IVC5), Ecuador (ECU), Jamaica (JAM), Venezuela (VEN), Nigeria (NIG), and Papa New Guinea (PNG)) – samples having different letters are significantly different ($p < 0.05$) from each other
Sum Q = sum of quercetin and quercetin glycosides, Sum Clov = sum of clovamide and mono- and dideoxy analogues, Sum P>4 = sum of procyanidin tetramers, pentamers, hexamers and heptamers, CA = catechin, EC = epicatechin, PB2 = procyanidin dimer B2, PC1 = procyanidin trimer C1.

Both cocoa beans from Venezuela and Ecuador, that were identified as extreme cases of polyphenol composition by PCA, showed the highest amount of total polyphenols in the fermented bean. Moreover, the high relative amount of catechin in Ecuadorian cocoa beans discriminates these from the rest. A 3-fold difference in the range of total polyphenol contents between fermented cocoa beans from different countries of origin was observed here, which emphasises the potential of selecting polyphenol-rich raw material as one strategy for producing chocolates with enhanced amounts of bioactive polyphenols. Nevertheless, relative polyphenol composition is rather similar among the studied samples.

3.4.4 Polyphenol content in various samples of dark and milk chocolate

Next, it was aimed to study both dark and milk chocolates from the same chocolate producer as well as from different suppliers in order to get an impression of intra batch variations and variations between manufacturing plants. Obviously, the differences between samples from different chocolate manufacturers result from selection of diverse fermented cocoa beans on the market, individual product recipes as well as selection of different process lines and conditions, whereas intra batch variations reflect more the ability of preserving desired quality attributes by the producer. Quality is usually based on sensory attributes in order to meet consumer acceptance, but in future preservation of bioactive compounds could be increasingly interesting for the food industry as well.

For this part of the study, eleven samples of dark chocolate, of those seven from the same supplier as well as ten samples of milk chocolate from only two diverse manufacturers, were purchased from local supermarkets and analysed in duplicate for polyphenol concentrations by HPLC-MS and colorimetric methods. As before PCA was performed for interpretational data analysis and visualisation highlighting samples with similar polyphenol composition (clusters) or samples having very different polyphenolic concentrations (outliers) as well as allowing exploration for key variables (polyphenol concentrations) that may serve for the characterisation of chocolates on the market. Figure 3.64a and 3.64b show the scores plot and loadings plot of the conducted PCA on the results of the analyses of dark chocolate samples.

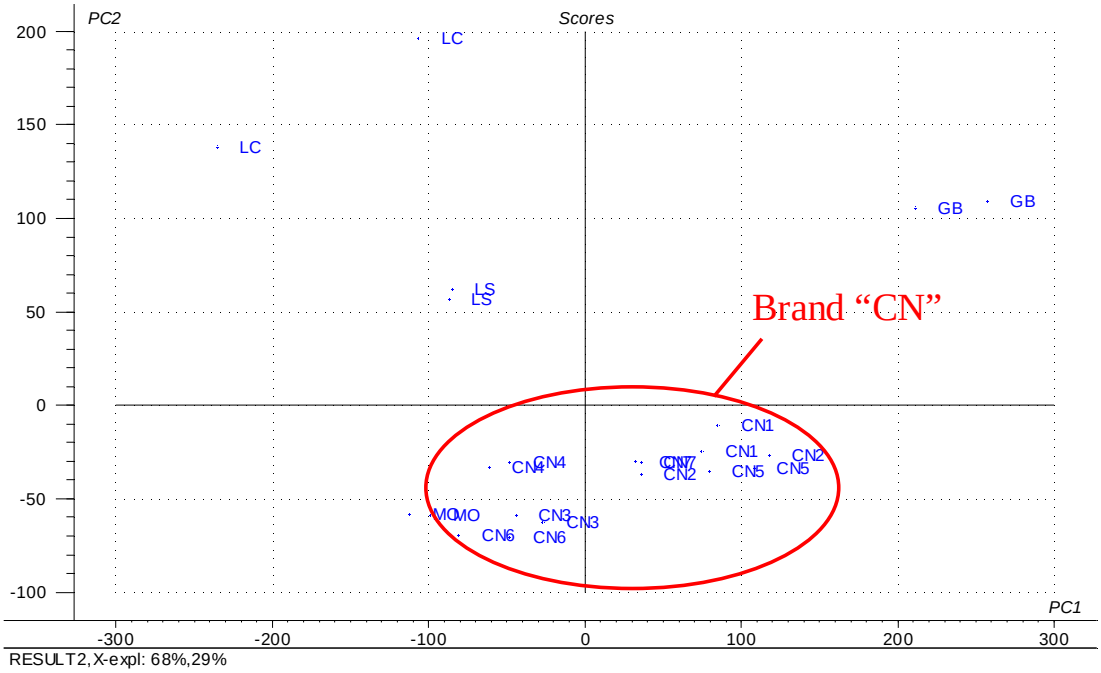


Figure 3.64a Result of principal component analysis – scores plot of 11 dark chocolate samples analysed in duplicate – PC1 explains 68% and PC2 29% of the total variance.

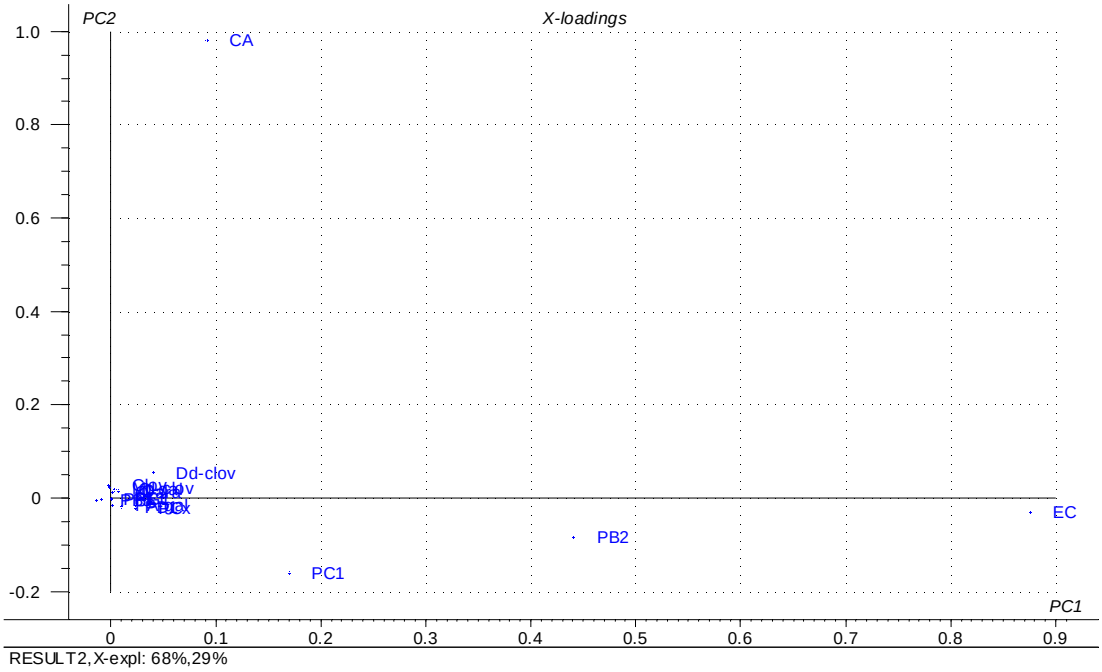


Figure 3.64b Result of principal component analysis – loadings plot of 11 dark chocolate samples analysed in duplicate – codes for polyphenols are the same as in figure 3.55.

While a high degree of similarity in polyphenol composition for the seven dark chocolates of the same supplier was striking, there were notable differences observed between this group of samples and dark chocolates produced by different chocolate makers (figure 3.64a). The loadings plot is presented here as well in order to highlight the explained variance of 29% by PC2 that is significantly higher than in above described PCA despite the similarity of key variables (epicatechin, procyanidins dimer to tetramer as well as catechin) for characterising dark chocolate samples. Catechin concentration is the main contributor to this PC and this denotes in turn that catechin is a strong variable for the description of differences in the studied dark chocolate samples. Thus, chocolates that are higher up in the scores plot of PC2 should have clearly higher catechin contents and vice versa.

Figure 3.65 shows the amount of total polyphenols in the dark chocolate samples calculated from summing up single polyphenol concentrations obtained from HPLC-MS analyses and the relative polyphenol composition, respectively.

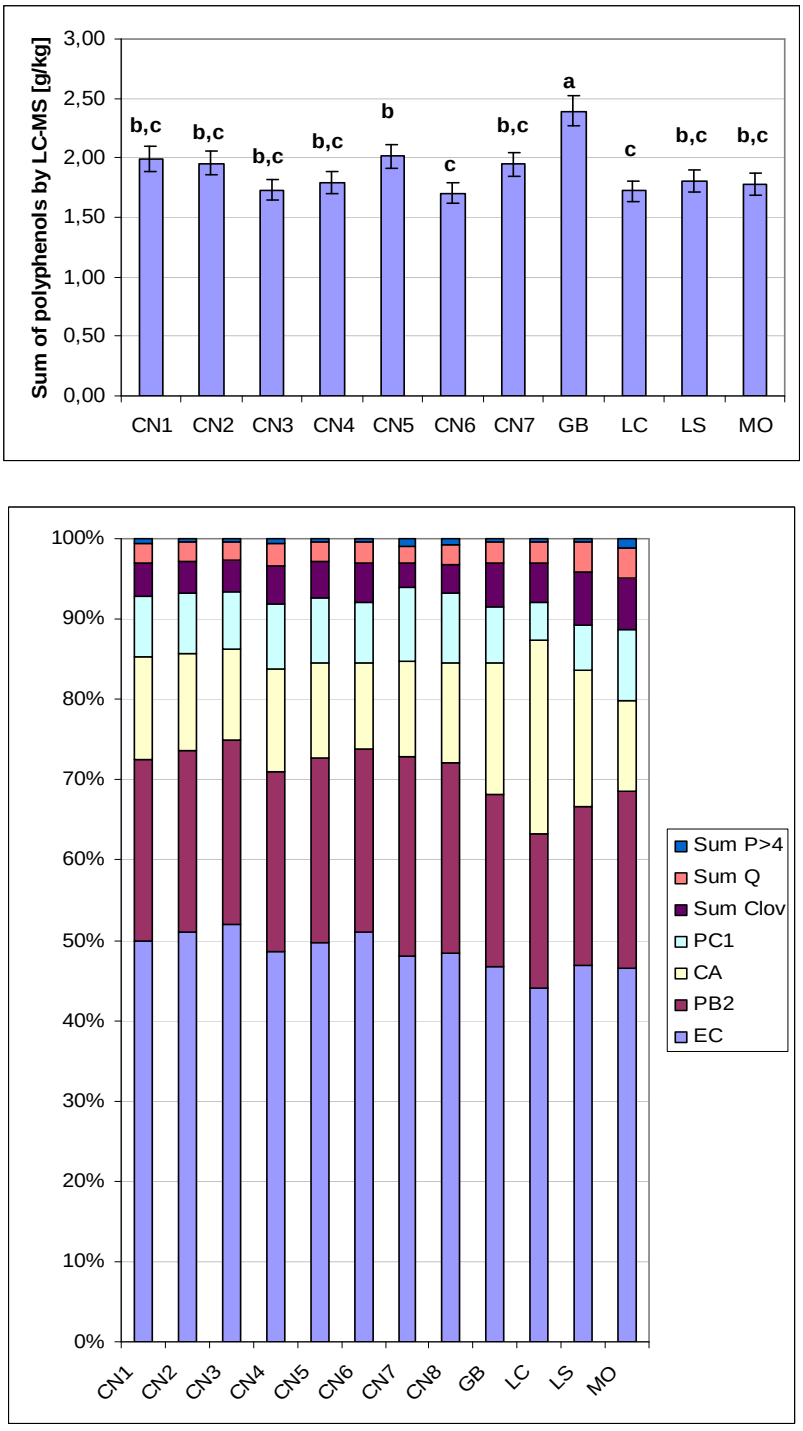


Figure 3.65 Polyphenol contents (top) and composition (bottom) in dark chocolates – samples having different letters are significantly different ($p < 0.05$) from each other.
Sum Q = sum of quercetin and quercetin glycosides, Sum Clov = sum of clovamide and mono- and dideoxy analogues, Sum P>4 = sum of procyanidin tetramers, pentamers, hexamers and heptamers, CA = catechin, EC = epicatechin, PB2 = procyanidin dimer B2, PC1 = procyanidin trimer C1.

The quantitative differences in total polyphenol content in the 11 samples studied here were small ranging from 1.8 to 2.4 g/kg. Selection of cocoa beans, individual recipe using 46 to 70% of 'cocoa solids' as stated on the product labels and the established process conditions may be the important variables determining the variations in contents and phenolic composition of the final dark chocolates studied in this work. Relative catechin contents were highest and epicatechin-catechin ratios were lowest for the sample with the highest total polyphenol content (GB), which could be an indication for a somewhat harsher cocoa sample treatment during chocolate making while using the highest amount (70%) of cocoa solids.

Figure 3.66 shows the results of PCA for the milk chocolate samples.

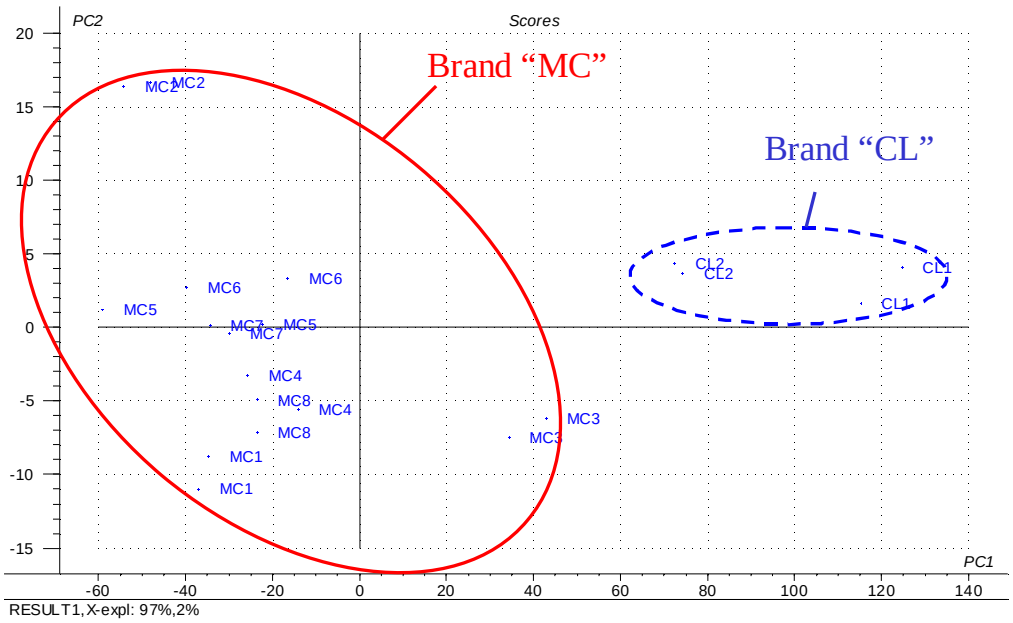


Figure 3.66 Result of principal component analysis – scores plot of 10 samples of milk chocolates analysed in duplicate – PC1 explains 97% and PC2 2% of the total variance.

The samples from the two different brands are clearly separated as illustrated by the red (continues line) and blue (dashed line) ellipses. As for PCA of the samples described

above, PC1 correlates positively with epicatechin, procyanidin B2 and C1 contents and PC2 correlates positively with catechin contents (PCA loadings plot not shown). However, PC1 explains already 97% of total variance or in other words, almost all variance of the samples is explained through this PC and the following PCs cannot be distinguished from the analytical noise. Consequently, only the position along PC1 (abscissa) in the PCA scores plot (figure 3.66) should be considered to distinguish the samples.

Consequently, the cluster brand “MC”, that appears visually to cover a wide range of polyphenol variation, actually varies little when considering only PC1 with the exception of sample MC3 that has higher values of PC1. Higher values of PC1 denote also higher values of positively correlating epicatechin as well as procyanidins B2 and C1.

Figure 3.67 shows polyphenol contents and patterns of the analysed milk chocolate samples.

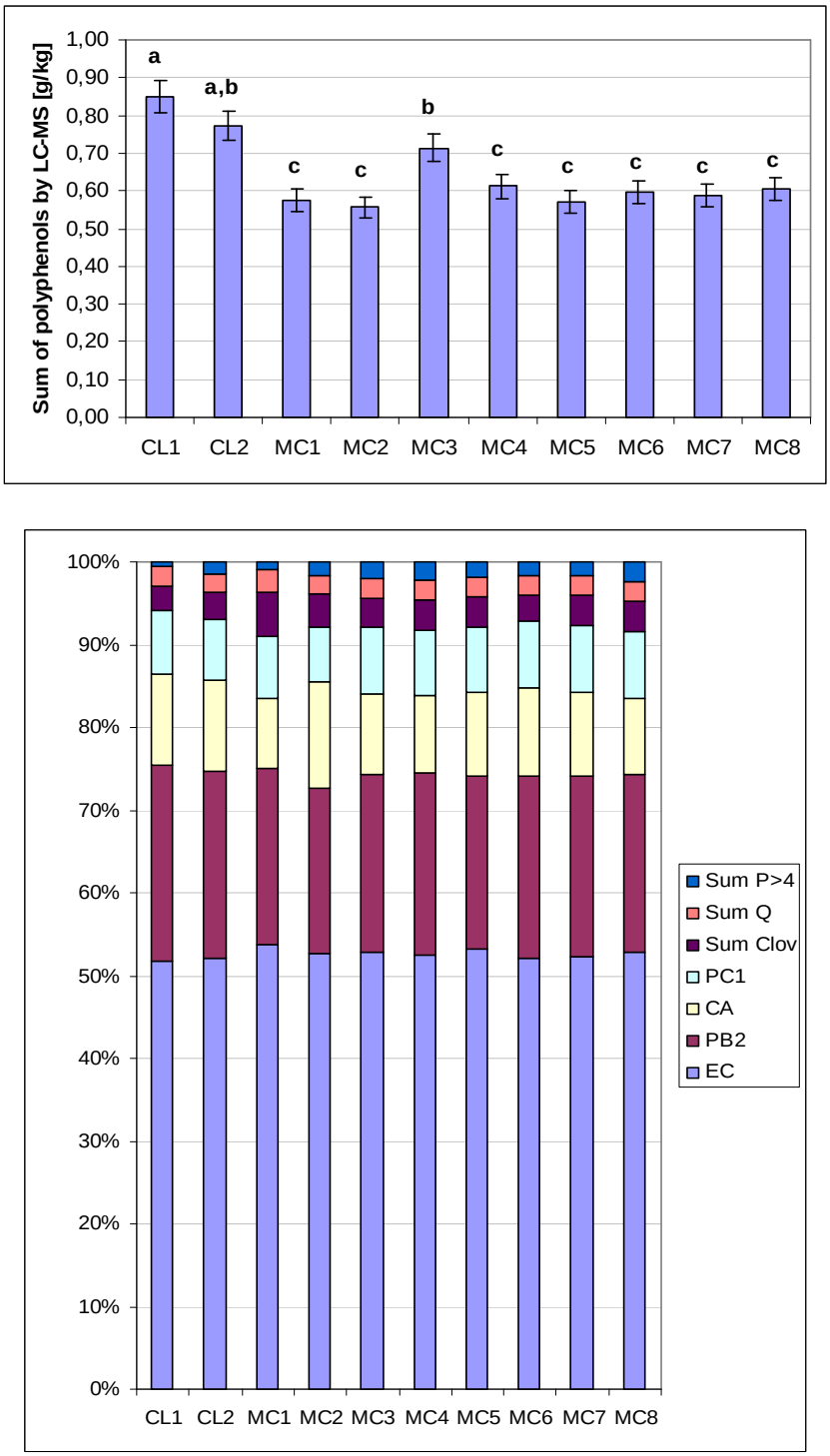


Figure 3.67 Polyphenol contents (top) and composition (bottom) in milk chocolates – samples having different letters are significantly different ($p < 0.05$) from each other.
Sum Q = sum of quercetin and quercetin glycosides, Sum Clov = sum of clovamide and mono- and dideoxy analogues, Sum P>4 = sum of procyanidin tetramers, pentamers, hexamers and heptamers, CA = catechin, EC = epicatechin, PB2 = procyanidin dimer B2, PC1 = procyanidin trimer C1.

As expected, the total polyphenol content of MC3 was significantly higher than those of the other samples of this brand, which is in agreement with the conclusion drawn above on epicatechin, procyanidin B2 and C1 from PCA, as those polyphenols represent approximately 75-80% of total polyphenols. Total polyphenol contents of the milk chocolate samples of brand “CL” were significantly higher than those of brand “MC”, which is in agreement of the more to the right oriented cluster in the PCA scores plot (figure 3.66). In contrast, the relative contribution of single phenolic compounds to total polyphenols were very similar for all samples independent from the chocolate producer (figure 3.67 bottom).

Taken together this suggests a higher amount of (fat free) cocoa solids in samples MC3 as well as CL1 and CL2 or the use of cocoa beans as starting material that are richer in polyphenols.

3.4.5 Quality control of analytical results

In order to assure constant analytical quality over the whole set of 112 samples (56 samples of chocolate or chocolate raw products analysed in duplicate) the sample of cocoa liquor that had already been used for method validation (section 3.3.2.2) was the quality control (QC) sample. All samples were coded and the order of analysis determined through random number selection. Analyses were then performed in 16 blocks of 7 samples plus one QC sample. Quality control charts were created by plotting individual polyphenol contents determined in each set for the QC sample against sample set number (1 to 16).

Constant analytical quality over the whole set of samples was proved, as no trend over time was observed for any individual polyphenol and less then the statistically expected 5% of the values laid outside the expanded measurement uncertainty (with a coverage factor of 2 corresponding to a 95% confidence level as determined during the method validation procedure).

Figure 3.68 represents an exemplary quality control chart illustrating the results for catechin concentration in the QC sample over the 16 blocks of analyses.

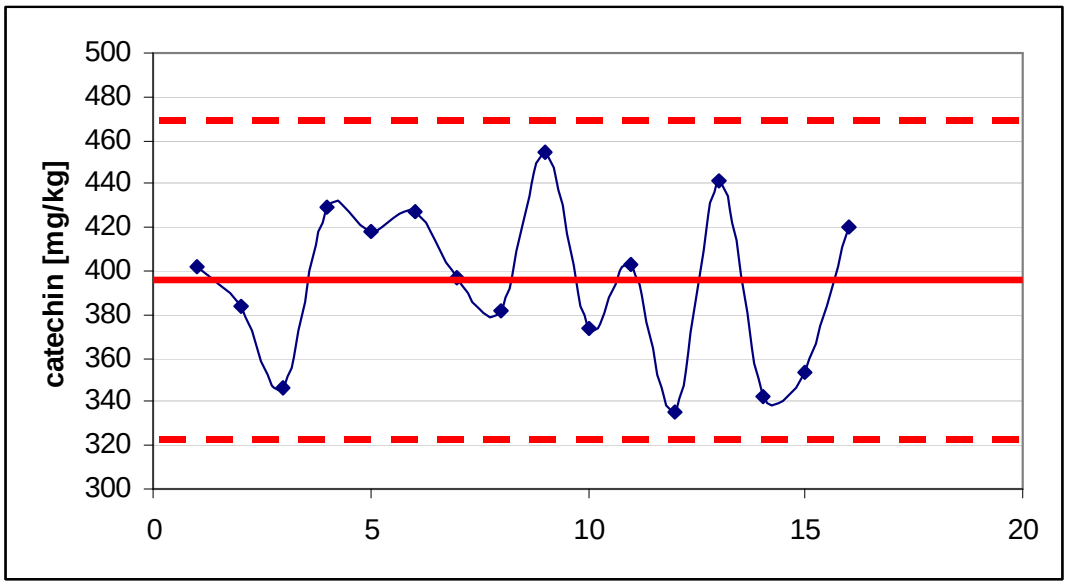


Figure 3.68 Quality control chart for catechin concentration in a cocoa liquor sample – red continuous line represents the average, red dashed lines represent mean value ± expanded measurement uncertainty with a coverage factor of 2 corresponding to a confidence level of 95%.

An additional proof of the maintained analytical quality over the whole set of analyses was derived from the constant (i.e., > 95% of the values were inside the expanded measurement uncertainty) area under the curve of the internal standard taxifolin, which had been added to all 128 analysed samples at constantly 500 µg (graph not shown).

3.4.6 Folin-Ciocalteu assay versus HPLC-MS

The Folin-Ciocalteu assay is widely used as a rapid and simple to perform measure of "total polyphenols" (Schofield et al., 2001). However, the development of the blue colour of the reaction products that is photometrically measured and quantified against catechin calibration solutions is based on redox reactions and is thus not specific for polyphenols (see discussion in chapter 1.4.3).

In order to investigate on the capacity of the Folin-Ciocalteu assay as a rapid estimation of total polyphenols all 128 samples analysed by HPLC-ESI-MS were analysed by the Folin-Ciocalteu assay as well and the results for total polyphenols (the sum of 22 phenolic compounds in the case of HPLC-MS analyses) compared.

Figure 3.69 shows a plot of total polyphenols determined by Folin-Ciocalteu assay versus sum of polyphenols by HPLC-MS.

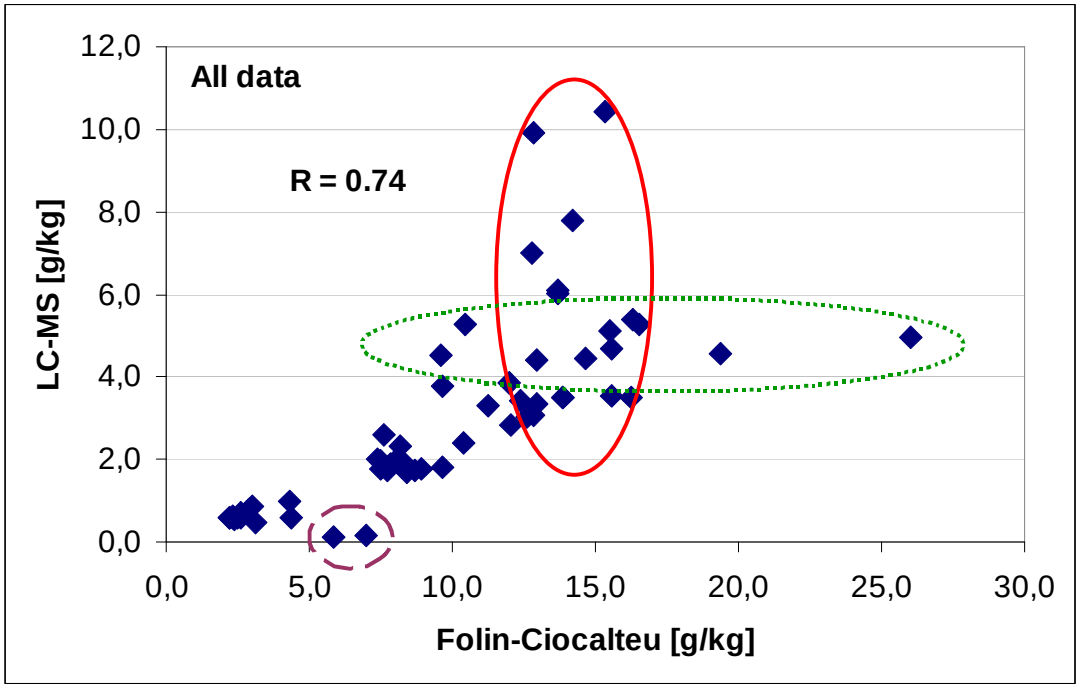


Figure 3.69 Correlation between total polyphenols as determined by the Folin-Ciocalteu assay and sum of all single measured polyphenols by HPLC-MS (R = 0.74, significant at $p<0.05$) – ellipses highlight subgroup of samples where the results obtained by the two methods clearly do not correlate at all.

Total polyphenols as measured by summing up single polyphenol concentrations from HPLC-MS analyses and determined colorimetrically by the Folin-Ciocalteu assay showed a significant ($p < 0.05$) positive correlation ($R = 0.74$). Nevertheless, this apparent good and significant correlation needs to be evaluated with care because subgroups of samples having approximately similar values according to HPLC-MS varied greatly in results from the Folin-Ciocalteu assay (green dotted ellipse) and vice versa (red continues-line ellipse).

Moreover, the two samples highlighted in the violet (long dashed line) ellipse are two cocoa liquor samples that had been kept at 100 °C for 72 hours in a laboratory oven,

which led to an almost complete degradation of all 22 polyphenols determined in the HPLC-MS analyses, whereas significant "reducing activity" was maintained as measured by the Folin-Ciocalteu assay (figure 3.70).

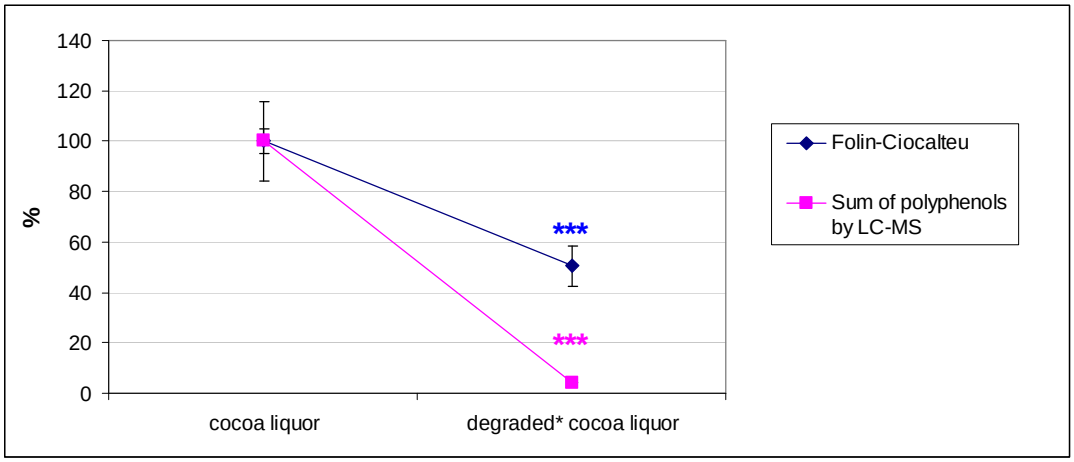


Figure 3.70 Relative changes of total polyphenol contents of cocoa liquor determined applying the Folin-Ciocalteu assay and by summing up single polyphenols measured by HPLC-ESI-MS in a cocoa liquor sample analysed before and after 72 hours heat treatment at 100°C in a laboratory experiment, respectively - error bars represent expanded measurement uncertainty with a coverage factor of 2 corresponding to a confidence level of 95%, *** refers to a statistical significant change ($p<0.001$).

It would be very interesting to understand what compounds in cocoa other than polyphenols were responsible for this remaining reducing activity. This would allow both to better understand the significance of the results of assays like the Folin-Ciocalteu assay and – probably even more interestingly – to investigate for other antioxidants in chocolate and other cocoa products that may be beneficial for human health, such as Maillard reaction products, partly oxidised and polymerised polyphenols (phlobaphenes).

Nevertheless, the investigation for such compounds would require lengthy procedures of isolation, purification and characterisation of unknown compounds as described in section 3.2.3, and was beyond the scope of this work.

3.4.7 Analyses of non-extractable polyphenols (NEPP)

In order to get an estimate on the amount of matrix bound high molecular weight procyanidins (i.e., non-extractable polyphenols, NEPP) in the various samples of cocoa beans, cocoa nibs, cocoa liquors, dark chocolates, and milk chocolates, that were not determined by HPLC-MS or Folin-Ciocalteu analyses, the modified proanthocyanidin assay as described in chapter 3.1.5 was applied on all residues of the aqueous acetone extraction throughout the analyses of the 128 samples.

Significant amounts of NEPP up to approximately 2.5 g/kg were detected in the residues reflecting ca 10 to 30% of the total polyphenol contents. Generally, levels of NEPP were higher in cocoa beans and cocoa nibs compared to chocolate samples and cocoa liquor. However, a significant ($p<0.05$) correlation between the total (extractable) polyphenol content, as calculated by summing up single polyphenol concentrations from HPLC-MS analyses, and the amount of NEPP determined by the modified proanthocyanidin assay (figure 3.71) suggest that the amount of NEPP is determined by the total polyphenol concentration of a cocoa sample rather than the type of cocoa product.

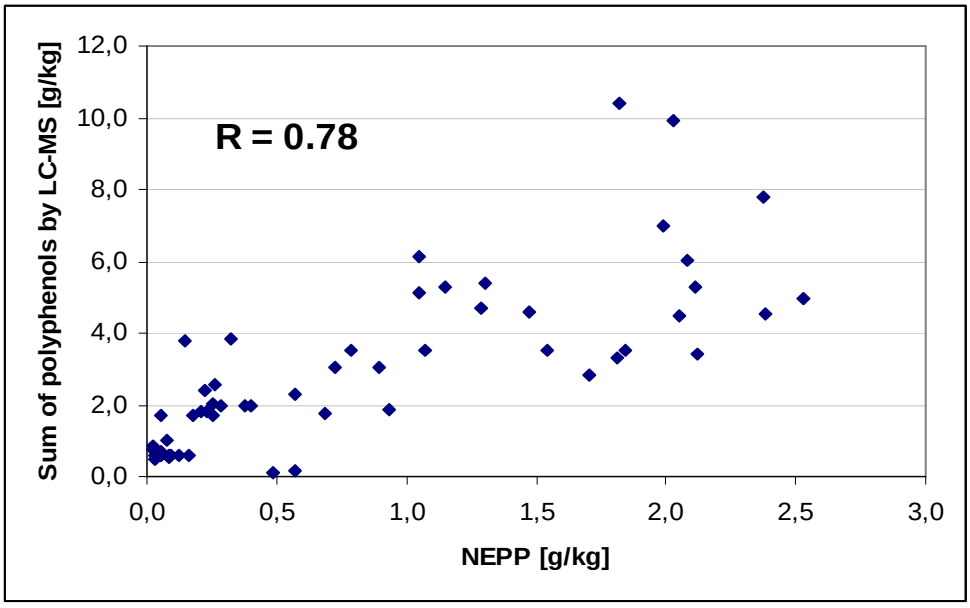


Figure 3.71 Correlation between non-extractable polyphenols (NEPP) as determined by the proanthocyanidin assay and sum of all single measured polyphenols by HPLC-MS (R = 0.78, significant at $p<0.05$).

Although the amount of NEPP as determined in this work by the modified proanthocyanidin assay must be considered a rather rough estimate, since the precision of this method was determined to be 15% (chapter 3.1.5), it is noteworthy that cocoa products contain significant amounts of high polymeric matrix bound procyanidins, which can still have important biological activity in the gastrointestinal tract or after microbial degradation and absorption in the colon as small molecular phenolic compounds.

3.5 Excursus A: Bioavailability of polyphenols from chocolate in humans

On important objective of the present work was to evaluate the possible health effects of polyphenols in chocolate. Whereas the quantitative relevance of chocolate polyphenols with respect to possible health effects was studied experimentally in the present work and the results discussed in chapter 3.4, there is also a need to consider the nutritional relevance, i.e. the fate of these compounds in the human body and their *in vivo* biological activity. This may in turn also allow for focussing on biological active phenolic compounds in future determination of polyphenol contents in chocolate, thus saving time and costs. Therefore, it was the objective in this part of the work to develop a method based on HPLC-ESI-MS capable of directly identifying human plasma and urinary polyphenol metabolites, which may be considered as the actual bioactive agents deriving from chocolate polyphenols *in vivo,* in a reasonable short analysis time after consumption of chocolate. As the presence of milk proteins has been suggested to reduce the antioxidant capacity of tea and chocolate polyphenols (Serafini et al., 1996 and 2003) both, dark chocolate and milk chocolate, were included for these preliminary studies.

The methodology used for this part of the work is described in chapter 2.5.3 and included the preparation of plasma from whole blood samples followed by extraction of phenolic metabolites from plasma and purification and concentration of these extracts by solid phase extraction (SPE). Urine samples were directly subjected to solid phase extraction. SPE extracts containing the polyphenol metabolites were then by HPLC-MS under virtually identical conditions as applied for qualitative analyses of polyphenols in cocoa liquor extract (chapter 3.2).

In a small pilot study two volunteers abstained from polyphenol-rich foods for two days, and after overnight fasting consumed 100 g chocolate or on a separate occasion 200 g milk chocolate both containing approximately the same amounts of epicatechin, catechin, procyanidin B2, higher procyanidin oligomers, hydroxycinnamic acid amides (clovamides), and quercetin compounds, respectively (described in chapter 2.5.2).

Plasma sample prepared from blood taken before and 2 and 6 hours after consumption as well as urine collected before, and in 3 hour intervals after consumption were analysed by HPLC-MS in search for various metabolites based on the results obtained by other reasearchers (reviewed in chapter 1.5.2).

26 phenolic compounds have been identified in cocoa in this work (section 3.2) and all probably result in several metabolites according to the mechanisms discussed above. However, in chocolate only four major polyphenols, epicatechin, catechin, procyanidin B2 and C1 make up 90% of all phenolic compounds. As a practical way forward exploratory investigation for metabolites was grouped in three phases:

1. Investigation for the four major polyphenols as well as their O-methylated, glucuronated and sulphated conjugates.

2. Investigation for minor polyphenols, such as quercetin, quercetin glycosides, clovamides, A-type procyanidins, and chlorogenic acid including their putative conjugates.

3. Investigation for all proposed microbial ring fission products as described above considering both free and conjugated forms.

First attempts to identify cocoa polyphenols and their metabolites eluting from the HPLC column by simple full scan mass spectrometric detection failed to highlight metabolites most likely due to insufficient selectivity and sensitivity of this approach in the highly complex mixture of endogenous and exogenous plasma and urinary compounds. Therefore, CID-MS^n experiments up to four stages (MS^5) were carried out with the ion trap mass analyser in various repeated runs on various urine and plasma samples.

The investigation for the four major compounds and their expected metabolites included a total of 32 compounds, for which single and multiple stage CID experiments were carried out. The procyanidin trimer C1 was not detected in any of the investigated forms and only trace amounts of intact procyanidin dimer B2 as sulphate and glucuronide conjugates were found suggesting an extremely low or even no bioavailability of intact procyanidins from chocolate.

In contrast, catechin and epicatechin as well as most of their anticipated metabolites were seen both in plasma and urine samples. However, the presence of catechin glucuronide and sulphate conjugates were the only forms of catechin detected with this methodology whereas for epicatechin all eight possible forms were confirmed, including free epicatechin, methylated epicatechin as well as their respective glucuronides, sulphates, and mixed sulfoglucuronides, respectively.

Figures 3.72 shows the CID-MS-MS spectrum of deprotonated epicatechin ($[M-H]^-$ m/z 289) in a urine sample, the identity of which was confirmed by comparison with a commercially available authentic standard. Identical mass spectra were also observed after performing CID-MS^n experiments on presumed deprotonated epicatechin glucuronide, sulphate or mixed sulfoglucuronide conjugates after isolating and further fragmenting m/z 289, thus suggesting neutral losses of conjugated acids. Figure 3.73 shows the respective reconstructed mass chromatograms for epicatechin and epicatechin conjugates. Finally, figures 3.74 and 3.75 show the corresponding results for O-methyl-epicatechin and O-methyl-epicatechin conjugates.

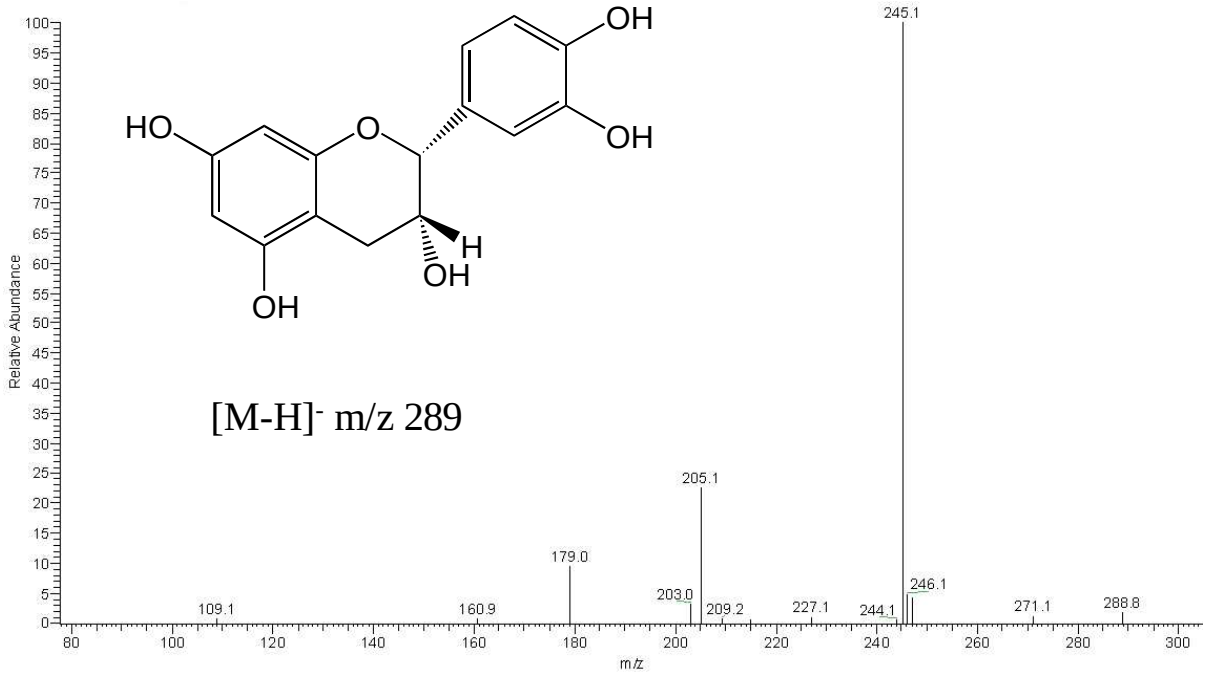


Figure 3.72 CID-MS-MS spectrum of deprotonated epicatechin or MS^n spectrum of deprotonated epicatechin conjugates (MS^{n-1} resulting in at least one product ion of m/z 289 corresponding to deprotonated epicatechin)

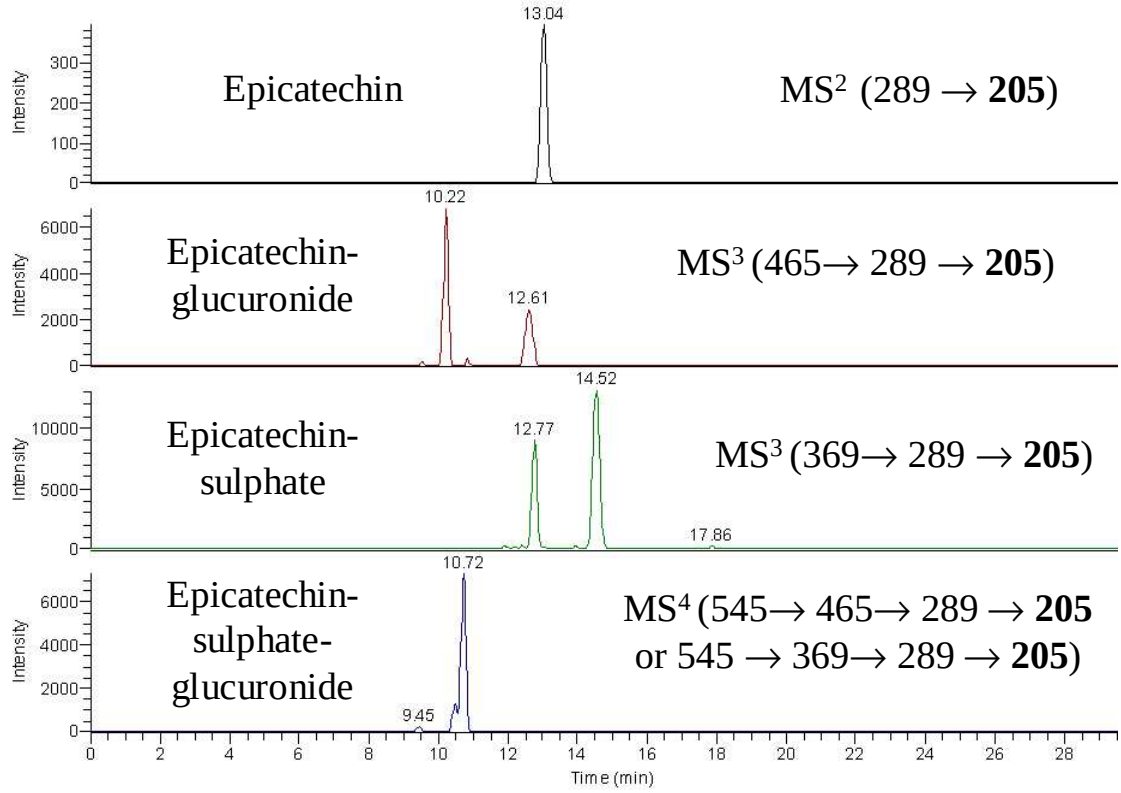


Figure 3.73 MS^n mass chromatograms of epicatechin and metabolites in urine – selected ion for extraction: m/z 205 - m/z 245 and m/z 179 were used for positive identification.

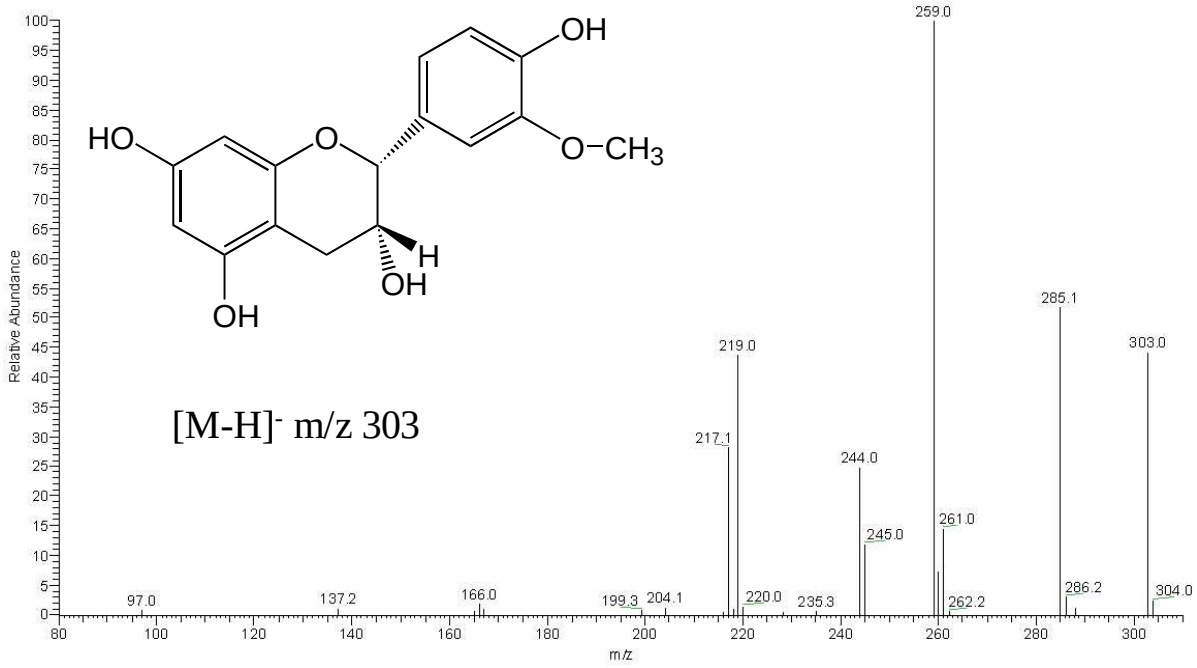


Figure 3.74 CID-MS-MS spectrum of deprotonated O-methylepicatechin or MS^n spectrum of deprotonated O-methylepicatechin conjugates (MS^{n-1} resulting in at least one product ion of m/z 289 corresponding to deprotonated O-methylepicatechin)

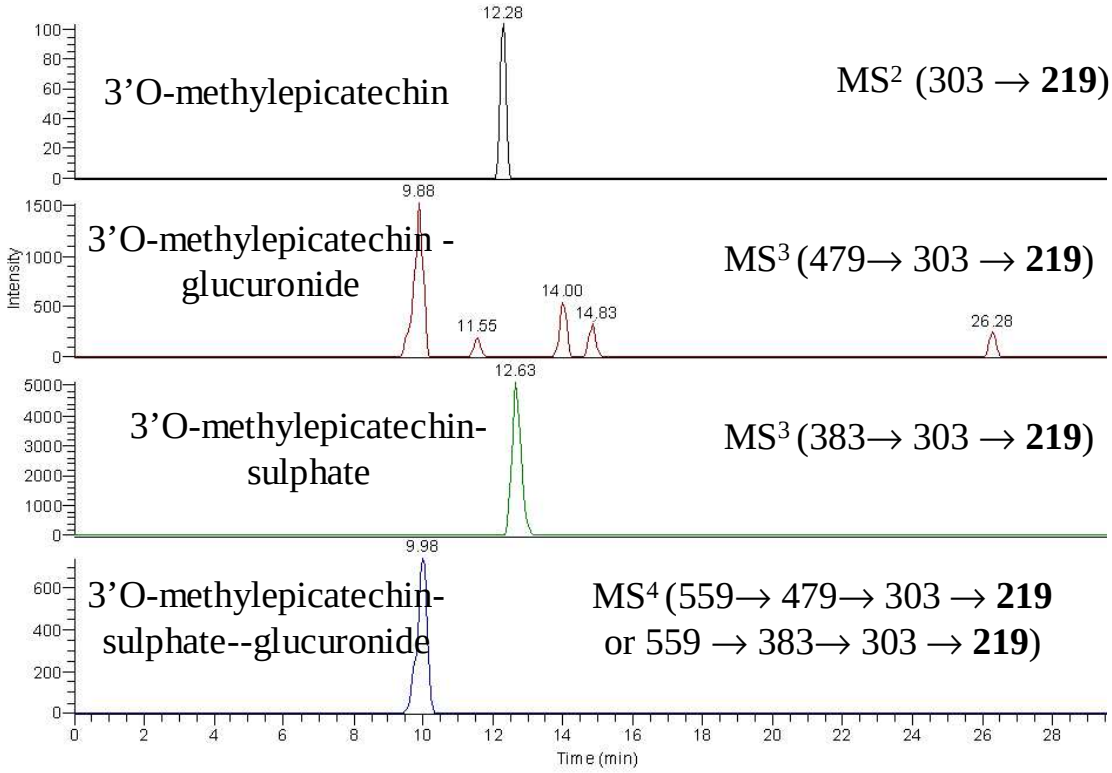


Figure 3.75 MS^n mass chromatograms of O-methylepicatechin and metabolites in urine – selected ion for extraction m/z 219 - m/z 259 was used for positive identification.

While epicatechin identity was possible to confirm by comparing the retention times and the CID-MS-MS spectrum of the ion in the urine or plasma sample to that of an authentic standard of epicatechin, this was not possible for any other urine or plasma metabolite of epicatechin, because such compounds are not commercially available. Consequently, O-methylepicatechin was only *tentatively* identified according to the mass of the deprotonated ion ($[M-H]^-$ m/z 303) as well as the CID-MS-MS spectrum thereof, which showed the same fragmentation pattern as free epicatechin with masses shifted up by 14 Da (-H substituted by $–CH_3$). In addition, typical losses for methyl groups of 15 Da corresponding to the loss of a radical ($CH_3\cdot$) as proposed by Cuyckens et al. (2000) and Wolfender et al. (2000) of the main fragment ions were observed in this spectrum or by a subsequent CID step (MS^3 303 → **259** → **244**, and 303 → **219** → **204**).

Glucuronides, sulphates and mixed sulphate-glucuronides of both epicatechin and O-methylepicatechin were identified by the mass of their deprotonated ions as well as by single and multiple CID experiments (figures 3.73 and 3.75). For instance, after single stage CID the deprotonated ion of epicatechin glucuronide ($[M-H]^-$ m/z 465) showed as the dominating product ion m/z 289 corresponding to deprotonated ion of the free epicatechin aglykon. In a second stage CID this product ion showed the exact fragmentation pattern as the deprotonated ion of epicatechin confirming the presence of an epicatechin based metabolite (figure 3.72).

From a quantitative point of view the main form present in urine and plasma was the sulphate for both free and methylated epicatechin. Quantitative occurrence was in decreasing order: sulphate > glucuronide > sulphate-glucuronide > free aglykon. It is striking that both epicatechin and O-methylepicatechin as free aglykons were only present in trace amounts in plasma as well as in urine whereas almost all of the intact epicatechin was present in a conjugated form.

Compared to the suggested effects for epicatechin in its original form found in cocoa the bioactivity of these *in vivo* metabolites might differ significantly, because the increased polarity of the conjugates compared to the aglykon greately reduces its ability to partition, which would limit its access to cells (Spencer 2003), possibly making a re-evaluation of the biological relevance of this polyphenol necessary. In fact, it has been

reported that uptake of epicatechin glucuronide into both cortical neurons and dermal fibroblasts was not detectable (Spencer et al., 2001a).

Non-methylated epicatechin and its metabolites appeared to be predominant over the respective O-methylated form in the present studies. Interestingly, O-methylepicatechin was equally effective *in vitro* as epicatechin at preventing oxidative damage in various different cell types as well as the oxidation of LDL (Schroeter et al., 2001; Spencer et al., 2001b).

Plasma levels of the epicatechin and its metabolites were higher at 2 hours compared to 6 hours as suggested from comparing respective ion intensities in the present study. The urine samples from 0 to 3, 3 to 6 and 6 to 9 hours had clear detectable levels of epicatechin and its metabolites whereas levels decreased continuously from 9 to 24 hours. There appeared to be no quantitative difference between levels after ingestions of similar amounts with dark chocolate or milk chocolate, respectively. However, a clear statement on quantities cannot be made in this work due to the absence of standard compounds for use as calibration reference and the lack of optimisation and validation of the method used in this party of the work for quantitative analyses.

Nevertheless, Heimann and Merfort (1998b) ,Scalbert et al. (2002) and Spencer (2003) summarised in their respective reviews that epicatechin recovery in urine in its intact form (i.e., free as well as methylated, glucuronated, and sulphated) was only 0.5 to 5% depending on the dose and the food matrix ingested, whereas much higher amounts (up to 40%) where reported as various colonic microflora metabolites. Thus, in agreement with the recommendation made by Scalbert et al (2002), it seems more appropriate to focus future research on polyphenol bioavailability and bioactivity mainly on the quantitatively dominant and numerous metabolites of the colonic microflora rather than on polyphenols found in plants and foodstuffs in order to determine the active fraction of phenolic compounds among all those circulating in the organism, and to determine their best dietary precursors and dietary sources.

Following the second of the three-step strategy for identification of cocoa polyphenol metabolites in this work, urine and plasma samples were explored by the HPLC-MS method for intact minor cocoa polyphenols including quercetin and two quercetin glycosides, three hydroxycinnamic acid amides (clovamides), A-type procyanidins, and

chlorogenic acid both in their free form as well as putative metabolites due to methylation, glucuronidation and sulphatation, respectively. No indication for the presence of any of the suspected substances were derived from HPLC separation with full scan mass spectrometric detection or by CID-MS^n experiments on target ion corresponding to anticipated deprotonated ions of such metabolites.

This was to be expected for A-type procyanidins because only trace amounts of B-type procyanidin dimer, that is present in several fold higher amounts in chocolate, was found in urine and plasma with the present experimental approach. Also the unlikely detection of chlorogenic acid or likely metabolites was expected due to its very low concentration in chocolate.

In contrast, it was thought likely to detect some form of quercetin as its bioavailability had been demonstrated previously (Heilmann and Merfort, 1998a). It may be that the predominant presence of quercetin linked to arabinose and galactose moieties in chocolate resulted in a significantly reduced bioavailability in the small intestine compared to free quercetin and quercetin bound to glucose, because the known enzymatic activity for the hydrolysis of quercetin-glucoside by cytosolic beta-glucosidase in intestinal cells may be significantly lower for other sugar residues. Moreover, ingested quercetin in the present pilot study, even when expressed as the sum of all quercetin compounds, was 20 fold lower than ingestion of epicatechin, which may result in concentrations of single metabolites in the plasma and urine samples, which fall below the detection capabilities of the selected HPLC-MS method.

To the author's knowledge, there is no information on the bioavailability of hydroxycinnamic acid amides (clovamides) from chocolate or any other source, such as red clover (Tebayashi et al., 2000). Nevertheless, the experiments carried out in this part of the work did not produce any evidence for bioavailibility of clovamides, as none of the identified cocoa clovamides or presumed metabolites were detected in urine or plasma. Since a hydrolysis of the peptide bound to lead tyrosine or dihydroxy-phenylalanine (DOPA) as well as caffeic or p-coumaric acid may be considered to happen, pre- or postabsorption experiments were also carried out to explore for these two hyroxycinnamic acids and possible conjugates. However, neither caffeic nor p-

coumaric acid nor any of their possible conjugates were found by the applied HPLC-MS strategies described above.

Following the third step of the strategy the objective was to identify as many of the proposed approximately 30 phenolic compounds (an overview is given in chapter 1.5.2) as possible both in the free or conjugated form in urine by the applied HPLC-MS method.

In the analyses carried out in this part of the study very high amounts of hippuric acid were found in urine both during the polyphenol-free diet and after chocolate consumption. Nevertheless, hippuric acid excretion increased two to three fold in the urine samples 6 hours after chocolate consumption compared to the urine sample collected before. If this increase observed in the studies carried out within this work were solely due to the polyphenols present in chocolate, hippuric acid would represent by far the most abundant cocoa polyphenol metabolite excreted in urine. Clifford et al. (2000) and Olthof et al. (2003) have also reported on hippuric acid as the major metabolite representing more than 50% of all metabolites formed after black tea ingestion. Nevertheless, hippuric acid (benzoyl-glycine) is a known metabolite of other metabolic pathways and it is difficult to judge, whether the increase was solely due to bacterial metabolisation of cocoa polyphenols.

Without assertion of completeness only a few other of the proposed microbial metabolites were tentatively identified by the HPLC-MS experiments carried out within this work, however concentrations appeared to be rather low. These tentatively assigned phenolic acids include: hydroxyphenylpropionic acid, hydroxyphenylacetic acid, hydroxy-methoxyphenylacetic (homovanillic) acid, hydroxybenzoic acid, and hydroxy-methoxybenzoic (vanillic) acid. The tentative assignments were based on MS-MS spectra compared to those of standard compounds (hydroxyphenylacetic acid, hydroxybenzoic acid, vanillic acid) or by comparing the spectra of similar compounds (hydroxyphenylpropionic acid and homovanillic acid) with those of the before mentioned standard compounds and subsequent identification of respective fragment ions.

In addition to the compounds detected and tentatively assigned from the experiments carried out within the present work, Rios et al. (2003) reported on increases in ferulic

acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, and 3-hydroxyhippuric acid after consumption of polyphenol-rich chocolate. It is striking that most significant increases of those metabolites have been found in urine samples from 9 to 24 hours and 24 to 48 hours after chocolate consumption (Rios et al., 2003). Here the samples primarily used for exploratory analyses were those collected between 3 and 12 hours after chocolate consumption, because the highest amounts of epicatechin metabolites were found in these fractions. Thus, microbial break-down products may appear predominantly in later fractions not investigated in the experiments carried out in the present work. This would explain the difficulty here in detecting and tentatively identifying microbial cocoa polyphenol metabolites.

Due to the lack of unambiguous identification of proposed microbial cocoa polyphenol metabolites as well as the possibly incomplete recovery of the main amount of those compounds by not collecting urine over at least 48 hours, no statement can be made from the results obtained in the present work on the influence of milk proteins on microbial metabolism of cocoa polyphenols in milk chocolate compared to dark chocolate.

In conclusion, the capability of the combination of HPLC and modern ion trap mass analysers for analysing a number of different metabolites of polyphenols in bio-matrices, such as blood plasma and urine in combination with reduced sample preparation and short analysis time, was demonstrated in this part of the work. Eight metabolites of intact epicatechin, each 2 catechin and procyanidin B2 conjugates in low amounts as well as 6 microbial polyphenol metabolites were identified by SPE-HPLC-MS analysis of human urine and plasma samples after ingestion of 100 g dark chocolate or 200 g milk chocolate, respectively. In contrast to earlier methodologies based on HPLC with ultraviolet, fluorescence, or electrochemical detection, tandem mass spectrometry allows for the direct identification and quantification of polyphenols conjugates avoiding enzymatic or conventional acid hydrolysis adding a major source of erroneous recovery.

Nevertheless, accurate quantification requires standard substances for calibration, which are not commercially available for most polyphenol metabolites. Lengthy and tedious isolation and purification procedures from complex matrices such as urine or in vitro

synthesis with isolated enzymes, microsomes or intact hepatocytes are required (Oliveira and Watson, 2000). Another interesting possibility is the synthesis or biosynthesis of stable isotope labelled polyphenols both for the use as internal standard compounds for isotopic dilution mass spectrometry techniques and unambiguous identification of polyphenol metabolites as well as for identifying metabolic routes in pharmacokinetic studies (Deprez and Scalbert, 1999; Rasku and Wähälä, 2000).

Due to the fact that some polyphenols form complexes with proteins it has been suggested that addition of milk reduces the bioavailability of polyphenols (Serafini et al., 1996 and 2003). However, from the preliminary data obtained in this part of the work it appears that epicatechin is equally well absorbed from dark and milk chocolate when correcting for the different amounts of polyphenols in the two types of chocolate. Nevertheless, studies with more subjects and accurate quantification of all metabolites including those from microbial metabolisation of simple and complex cocoa polyphenols are needed in order to investigate the influence of milk proteins and other food compounds on the fate of cocoa polyphenols in the human gastrointestinal tract.

3.6 Excursus B: In-vivo antioxidant activity of polyphenols from chocolate in humans – the biomarker approach

Much of the recent attention that polyphenols have gained is due to their suggested antioxidant activity and the possible implications in human health, such as in the treatment of age-related diseases including cardiovascular diseases and cancer. However, most data on antioxidant activity of polyphenols derive from *in vitro* or *ex vivo* studies on the compounds in its original form prior to metabolic changes in humans (discussed in chapter 1.5.3.1). As it would be practically very difficult to study each of the many possible metabolites in such studies, predominantly, because such substances are not commercially available, a more promising strategy is to compare diets or foods with varying amounts of polyphenols directly in clinical studies. For this, biomarkers are needed that are both patho-physiologically and analytically valid. The two most prominent markers for oxidative DNA damage (8-hydroxydeoxyguanosine, 8-OHdG) and lipid peroxidation (15-F_{2t}-isoprostane, 15-F_{2t}-IsoP) were discussed in chapter 1.6 with respect to patho-physiological validity and available methodology for their quantitative determination in urine. However, most applied methodologies are time-consuming and require several steps of sample preparation prior to instrumental analysis by gas chromatography-mass spectrometry or high-perfomance liquid chromatography-electrochemical detection, respectively.

Therefore, the objective of this study in collaboration with a diploma thesis (Lanfer, 2002) was to set-up and validate analytical methodologies based on recently available high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS) and enzyme immunoassay (EIA) for urinary 8-OHdG and 15-F_{2t}-IsoP and compare these methods with respect to analytical performance. In addition, intra-individual variability of normal levels for both markers was studied in two healthy subjects in order to investigate for appropriate number of subjects and/or appropriate sampling frequency in eventual future intervention studies.

3.6.1 High-performance liquid chromatography-tandem mass spectrometry for the measurement of urinary 8-hydroxydeoxyguanosine

A methodology based on single step solid phase extraction (SPE) for purification and concentration followed by HPLC with ion trap tandem mass spectrometry was developed and validated as part of this work for the analysis of urinary 8-OHdG. In addition, this method was compared to a commercially available EIA assay for urinary 8-OHdG, which was contemporaneously established in the context of a diploma thesis project (Lanfer, 2002).

For solid phase extraction (SPE) both reversed phase C18 and styrene divinyl benzene (SDVB) polymer cartridges were evaluated for clean-up and concentration of 8-OHdG from urine. The C18-cartridges were superior in terms of removing matrix compounds co-eluting with 8-OHdG from the HPLC column and thereby allowed for more accurate ionisation in the mass analyser. Possibly in part due to this removal of interfering matrix compounds, C-18 SPE sample clean-up showed better repeatability and was thus selected for further use. However, an approximately 50 fold concentration by SPE was necessary for obtaining sufficient amounts of 8-OHdG for HPLC-MS-MS analyses resulting in a 50 fold concentration of matrix compounds as well. This resulted in significant ion suppression in the mass analyser during electrospray ionisation as was deduced from apparent low recovery in preliminary studies. Therefore, an additional step for removing matrix compounds by ethyl acetate-heptane (1/1 v/v) was included following the initial washing procedure in the SPE procedure using 25 m*M* potassium dihydrogen phosphate and 3 ml of water prior to the final elution of 8-OHdG with methanol.

In addition, the mobile phase in the HPLC separation consisted of a very high water content and was set to an isocratic water/acetic acid/acetonitrile solution (97.8/0.2/2; v/v/v) until elution of 8-OHdG prior to after-run purification of the column with high proportions (50%) of the organic solvent. This resulted in an efficient HPLC separation of 8-OHdG from interferences, which mostly remained on the column till the post-run washing that was diverted to waste. The almost undisturbed ionisation of the analyte allowed accurate quantification and moreover, the ion source was not contaminated by

coeluting matrix compounds leading to a highly rugged method suitable for long uninterrupted sequences.

Figure 3.76 shows a typical mass chromatogram of the main fragment ion of protonated 8-OHdG ($[M+H]^+$ m/z 284) after CID-MS-MS in the ion trap of the mass analyser corresponding to the free protonated base 8-hydroxydeoxyguanine ($[M\text{-ribose}+H]^+$ m/z 168).

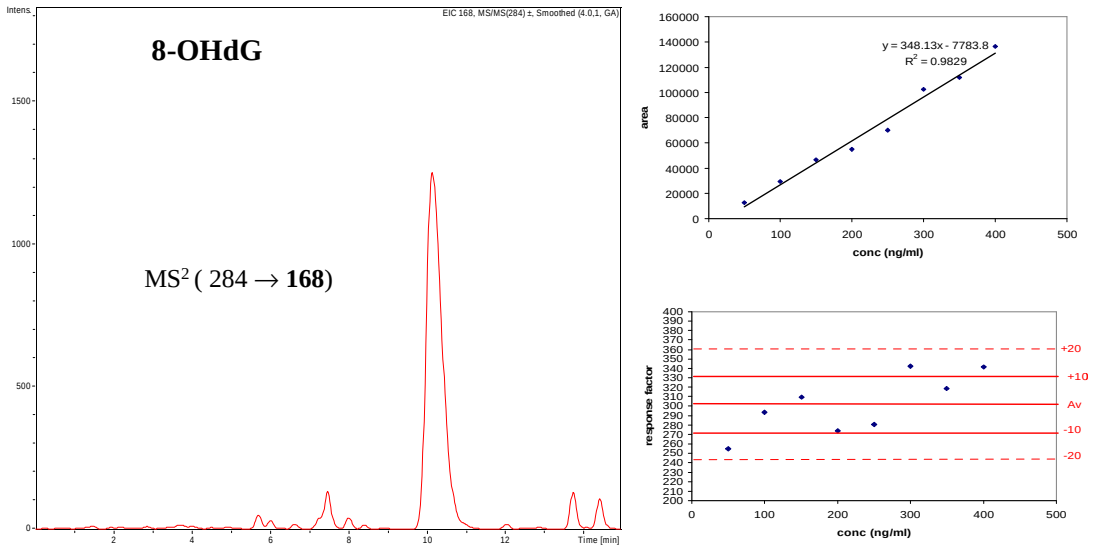


Figure 3.76 Typical mass chromatogram of the main fragment ion of protonated 8-OHdG after CID-MS-MS (m/z 284 → 168) of a urine sample (left) as well as the standard curve (top right) and response factors versus concentration (bottom right) proving linearity of the method.

The 8-OHdG standard curve of the HPLC-MS method was found to be linear in the concentration range of the curve ($R^2 > 0.98$). The standard curve and the corresponding plot of response factors (RF) versus concentration are shown on the right in figure 3.76. According to linear regression analysis the assumptions for linearity were fulfilled: the slope parameter was significantly different from zero ($p < 0.05$), while the y-intercept showed no significant difference from zero ($p > 0.05$) and the residuals were normally distributed ($p > 0.05$). The method was thus proven linear over the chosen application range from 1 to 8 ng/ml 8-OHdG in urine with a limit of detection of 0.1 ng/ml.

Precision was determined by analysing the same urine sample of a male volunteer 10 times with the whole method starting from the sampling procedure. The resulting coefficient of variation (CV%) was 10.8% for the urine sample having 2.34 ± 0.25 ng/ml 8-OHdG.

Recovery studies at three spiking levels (1, 2, and 3 ng/ml) revealed incomplete recovery, although at very constant rates of 85 ± 1%. The addition of an isotopically labelled internal standard for isotope dilution mass spectrometric detection should solve the problem of incomplete recovery. However, such an isotopically labelled 8-OHdG is currently not available on the market and thus the technically demanding synthesis and purification from available precursor compounds would be required. Nevertheless, for studies with cross over design, in which subjects are their own controls, a constant recovery even below 100% in combination with high precision is sufficient for the purpose of studying changes in the level of 8-OHdG through a dietary intervention.

In conclusion, the combination of structural information for unambiguous compound identification and the virtual absence of interfering matrix compounds in the mass selective detection of the present method based on a one step SPE followed by HPLC-MS-MS allowed for reproducible quantification with high compound specificity. This demonstrates the high potential of HPLC tandem mass spectrometry for the high-throughput analysis of urinary 8-OHdG as a marker of oxidative DNA damage in the low ng/ml range in a complex biological matrix such as urine.

When compared to a commercially available EIA kit the HPLC-MS-MS method was superior in terms of specificity, sensitivity and trueness while the EIA showed advantages in the simplicity and velocity of use (for details see Lanfer, 2002).

3.6.2 High-performance liquid chromatography-tandem mass spectrometry for the measurement of urinary 15-F_{2t}-isoprostane

A methodology based on high-performance liquid chromatography isotope dilution tandem mass spectrometry was developed and validated within the present work for the analysis of urinary 15-F_{2t}-isoprostane. In addition like for 8-OHdG, this method was compared to two commercially available EIA assay for urinary 15-F_{2t}-IsoP, which were

contemporaneously established in the context of the diploma thesis project (Lanfer, 2002).

For sample clean-up and concentration of 15-F_{2t}-IsoP from urine solid phase extraction (SPE) both reversed phase C18 from two suppliers and styrene divinyl benzene (SDVB) polymer cartridges were evaluated. Preliminary result from pilot studies in this laboratory showed values for urinary 15-F_{2t}-IsoP of approximately 200 to 300 pg/ml urine. These concentrations were approximately 10 times lower than those for urinary 8-OHdG. Accurate MS-MS quantification of 15-F_{2t}-IsoP required absolute amounts of 1 ng or more injected onto the column. Consequently, a concentration of 100 to 200 fold was necessary in order to obtain sufficient quantities. However, the established procedures following a single step SPE using either of the C18-cartridges or the styrene divinyl benzene polymer-based cartridges and subsequent HPLC-MS-MS analysis was insufficient with respect to repeatability and accuracy. It is believed that this was due to either overloading of the SPE columns and/or ion suppression in the mass analyser due to the 200 fold concentration of matrix compounds that were not removed by the SPE step.

In contrast to the high concentration requirements for the present HPLC-MS-MS set-up, the methods based on SPE followed by HPLC-MS-MS reviewed in chapter 1.6.2 usually required only a 10 fold concentration prior to the LC-MS-MS analysis. All of these described methods made use of triple quadrupole mass analysers in the multiple reaction mode (MRM). With this, deprotonated ions of 15-F_{2t}-IsoP are highly selectively isolated in a first quadrupole ion filter, transferred to a hexapole collision cell (second “quadrupole”) for collision induced dissociation (CID) resulting in specific product ions, one or two of which are again highly selectively isolated by the third quadrupole ion filter for final ion detection. On the contrary, the mass analyser used here was based on a more recent development of the ion trap technique. Here, the tandem mass spectrometry is achieved by “trapping” the target ions first, followed by CID in the ion trap and subsequent selective release of the product ions towards the ion detector for a product ion scan. Reconstructed ion chromatograms from the full product ion scan of selected specific daughter ions of deprotonated 15-F_{2t}-IsoP are then used for detection and quantification by means of MS-MS. Possibly, this ion trap analyser approach is less selective and/or less sensitive compared to the MRM technique of a

triple quadrupole machine, which in turn would explain the observed difficulties in accurate detection as well as the 10 to 20 fold higher pre-concentration need of the HPLC-MS-MS analysis method established here.

As additional steps applying silica based SPE, TLC or HPLC for clean-up would have abolished the advantage of HPLC-MS-based over GC-MS-based methodologies of short and less tedious sample preparation, commercially available immunoaffinity columns were used for highly selective extraction of 15-F_{2t}-IsoP from urine. The application of isotopically labelled 15-F_{2t}-IsoP-d_4 as internal standard added to urine prior to immunoaffinity clean-up (IAC) allowed control of recovery as well as accurate quantification by isotope dilution MS-MS analysis. Figure 3.77 (left) shows typical mass chromatograms of the main fragment ions (m/z 193, 255) of deprotonated 15-F_{2t}-IsoP ($[M-H]^-$ m/z 353) as well as the corresponding product ions (m/z 197, 259) of deprotonated internal standard 15-F_{2t}-IsoP-d_4 ($[M-H]^-$ m/z 357) after CID-MS-MS in the ion trap of the mass analyser in human urine.

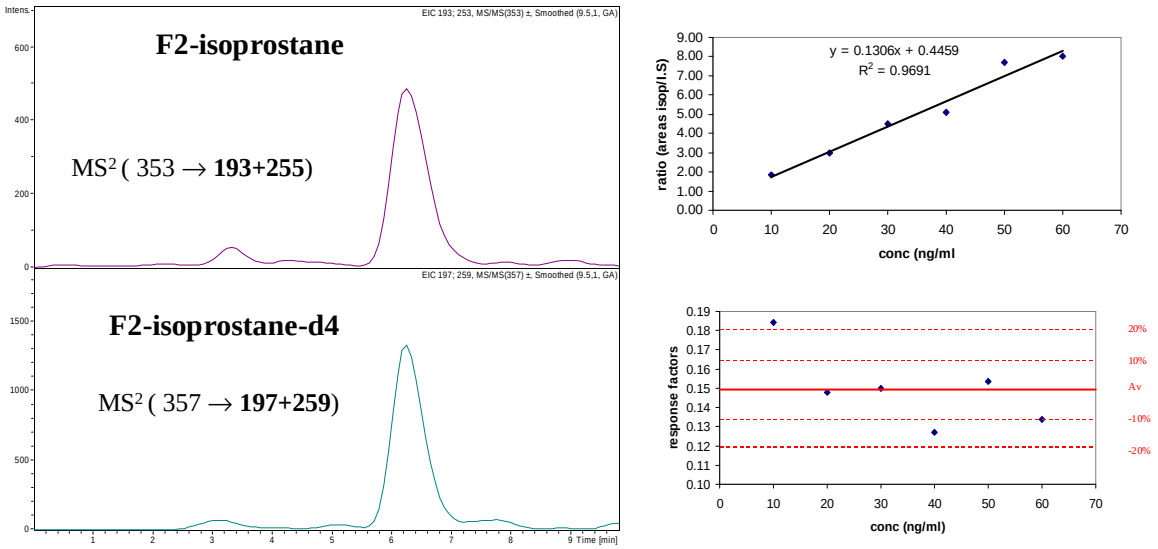


Figure 3.77 Typical mass chromatogram of the sum of the main fragment ions after CID-MS-MS of deprotonated 15-F_{2t}-IsoP (top left: m/z 353 → 193 + 255) and deuterated internal standard 15-F_{2t}-IsoP-d_4 (bottom left: m/z 357 → 197 + 259) of a urine sample as well as the standard curve (top right) and response factors versus concentration (bottom right) proving linearity of the method.

As demonstrated by the absence of interfering matrix compounds in the mass selective detection of both analyte and internal standard the developed method based on IAC and HPLC-isotope dilution tandem mass spectrometry allowed for reproducible quantification with high compound specificity.

The 15-F_{2t}-IsoP standard curve of the HPLC-MS method was found to be linear in the concentration ranges of the curve ($R^2 = 0.97$). The standard curve and the corresponding plot of response factors (RF) versus concentration are shown in figure 3.77 (right). According to linear regression analysis the assumptions for linearity were fulfilled: the slope parameter was significantly different from zero ($p < 0.05$), while the y-intercept showed no significant difference from zero ($p > 0.05$) and the residuals were normally distributed ($p > 0.05$). The method was thus proven linear over the chosen application range from 50 to 300 pg/ml 15-F_{2t}-IsoP in urine with a limit of detection of 20 pg/ml.

Precision was determined by analysing the same urine sample of a male volunteer 10 times with the whole method starting from the sampling procedure. The resulting coefficient of variation (CV%) was 6.4% for the urine sample having 271 ± 17 pg/ml 15-F_{2t}-IsoP. Recovery studies at two spiking levels (100 and 200 pg/ml) demonstrated complete recovery of 94 ± 11%. The complete recovery in contrast to the incompleteness for 8-OHdG described above was believed to be a result of the addition of isotopically labelled 15-F_{2t}-IsoP to the urine samples as internal standard for compensation of analyte losses during IAC as well as for isotope dilution mass spectrometric detection.

In conclusion, the highly selective extraction of 15-F_{2t}-IsoP from relatively large amounts of urine (usually 20 ml were applied) by IAC together with the use of an isotopically labelled internal standard allowed fast (HPLC run time is only 8 minutes) and accurate quantitative analysis of urinary 15-F_{2t}-IsoP with the present methodology.

This method was also compared to two different commercially available EIA kits for the measurement of urinary 15-F_{2t}-IsoP (Lanfer, 2002). However, only one of these kits proved to be sufficiently sensitive for normal urinary 15-F2t-IsoP levels. The other EIA required sample clean-up using immunoaffinity columns similar to those used for the present HPLC-MS-MS method prior to the administration to antibody-coated wells for photometric measurement. As the EIA method required additionally over-night

incubation the HPLC-MS-based method was the by far more rapid one. HPLC-MS showed further superiority in specificity, precision and trueness due to the highly selective IAC combined with specific and accurate quantification by isotope dilution MS-MS, although the EIA delivered results of good accuracy as well and was 5 to 10 times more sensitive (Lanfer, 2002). Consequently, both methodologies delivered accurate determination of urinary 15-F_{2t}-IsoP levels and only the exact purpose of the study and the expected variation in 15-F_{2t}-IsoP levels can determine which method best to use.

3.6.3 Biological variation of urinary levels of 8-OHdG and 15-F_{2t}-IsoP

In order to study the biological variability of normal levels for both markers, urinary 8-OHdG and 15-F_{2t}-IsoP were studied in two healthy subjects (1 male and 1 female) on 17 consecutive days. Both the extent of intra-individual variability within days (trial A) and between days (trial B) were studied. Trial B included four intervention days (day 10-13) to investigate the short-term effects of physical exercise, of a low and high antioxidant diet and of increased consumption of chocolate, a particular rich source of polyphenolic antioxidants, on the marker levels. For the determination of the urinary biomarkers previously tested EIA methods were used. Urinary creatinine was assessed by capillary electrophoresis with diode-array-detection (CE-DAD) (for details see Lanfer, 2002).

There was no significant short-term effect of the interventions on the marker levels, but mean creatinine excretion was significantly higher on the day when subjects performed strong exercise. The highest intra-individual coefficients of variance (CVs) within and between days (without intervention days) were 38 % and 30 %, respectively, for 8-OHdG as well as 47 % and 35 %, respectively, for 15-F_{2t}-IsoP. The CVs, which include a biological and an analytical variation, even increased by standardisation on creatinine as well as when the data of the intervention days were included. The highest CV obtained for urinary creatinine within a day was 16 % and between the days 12 %.

In good accordance with the values observed here, Helmersson and Basu (2001) examined 15-F_{2t}-IsoP excretion rate and day to day variation in 13 subjects over ten days and observed an intra-individual variation of 42% in urinary 15-F_{2t}-IsoP. Pilger et al. (2001) found a mean intra-individual variation of 48% (range 18 to 106%) on 6

consecutive series of measurement of urinary 8-OHdG in 68 healthy probands. Later, the same group reported **inter**-individual variation in urinary 8-OHdG levels of 54% for 24-hour urine levels in 67 healthy subjects, 58% when corrected for creatinine, and 60% in spot urine samples of 148 healthy participants (Pilger et al., 2002).

In view of the high intra-individual variations in levels of urinary 8-OHdG and 15-F_{2t}-IsoP it appears that the intra-individual variability of the urinary markers has been underestimated as yet. Two questions have to be answered before starting a study:

- What change in marker levels is biologically relevant?
- How many subjects does the study need in order to show this biologically relevant change given that the expected variance (combined biological and analytical variance) is known?

The answer to the first question might vary depending on the specific situation of the study population (healthy subject versus subjects suffering from a state of high oxidative stress) and the objective of the study (e.g., effect of antioxidant supplementation or comparison of marker levels in a group of subjects with a oxidative stress-related disease and a healthy control group). For instance, Loft and Poulsen, (1999) suggest that even small changes in the oxidation rate of 10 % may be biologically important.

For answering the second question, additional knowledge of the expected variation in observed marker levels in each group of samples to be compared is required. Since the intra-individual variation was examined in the present studies for urinary 8-OHdG and 15-F_{2t}-isoP, respectively, and ranged from 30-47% statistical power analyses were carried out in order to determine the required number of subjects to participate in possible future studies.

Figure 3.78 shows two exemplary calculations of the required number of subjects per group for a cross over design (left) as well as a parallel design (right). An expected variation in marker levels of 40 % as determined in this study was assumed and the desired minimal change to be shown in this study was set to 10% as proposed by Loft

and Poulsen (1999). Statistical significance was hypothetically to be examined at $p < 0.05$ (α-error) and the minimum statistical power was set to 0.8.

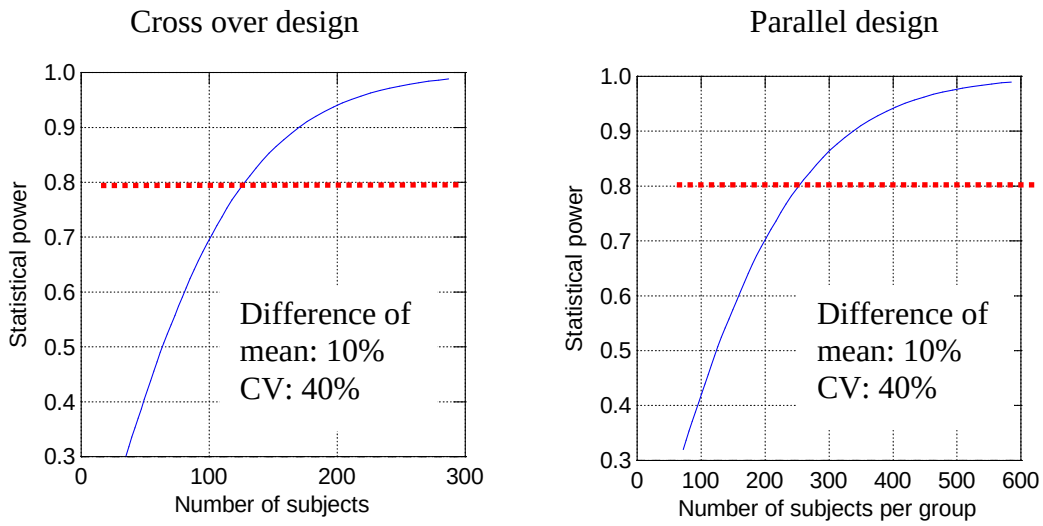


Figure 3.78 The relation of the number of study subjects and statistical power assuming an expected difference of mean values of 10% as well as a combined analytical and biological variability of 40% CV in a cross over design (left) and a parallel design (right), respectively – the red dash line indicates a statistical power of 0.8, which is usually considered as minimum required power.

As can be depicted from the horizontal dashed line, at least 128 subjects in a cross-over design (figure 3.80 left), where subjects are their own controls, and even 252 subjects per group in a parallel design, with two independent groups (exposure and control) (figure 3.80 right), would be required according to a statistical power analysis.

Of course, alternative to the increase in the number of subjects in a study, strategies such as repeated sampling of urine at various time points of control and exposure period or group as well as replicate analytical measurements may be applied in order to reduce the variance of means. How the variability influences the number of samples (subjects) determinations is illustrated in figure 3.79. In addition, this graph also highlights the inter-relations between effect size, biological variability, and number of subjects that need to be considered in the planning of clinical studies using the biomarker approach.

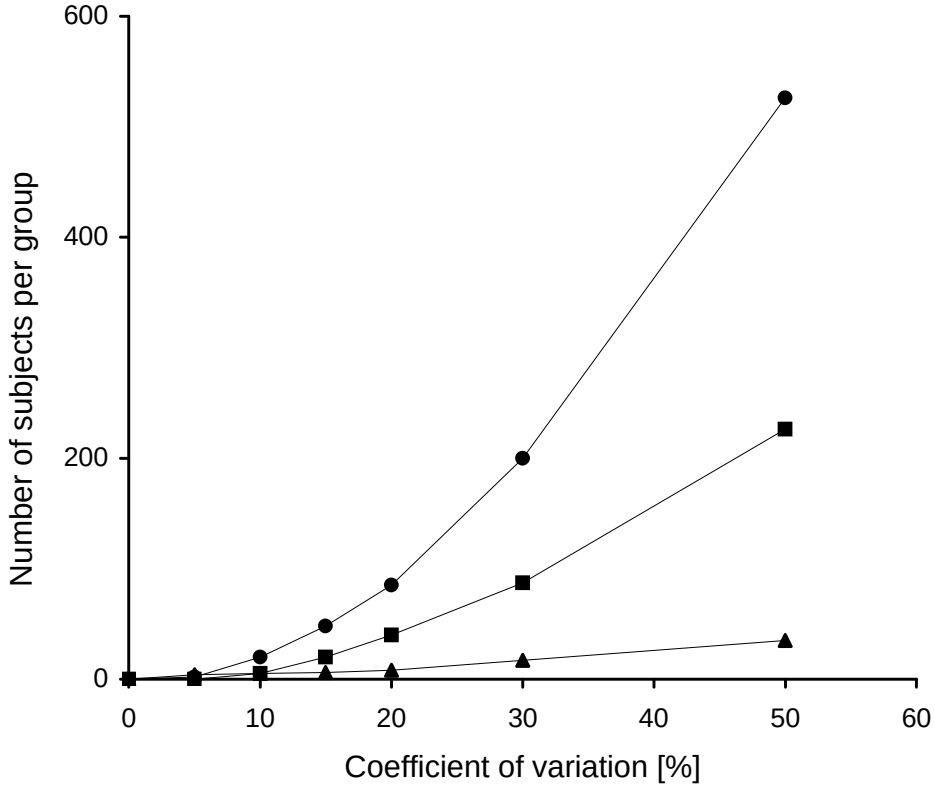


Figure 3.79 Influence of variability (CV) on the number of subjects required in order to demonstrate a difference between two different groups (parallel design). The difference is set at 10 % (●), 15 % (■), or 40 % (▲). Graph adapted from ESCODD (2000).

In fact, in some cases it seems possible that the lack of effects in intervention trials (reviewed in Lanfer, 2002), in which urinary 8-OHdG or 15-F_{2t}-IsoP were measured before and after supplementation with antioxidants could be due to the limited number of subjects involved in the studies. The considerations in the present work strongly suggest that statistical power calculations based on the estimated combined biological and analytical variance and expected effects to be demonstrated for each marker be included in the planning of clinical trialsm as well as the determination of the sampling strategy aiming to investigate for targeted biologically relevant differences in biomarker levels.

4 Conclusions and outlook

Twenty-six phenolic compounds have been identified or tentatively identified in chocolate and cocoa within this work by means of high-performance liquid chromatography-mass spectrometry (HPLC-MS) as well as isolation and purification of compounds by combinations of flash chromatography, gel filtration chromatography, high-speed counter-current chromatography (HSCCC) and semi-preparative HPLC with subsequent structure elucidation by means of nuclear magnetic resonance (NMR) spectroscopy and various mass spectrometric experiments, such as single and multiple stage collision induced dissociation (CID) mass spectrometry (MS-MS and MS^n) and hydrogen/deuterium exchange mass spectrometry (H/D-MS). Two phenolic compounds – caffeoyltyrosine and p-coumaroyl-dihydroxy-phenylalanine – were isolated and unambiguously identified for the first time in cocoa. Although a very promising method based on micellar electrokinetic capillary chromatography (MECC) had been successfully developed as well, the HPLC-MS-based methodology was finally selected because of the greater selectivity of the mass spectrometric detection compared to the solely available diode array detection (DAD) system of the MECC system. This qualitative methodology was thus extended and thoroughly validated for quantifying 22 quantitatively relevant polyphenols in cocoa beans, cocoa nibs, cocoa liquor, dark chocolate, and milk chocolate. Compared to existing methodologies in the literature, this multi-compound method represents a significant extension in numbers of cocoa polyphenols included in combination with high selectivity and short analysis time for enhanced throughput.

Subsequently, the method was successfully applied for the analysis of 22 phenolic compounds in 128 chocolate and chocolate raw products samples in effectively only eight days of laboratory analysis. The multivariate analysis of this huge amount of data allowed the identification of fermentation (selection of beans with different degrees of fermentation), roasting, and – if applied – alkalising as key process parameters in the chocolate making process responsible for the decline and change in profile of polyphenols from the cocoa bean to the final product chocolate. Moreover, it was possible to identify the oligomeric procyanidins as the most labile compounds and to suggest oxidative degradation, epimerisation and deglycosidation as responsible

mechanisms for the observed changes. This study demonstrates the usefulness of the developed multi-compound method in combination with multivariate analyses for gathering a lot of information together with a reduction of time and cost for laboratory analyses. Moreover, it allows chocolate producers to target the identified key process parameters as well as particular groups of cocoa polyphenols for developing cocoa products with desired polyphenol contents and patterns. Obviously, the prioritisation on particular cocoa polyphenols depends not only on the quantitative relevance, but to a great extend on the *biologic* relevance for human health, which includes their bioavailability and bioactivity in the human body.

Both the experiments performed here and the literature survey revealed that chocolate polyphenols are absorbed from the gut, at least the monomers catechin and epicatechin and to a much lesser extent the procyanidin dimer B2, whereas higher oligomers do not enter the blood stream in their intact form. It was shown here, that in blood and urine almost all of the absorbed polyphenols are either methylated and/or conjugated to glucuronic and sulphuric acid. Thus, these metabolites should be considered as the actual compounds *in vivo*, for which biological activity should be studied in mechanistic *in vitro* experiments. In contrast, the original compounds as they apear in plants have been almost exclusively studied to date. None of the other identified chocolate polyphenols have been detected in either blood or urine with the experimental design chosen within this work nor has to the authors' knowledge such an absorption been described elsewhere in the scientific literature. Quercetin and quercetin-glucoside are known to be absorbed from other foods. However, most of the chocolate quercetin is bound to galactose or arabinose, for which very low absorption rates have been suggested. Thus, the absorbed quantity of quercetin from chocolate might have been below detection limits of the HPLC-MS methodology applied. Unfortunately, no information on the bioavailability of the interesting group of hydroxycinnamic acid amides (clovamides) isolated, purified and characterised within this work, was obtained by the performed bioavailibility studies, as the most obvious conjugates (methyl-, glucuronide, and sulphate) of the intact compounds and of the phenolic part (caffeic and coumaric acid) were absent in blood and urine of the human subjects taken part in the study or at least below the method detection limit. To study the fate of this group of phenolic compounds in the human body is clearly a future research objective, as they

had been demonstrated elsewhere to have equal or stronger antioxidant activity than epicatechin, at least *in vitro* or *ex-vivo*. Clearly, an until short time ago widely ignored pathway for the biggest part of chocolate polyphenols, in particular of the higher molecular weight procyanidins, of entering the human blood stream is through breaking down to smaller molecules (i.e., to monomeric catechins and various phenolic acids) by the colonic microflora and subsequent absorption. In the present work, only few and minor quantities of the proposed breakdown products were found, since the detection and identification of putative chocolate polyphenol metabolites was focused mainly on blood taken after two hours as well as urine collected three to twelve hours after consumption of chocolate by the volunteers, because in these fraction the highest concentrations were assumed from the experience reported by other researchers. However, it is now clear that significant amounts of colonic metabolites cannot be expected before six hours in blood and nine hours in urine. In fact, notable proportions of these metabolites had been detected in urine collected between 24 and 48 hours after ingestion, a fraction that was not collected in the present design. This is particularly relevant when interpreting the results from the numerous short-term studies that suggest health beneficial effects of cocoa polyphenols by measuring *in vivo* or *ex vivo* indicators after two hours of ingestion, which surely cannot be the results of bioactivity of those likely to be more abundant colonic metabolites compared to quickly absorbed intact polyphenols.

The in the scientific literature suggested health benefits of chocolate, cocoa, as well as enriched or isolated cocoa polyphenols include mainly protection against reactive oxygen and nitrogen species (antioxidant activity), cancer, cardiovascular diseases (CVD), together with diseases that involve malfunction of the immune system or excessive and chronic inflammation. Clearly, the strongest emerging evidence exists for potential benefits in CVD including those derived from reduced excess and chronic inflammation, since several short to mid-term human studies have been conducted recently. The studied mechanisms for such protection include the reduction of platelet activity and blood pressure, probably via induction of nitric oxide biosynthesis, as well as the inhibition of the biosynthesis of proaggregatory, vasoconstrictive, proinflammatory eicosanoids and enhanced synthesis of eicosanoids that are antiaggregatory and antiinflammtory vasodilators, all of which contribute to a reduction

in thrombosis risk. However, it should be notified that many of these effects have been observed with specifically designed cocoa or chocolate, rich in polyphenols compared to a control cocoa product manufactured to be polyphenol-poor. Although this design allows very well studying the effect of polyphenols it is impossible to identify whether the consumption of chocolate – extra polyphenol-enriched or not – exert the same beneficial vascular effects. Moreover, well-designed, randomised, controlled, and - if feasible - double-blinded long-term studies involving large study groups are still lacking and available results from two to six-week studies indicate very modest changes or even cannot confirm short-term findings at all. Thus, the biological relevance of small changes is an open question. Consequently, although the beneficial effects on cardiovascular health have been shown in several clinical studies, the results can only be considered preliminary due to the short durations and small study groups selected. In contrast, the evidence for the proposed antioxidant, anticarcinogenic and immunemodulating activities of cocoa polyphenols derive only from *in vitro*, *ex vivo* and animal studies. Although there are many such studies, the implication of these findings for the suggested health benefits in humans *in vivo* remains to be elucidated in future.

So far the protection against reactive oxygen species (ROS) by antioxidant cocoa polyphenols in humans has mainly been studied in short-term designs by investigating *ex vivo* antioxidant capacity of blood plasma or low-density lipoprotein (LDL) oxidisability with unknown relations to actual whole body oxidative damage and related degenerative diseases. Therefore, the development and validation of fast state of the art methodologies based on HPLC-MS and enzyme immunoassay (EIA) suitable for widely accepted urinary markers of oxidative DNA damage (8-hydroxydeoxyguanosine) and lipid peroxidation (15-F_{2t}-isoprostane) in the present work is considered an important contribution towards the implementation of these biomarker studies in the research on the *in vivo* antioxidant activity of polyphenols, particularly in larger human intervention studies. In addition to the thorough analytical validation process allowing the quantification of the analytical error, expressed as measurement uncertainty, intra-individual (biological) variations in urinary marker levels were studied as well in a preliminary study in order to estimate the expected error resulting from sampling. The exact knowledge of both analytical and sampling errors is considered crucial in view of

the planning of sufficiently powered human studies. Whereas these two markers will allow more soundly to study effects of polyphenols from chocolate or other sources on antioxidant protection against ROS-mediated damage, it is envisaged that future large long-term studies will include a wide variety of such surrogate markers that show established relations to various diseases or health status indicated by risk factors for such diseases, as well as the inclusion of metabolomics techniques studying metabolic changes without prejudication of a particular disease outcome, all of which will clearly give a greater insight in the actual *in vivo* effects of polyphenols from chocolate or of regular chocolate consumption, respectively.

Important issues remain the amount of chocolate that needs to be regularly consumed within varying diets for obtaining an effect and the nutritional implications that a significant increase in chocolate consumption, both on an individual and a population-wide basis, may have. In fact, the discussion on the potential adverse health implications of high or increased chocolate consumption revealed a possible role in the contribution to the worldwide epidemic of obesity with all its impacts on chronic diseases including CVD, cancer, diabetes and so forth due to the high energy-density of chocolate as main issue. In contrast, a modest cariogenicity of chocolate and moderate direct effects of cocoa butter on CVD, which are still under debate, appeared to be less matter of concern. In fact, with respect to CVD it should be a future research objective to study the effects of chocolate as a whole, containing both potentially protective polyphenols and potentially detrimental saturated fats, in particular over extended time intervals not forgetting to monitor weight development in free living subjects because of the indirect effects of overweight and obesity on CVD.

As the current evidence does not sufficiently sustain a health beneficial effect of cocoa polyphenols or of chocolate as a whole we should be careful to recommend or encourage people to enhance chocolate consumption, e.g. by allowing health claims in advertisement and/or food labelling, in view of the dramatically increasing incidence of obesity and its health implications in today’s sedentary societies.

5 Summary

Recently, chocolate and cocoa have gained much attention owing to suggested health beneficial effects, such as in the treatment of cardiovascular diseases, cancer, as well as diseases related to an imbalance between oxidative attack and antioxidant defences, malfunctioning of the immune system or uncontrolled inflammation, which are believed to be mediated by bioactive polyphenols that are present in significant amounts in chocolate and other cocoa products. However, reported concentrations of polyphenols in chocolate and cocoa vary significantly and often cover only a few compounds, there is little knowledge on bioavailability and metabolism of cocoa polyphenols, and finally, human intervention studies using hard endpoints or validated and accepted surrogate markers providing more conclusive data for the suggested health effects *in vivo* are still lacking.

In this work, methodologies for both the qualitative and quantitative analysis of polyphenols in chocolate, both milk and dark chocolate, as well as chocolate raw products, such as cocoa beans, cocoa nibs, cocoa liquor and chocolate masses were developed, validated and applied to study changes in polyphenol contents and composition during different chocolate manufacture procedures. Twenty-six phenolic compounds, including various flavan-3-ols (catechins), oligomeric procyanidins both with a single (B-type) and a double linkage (A-type), quercetin both in the free form (aglykon) and linked to glycosides, and hydroxycinnamic acid derivates were tentatively identified by high-performance liquid chromatography-electrospray-ionisation-mass spectrometry (HPLC-ESI-MS) with an ion trap technology performing single as well as multiple collision induced dissociation (CID) experiments also known as MS-MS (MS^2) and MS^n experiments. In addition, several isomers of oligomeric procyanidins, the exact stereo-chemical structure of which remained unclear, were also observed in the HPLC-MS experiments performed within this work. The identity of most compounds was verified by comparison of retention times on the HPLC column and of MS or MS^n spectra with those of commercially available authentic standards. In addition, six cocoa polyphenols that are not commercially available were isolated and purified by a combination of several chromatographic techniques, including flash chromatography, high-speed countercurrent chromatography, size exclusion

chromatography, and semi-preparative HPLC. The structures of theses isolated compounds were unambiguously elucidated by means of several one-dimensional and two-dimensional nuclear magnetic resonance experiments, HPLC-ESI-MS, as well as hydrogen/deuterium exchange mass spectrometry and tandem-mass spectrometry. Two of the isolated phenolic compounds, caffeoyltyrosine and p-coumaroyl-dihydroxy-phenylalanine were described for the first time in cocoa.

Subsequently, the methodology based on HPLC-ESI-MS was further elaborated and validated for the quantitative analysis of twenty-two quantitatively relevant polyphenols in chocolate and chocolate raw products. Samples of fermented cocoa beans from various origins, cocoa nibs, cocoa liquor, and final chocolate mass were taken from different chocolate making process lines, the difference of which was mainly be described by the harshness of the heat treatment (roasting) and the presence or absence of an alkalising procedure. These samples were then analysed by the multi-compound HPLC-ESI-MS method. From multivariate statistical analysis (principal component analysis) of the data it was evident that fermentation (selection of beans with different degrees of fermentation), roasting, and – if applied – alkalising were the key process parameters in the chocolate making process responsible for decline and change in profile of polyphenols from the cocoa bean to the final product chocolate. Moreover, it was possible to identify the oligomeric procyanidins as the most labile compounds and to suggest oxidative degradation, epimerisation and deglycosidation as responsible mechanisms for the observed changes.

In another part of the present work, a method based on solid phase extraction and analysis by high-performance liquid chromatography-tandem-mass spectrometry (HPLC-MS-MS) was developed for the analysis of cocoa polyphenols as well as their metabolites in human plasma and urine and applied to samples taken during a small pilot study with two human subjects consuming both dark and milk chocolate on different occasions after polyphenol-free diet and overnight fasting. Epicatechin, the major polyphenol in chocolate, was shown to be absorbed from chocolate and was present mainly as its metabolites following methylation, glucuronidation, and sulphatation of the original cocoa compound, respectively. In addition, several fold lower amounts of two additional quantitatively relevant cocoa polyphenols catechin and procyanidin B2 were observed in human plasma and urine, although in their conjugated

forms only. From the results obtained within this preliminary study it appeared that the presence of milk proteins in milk chocolates had no influence on the presence and amounts of the observed cocoa polyphenol metabolites compared to those derived from dark chocolate consumption. Nevertheless, there was no evidence for absorption of any higher oligomeric procyanidin in its intact form from either milk or dark chocolate. However, several lower molecular phenolic acids were observed in the urine samples, which might be an indication for metabolism of un-absorbed cocoa polyphenols by the colonic microflora followed by absorption of breakdown products.

In a further part of this work, which included the collaboration with a supervised diploma thesis project, fast state of the art methodologies based on HPLC-ESI-MS and enzyme immunoassay (EIA), suitable for high-through-put analyses in large human intervention studies, were developed and validated for the quantitative analysis of urinary markers of oxidative DNA damage (8-hydroxydeoxyguanosine), linked to the development of cancer, and lipid peroxidation (15-F_{2t}-isoprostane), related to the development of cardiovascular diseases, respectively. While the thorough analytical method validation allowed quantifying the analytical error (measurement uncertainty), this work also included a preliminary study for the estimation of intra-individual variation of urinary levels of theses markers in two subjects, both within a day and over 17 consecutive days, as this information is important for the planning of accurately powered intervention studies.

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