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Review

Chromatography of tea constituents

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ABSTRACT

Modern chromatographic techniques such as high-performance liquid chromatography are currently the most helpful approach to the routine analysis of and research of non-volatile tea constituents. Using these techniques some errors in the more classical analytical techniques could be detected. Unfortunately, some of these methods of analysis are still in widespread use, even as official methods. However, knowledge of especially the polyphenols in tea is still lacking, and for many of the minor polyphenols no chromatographic methods for the determination exist.

CONTENTS

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1. INTRODUCTION

Tea is one of the most popular beverages in the world. In China and Japan usually the green (non-fermented) tea is consumed, whereas in India and most European countries black (fermented) tea is preferred. The major tea-producing countries are India, China, Japan, Sri Lanka, Indonesia and Central African countries. Tea production was about 2.2 million tons in 1984 [1]. Until now the quality assessment of tea has been performed worldwide by professional tea tasters [2]. For a long time, attempts have been made to correlate their findings with contents of chemical constituents.

The objectives of chemical analysis of tea cover at least three different areas: (a) to find a constituent or a group of tea constituents which are a measure of tea quality; (b) to optimize tea technology; and (c) to correlate the health effects of tea with certain tea constituents.

As regards the non-volatile constituents, in the past in most instances groups of tea constituents (*e.g.*, TFs, TRs) have been determined as a sum parameter using non-chromatographic methods. Chromatographic methods (PC, TLC) were developed in the 1950s and 1960s. Since 1976, many HPLC methods have been developed for tea alkaloids and pigments.

In this review we compare the more recently developed chromatographic methods (*e.g.*, HPLC) for the determination of natural tea constituents and more classical approaches. The determination of, *e.g.*, pesticide residues is not covered. More general information about tea (horticulture, technology, chemical composition) can be obtained, *e.g.*, from reviews by Bokuchava and Skobeleva [3,4] and Wickremasinghe [5] and from monographs [6–8].

2. ANALYSIS OF TEA CONSTITUENTS

2.1. Alkaloids

The different health effects of caffeine have been compiled recently by James [9]. Another recent paper deals with the metabolism of methylxanthines in tea and other beverages, including analytical aspects [10]. Black tea contains normally 1.5–4% of caffeine, 0.2–0.4% of theobromine and about 0.02% of theophylline.

2.1.1. Non-chromatographic methods

The most widely used non-chromatographic method is a modified Levine procedure [11]. The caffeine is extracted from ammonia-containing tea solutions, clean-up is performed on two columns (acidic, alkaline) and caffeine is eluted from the column using chloroform. Absorbance is measured at three different wavelengths, which has to be done to subtract interferences from other compounds. This procedure leads to reproducible results.

Gravimetric determination after extraction of the brews [$Ba(OH)_2$ added] with chloroform has also been used [12]. A similar procedure, using $Pb(OAc)_2$, KOH and $KMnO_4$ solutions, treatment with H_2SO_4 and iodine, followed by titration with $Na_2S_2O_3$, was reported [13].

2.1.2. HPLC methods

Numerous HPLC methods for the detection of tea alkaloids have been published, but in most countries the official methods are still non-chromatographic.

Clean-up for HPLC analysis is performed, *e.g.*, using heavy magnesium oxide [14] in a modified Kjeldahl apparatus [15] or in a shaking water-bath for 20 min [16]. In an international interlaboratory test an HPLC system with RP-18 phases and methanol water (*ca.* 40:60, depending on the column used) was recommended. This procedure is simple and rapid to perform and leads to reproducible results, even for decaffeinated teas. It has been tested successfully in international and national ring tests [16].

Another clean-up procedure prior to HPLC is the use of a Sep-Pak C_{18} cartridge and a PVPP column for aqueous extracts of green tea as carried out by Kuwano and Mitamura [17]. The eluate was analysed by HPLC, using an RP-18 (Nova-Pak C_{18}) column and acetonitrile–water (20:80) as the mobile phase and monitoring at 254 nm.

Alternatively, if theobromine also should be detected, a clean-up by means of ion exchange (Dowex 1-X4) and elution with methanolic phosphate buffers has been proposed and is currently being tested as a German standard method [18,19]; the HPLC elution systems can be water–methanol or water–acetonitrile.

Kunugi *et al.* [20] used an Extrelut column clean-up with 8 *M* H_2SO_4 and 2 *M* NaOH (elution with

dichloromethane) and chromatography on a Li-Chrosorb Si 60 column. Water-saturated dichloromethane–ethanol (97:3) was used as a solvent. Some workers have described a clean-up by column chromatography using Al_2O_3 or MgO phases to remove polyphenols prior to RP-HPLC [21,22]. In most papers RP-HPLC separations are described. It is also possible to use normal-phase HPLC on silica layers [eluent, dichloromethane–methanol, (90:10)] as described, *e.g.*, by Vergnes and Alary [23].

A comparison between a spectrophotometric method and HPLC after chloroform extraction and water extraction–PVPP clean-up has been made [24]. Unfortunately, it was impossible to compare the results of the different methods owing to the different extraction conditions used (water and chloroform extractant, respectively).

HPLC (10-μm Bondapak C_{18} column with acetonitrile–2% acetic acid as eluent) and GLC (column: OV-17 on Chromosorb W HP; temperature programming) determinations have been compared [25]. Clean-up was done by liquid–liquid extraction (chloroform containing 5% isopropanol from alkaline brews). HPLC gave better resolution and a better sensitivity. A nitrogen–phosphorus detector for the sensitive GLC determination was also used [26] together with a packed column (column: OV-101 on Chromosorb W; isothermal). Clean-up was effected by partitioning into toluene. The data were similar to those obtained by a spectrophotometric procedure (Chinese standard method, including treatment with lead acetate). A similar spectrophotometric method was described by Chen [27].

In general, the non-chromatographic methods use hazardous chemicals to a greater extent (chloroform, lead salts and other heavy metals), and they are more tedious than, *e.g.*, HPLC methods. GLC methods can also be used, but an extraction is necessary. If only caffeine is to be determined an HPLC procedure after MgO treatment, filtration and dilution should be the method of choice. The pretreatment of the sample depends on what information is required. If one wants to determine the amounts of caffeine ingested by the consumer, a normal tea brew of unground material should be prepared, followed by a suitable clean-up prior to HPLC. The standard procedures normally determine the total extractable caffeine, so grinding and longer extraction times are included.

2.2. Volatile flavour compounds

As there are a large number of papers that deal with the composition of tea aroma and the analytical aspects, this section will only try to give some representative examples of the analytical concepts used. About 500 different aroma compounds have been identified in black tea [6,7,28,29]. According to Flament [29], there are 37 different hydrocarbons, 46 alcohols, 55 aldehydes, 57 ketones, 55 esters, 71 acids, 16 lactones, various furans, pyrroles and others. In recent years reviews on the composition and analysis of tea aroma have been compiled by, *e.g.*, Bokuchava and Skobeleva [30], Schreier [28] and Flament [29]; information on the biogenesis of the tea volatiles can be obtained from the same sources. Some aroma compounds in teas, such as alcohols and linalools, are biosynthesized directly by the tea plant (primary products); most of tea volatiles are formed during processing from precursors such as carotinoids, amino acids and lipids (secondary products) [6].

An aroma impact compound has not been identified as yet. Sanderson [31] stated in 1975 that, "The possibility that an as yet unidentified aroma constituent is the primary determinant of the character of black tea aroma is not entirely excluded".

Nowadays the method of choice is capillary GLC, as in analysis of flavours of other foods and beverages. Bokuchava and Skobeleva [30], who summarized the older attempts to determine tea aroma compounds, stated that there was no efficient method for tea aroma analysis before the introduction of GLC. The main effect on the results is due to the sample preparation used. A specific treatment according to volatility or the chemical nature of the compounds is necessary. This will be briefly outlined using some examples.

Heins *et al.* [32] employed a static headspace technique for identifying aroma components in dry tea leaves. They used GLC–MS, with the mass spectrometer disconnected during the injection of the (10-ml) vapour sample on to the capillary column (cooled by dry-ice). Using this method, 36 compounds from dry tea leaves were identified. Another headspace technique was published by Reymond *et al.* [33], who compared the fraction of highly volatile tea constituents. Wickremasinghe *et al.* [34] produced aroma concentrates of Ceylon black teas al-

ternatively by steam distillation and adsorption on charcoal of the headspace vapour over the tea brew and eluted with diethyl ether. The former method proved to be superior to the latter, which was partly due to artefact formation on the charcoal. Identification of the compounds was carried out by GLC and GLC–MS. As one result, variations in the aroma pattern of flavoury and non-flavoury tea were detected. A dynamic headspace technique combined with concentration of the volatiles was also used by Vitzthum and Werkhoff [35].

Solvent extraction procedures prior to GLC were used by other workers, *e.g.*, Yamanishi *et al.* [36]. They separated essential oils from black teas (isolated by solvent extraction using diethyl ether) by treatment with acidic and basic solutions, partitioned into a carbonyl-free and a carbonyl fraction and determined alcohols and carbonyls (such as benzaldehyde and phenylacetaldehyde).

Steam distillation was used, *e.g.*, by Yamanishi *et al.* [37]. They employed two traps for the steam volatiles (temperatures −15 and −78°C). The distillates were extracted with isopentane and diethyl ether and concentrated before analysis by GLC on packed columns. For the identification retention times, GLC–MS and IR were used; 57 compounds could be identified and their relative amounts were obtained. Similar procedures have been published (*e.g.*, 38–41]).

In most instances no real quantification of the steam volatiles was carried out. Relative amounts of the volatiles were obtained by calculating the ratio of peak areas and the internal standard area. Suitable internal standard compounds were, *e.g.*, ethyl caproate or ethyl decanoate.

Some workers (*e.g.*, refs. 42–44) use a so-called flavour index (FI). This is defined as the ratio of GLC peak areas (calculated *versus* an internal standard) of two groups of flavour compounds. The first group (VFC I) consists of the compounds with an undesirable flavour formed by twelve substances including hexanal, (*E,Z*)- and (*E,E*)-2,4-heptadienal,(*Z*)-3-hexenol,(*Z*)-3- and (*E*)-2-hexenal, *n*-pentanol and *n*-hexanol. The second group (VFC II), which imparts a sweet and flowery aroma to tea, includes twenty compounds such as linalool and its oxides, benzaldehyde, geraniol, α-terpineol, methyl salicylate, benzyl alcohol and β-ionone. Isolation is performed using SDE in a modified Likens–Nickerson apparatus. This concept was used to detect the influence of plucking on tea aroma [42], seasonal variations and the use of nitrogen fertilizers [43] or the effects of the altitude of the tea estate [44]. Similar approaches, such a terpene index, have also been made [45].

These analytical techniques have been applied in various studies on the influence of horticultural and technological variables such as plucking season and technique, use of fertilizers and withering on the formation of tea aroma. A Japanese group published a method for differentiating various tea clones by means of a terpene index [45].

Mick and co-workers [46–49] used successive extraction, vacuum distillation, liquid–liquid extraction with pentane and dichloromethane and column chromatography on silica gel to isolate four purified fractions of different polarity, which were analysed by capillary GLC on Carbowax or by GLC–MS. They also used GLC–MS to differentiate between two qualities of black tea (Darjeeling orange pekoe and Indian broken). A real quantification of some tea aroma components was performed.

A new aspect is due to the fact, that some volatile aroma constituents occur in form of enantiomers with different aroma values. Moreover, because of the different enantiomer patterns in natural and synthetically prepared aromas, adulteration can be detected. Werkhoff *et al.* [50] used enantioselective GLC on heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin in polysiloxane for the separation of *trans*-α-damascenone and *trans*-α-ionone enantiomers in tea and other foodstuffs.

According to Kinugasa and Takeo [51], who used GLC–MS (electron impact mode; column 50-m Carbowax 20M), the decomposition of unstable compounds (hexenal and esters) and the liberation of more volatile compounds (e.g., linalool and geraniol) from precursors lead to imbalances in aroma pattern and off-flavour.

Hence powerful methods for the analysis of tea volatiles are available. The problem is the assessment of the analytical data, because a generally accepted and convincing concept for the use of the data does not exist so far.

2.3. *Polyphenols*

2.3.1. *Flavanols (catechins)*

Polyphenols are the most abundant group of compounds in tea leaf. Among these, the flavanols (catechins) constitute the quantitatively major component, with up to 30% of the dry matter of fresh leaf. Fig. 1 gives the structures of the main flavanols of tea. Flavanols make an important contribution especially to the bitter and astringent taste of green tea. They are also of interest because of their physiological effects, in particular the capillary strengthening activity and antiartherosclerotic effect and the anticarcinogenic [52] and the bacteriostatic effects on several microorganisms. Acting as antioxidative agents or free radical scavengers, flavanols inhibit the Maillard reaction [53].

During the manufacture of black tea the flavanols are easily oxidized by polyphenol oxidase and further reactions lead to TFs and compounds of higher molecular mass. The amount and proportion of various flavanols, depending on the leaf age, are directly correlated with the quality of the final beverage. The finest teas are made from young tea shoots containing the highest flavanol levels.

Therefore, this group of tea constituents has been the subject of intensive studies for a long time. The first objective following the identification of the main flavanols of tea was to determine their distribution in different parts of the tea plant and their fate during the processing of black tea. For these investigations, *e.g.*, Roberts *et al.* [54] and Bhatia and Ullah [55] used 2-D PC after extracting the

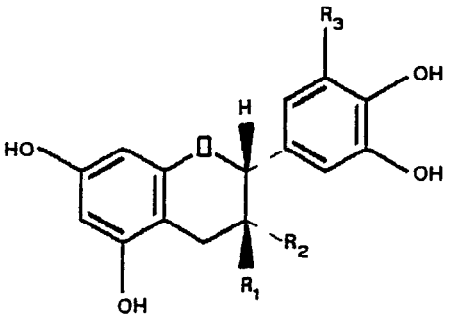

Fig. 1. Structures of flavanols in tea. R_1 = OH; R_2 = R_3 = H, (+)-catechin; R_1 = R_3 = H; R_2 = OH, (−)-epicatechin; R_1 = R_3 = H; R_2 = galloyl, (−)-epicatechin gallate; R_1 = H; R_2 = R_3 = OH, (−)-epigallocatechin; R_1 = H; R_2 = galloyl; R_3 = OH, (−)-epigallocatechin gallate.

powdered green leaf with boiling methanol. After detecting the spots by their purple fluorescence under UV light in the presence of ammonia vapour, they were eluted with cold water and the absorbance of filtrates were measured at 275 nm.

2-D TLC was applied by Forrest and Bendall [56]. They used cellulose layers and developed in water or 5% aqueous methanol in the first direction and 1-butanol–acetic acid–water (4:1:5) in the second direction. Identification of polyphenols was carried out by means of co-chromatography, colour reactions and UV spectra. For quantification two different colour reactions were applied: (1) with benzidine–sodium nitrite hydrochloric acid and (2) with vanillin–sulphuric acid, followed by measuring the absorbance at 450 and 500 nm, respectively. This method has been applied to flavanol analysis by several other groups [57–59]. TLC analysis on polyamide layers using different solvents and spray reagents for detection has also been described [60,61].

Because PC and TLC methods are time consuming and involve problems in quantification, GLC was introduced to overcome these disadvantages. In 1969 Pierce *et al.* [62] presented a method for the analysis of tea flavanols by GLC of their trimethylsilyl derivatives. Extraction of fresh or dried tea leaves with pyridine was followed by silylation with bistrimethylsilylacetamide (BSA). Two sets of isothermal conditions and a complex calibration procedure were necessary to separate and analyse the derivatives on a column packed with 3% OV-1 on 60–80-mesh Gas-Chrom Q. Collier and Mallows [63] improved the GLC method. They developed a temperature-programmed method for separation so that the complete analysis required only one run. Studies with different extraction solvents and conditions favoured ethyl acetate for selective and complete extraction of the flavanols from aqueous tea infusions. Solvent extraction is necessary to avoid interferences with other tea constituents. Similar GLC methods for the separation and determination of the flavanols of tea were applied by other workers [64–67].

Liquid chromatography was first employed for the preparative isolation of flavanols from green tea. Flavanols were extracted from tea leaves with boiling water and aqueous acetone and separated on Sephadex LH-20 columns [68–73]. Some appli-

cations of other stationary phases, *e.g.*, styrene–divinylbenzene copolymer or methacrylate esters [74], MCI gel CHP-20P or Bondapak C_{18} Porasil B [72] and high-porosity polystyrene gel Diaion HP20 [69], have also been described. The flavanol fractions were eluted with aqueous solutions of methanol, ethanol, acetone or their mixtures in varying compositions and monitored at 280 nm. This technique was also used for the isolation of minor flavanols in tea such as acetylated or 3-O-methyl flavanols [68,69,72].

Investigations on the effect of the extraction method on the determination of flavanols [75] showed that 90% aqueous methanol is the most efficient solvent compared with 90% ethanol, 90% acetone and water-saturated ethyl acetate. When comparing results from different methods, it is very important to consider the extraction technique.

Hoefler and Coggon [76] introduced HPLC with reversed phases into the analysis of flavanols in tea. For green tea the sample clean-up procedures could be reduced to a minimum. Even the direct injection of aqueous tea solutions after filtration through a membrane (cellulose, 0,45 μm) gave the desired separation. Moreover, the accurate and easy quantitative analysis inherent in GLC also applies in HPLC, which became the method of choice for the determination of non-volatile tea constituents. The authors used an RP-18 column (μBondapak C_{18}, 10 μm, 30 cm × 4 mm I.D.), flow-rate 2 ml/min and a UV detector with a 280-nm filter for separating the five flavanols EGCG, ECG, EGC, EC and catechin within 35 min. The mobile phase was acetic acid–methanol–DMF–water (1:2:40:157). This method has frequently been used for investigating green tea or monitoring the purity of preparatively isolated flavanols which were needed for model fermentation systems [77–80]. Several slightly modified HPLC methods have appeared [66,68,81–83]. Hirose and Tamada [81] analysed fresh green tea leaves using the described method with acetonitrile instead of methanol in the eluent. A Japanese patent [83] used acetone–THF–water (12:10:78). More differences could be found concerning the sample preparation, which should be mentioned in any case together with the chromatographic technique. Separation with gradient elution systems was carried out by Ma and Wang [84] and Liang *et al.* [85] with acetic acid–methanol–water (1:1:98) and acetic acid–methanol–DMF–water (1:1:50:48). However, the chromatograms showed no real improvement over isocratic elution.

A Japanese group developed another simple method for the determination of flavanols in infusions of green, oolong and even black tea by means of HPLC with gradient elution. Their solvents contained phosphoric acid, acetonitrile and DMF. A good separation within 35 min was achieved with an RP-18 column (Ultron-N C_{18}, 15 cm × 4.6 mm I.D.) held at 43°C [86]. As expected, the flavanol concentration in green teas was higher than in oolong and especially in black teas. Unfortunately, no HPLC separation of a black tea was published.

An RP-HPLC method for the determination of the main flavanols of tea together with caffeine, theobromine, gallic acid and theogallin has been reported recently [87]. For rapid and easy sample preparation an SPE technique with RP-18 cartridges was employed. The method is suitable for green, oolong and black tea and for tea extracts. A gradient method with eluents containing only 2% aqueous acetic acid and acetonitrile allowed a separation of the mentioned compounds in about 20 min. Fig. 2 shows a chromatogram of a green tea extract.

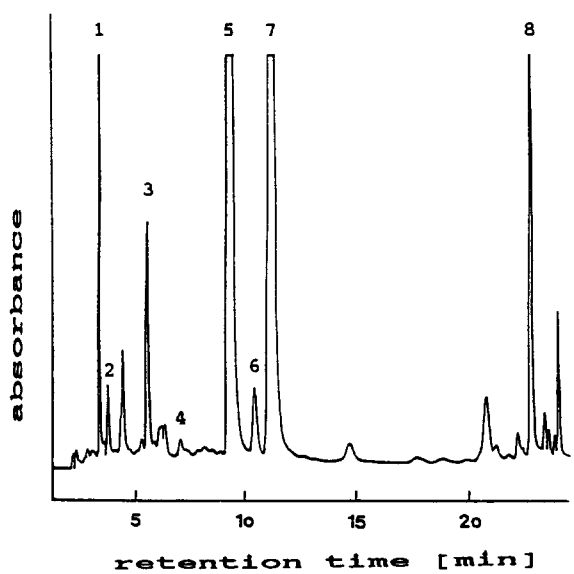

Fig. 2. HPLC profile of a green tea (measured by absorbance at 278 nm). 1 = Theogallin; 2 = gallic acid; 3 = EGC; 4 = catechin; 5 = caffeine; 6 = EC; 7 = EGCG; 8 = ECG. From ref. 87, with permission.

Comparison of different extraction methods, (1) a normal tea brew with boiling water, (2) a methanolic extract of ground tea and (3) the official German method for the determination of soluble solids (1 h boiling under reflux) [88], revealed a great influence on the flavanol yield. The official German method led to degradation of flavanols while methanolic extracts gave the highest yields. The use of DAD improved and simplified the confirmation of peak identity and purity [80,89], so that sample clean-up procedures became less important.

An interesting HPLC method using postcolumn derivatization with 4-dimethylaminocinnamaldehyde in the presence of concentrated sulphuric acid allowed the selective detection of flavanols [90]. A 200–40 000-fold sensitivity increase was observed for EC compared to other phenols. However, this sensitive method is time consuming and not necessary in analysis of the major flavanols of tea.

2.3.2. Flavonols and glycosides

The flavonols in tea are present as aglycones (traces) and to a much higher extent as their glycosides. In tea leaves all the flavonol glycosides derive from the aglycones myricetin, quercetin and kaempferol. Their structures are given in Fig. 3. The carbohydrate moieties, in most instances located at position 3 of the aglycone, consist of various combinations of glucose, galactose, rhamnose and, in a single instance, fructose. Mono-, di- and triglycosides have been observed. Flavonol glycosides are interesting compounds because of their positive physiological activities, especially their capillary

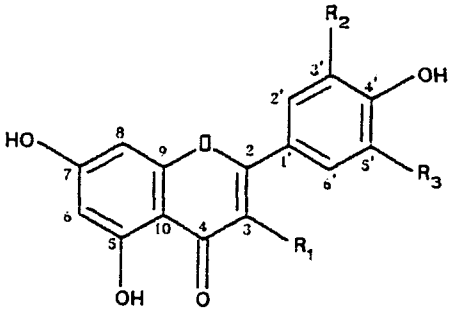

Fig. 3. Structures of flavonol and flavone aglycones in tea. R_1 = OH; R_2 = R_3 = H, kaempferol; R_1 = R_2 = OH; R_3 = H, quercetin; R_1 = R_2 = R_3 = OH, myricetin; R_1 = R_2 = R_3 = H, apigenin.

strengthening effect (so called vitamin P activity). Owing to their yellow colour, the flavonol glycosides contribute to the colour of the tea brew in addition to TRs and TFs. Moreover, the colour of the green tea brew is caused almost exclusively by flavonol glycosides. For this reason, most of the investigations on flavonol glycosides deal with green tea or fresh tea shoots.

The first investigations on flavonols and their glycosides in tea were carried out by the groups of Roberts and Oshima and Nakabayashi. The Japanese workers [91] separated 23 flavonols, flavones and their glycosides by means of 2-D PC from extracts of tea leaves (var. *assamica*). Nine of them could be identified as derivatives of kaempferol and some others as glycosides of quercetin, including rutin. For the latter they published a spectrophotometric method for its semiquantitative determination [92]. They also used column chromatography on magnesium or zinc silicate for isolation. Roberts *et al.* [93] used 2-D PC to separate the flavonol glycosides and aglycones from Indian fresh tea leaves and black tea. Dried tea was ground and three volumes of water were added. The extract was centrifuged and applied to the paper. Chromatograms were run with 1-butanol–acetic acid–water (4:1:2.2) in the first direction followed by acetic acid (2%). This chromatographic system became a standard system for the separation of flavonoids from tea and was used frequently during the next few decades. Roberts *et al.* [94] also applied this system to taxonomic investigations on different camellia species. Spot detection was carried out either by fluorescence in UV light or for sufficiently high concentrations, by spraying with iron(III) chloride–potassium hexacyanoferrate(III) reagent, which gave blue reaction products with quercetin and myricetin derivatives. Acetic acid (2%) as the second solvent could be replaced with some advantage by 2% aqueous boric acid. This did not lead to any changes in R_F values but, owing to the formation of borate complexes, most of the flavonols showed up as bright yellow spots intensifying under UV light without any spray reagent being necessary. Some flavonol glycosides that remained unresolved under these conditions could be separated using 80% aqueous phenol followed by 1-butanol–acetic acid–water as solvent. Imperato [95] isolated three flavonol glycosides from a tea brew using preparative PC employing

mixtures of 1-butanol–acetic acid–water (4:1:5, upper phase), 5% acetic acid and 1-butanol–ethanol–water (4:1:2.2) as solvents.

The Georgian team of Chkhikvishvili and co-workers [96,97] used column chromatography and TLC for the isolation and separation of various flavonoids from tea. Generally, a clean-up of the extract prior to analysis, *e.g.*, by PC, TLC, UV, ^{1}H NMR or MS, is essential. Suitable clean-up techniques are column chromatography on silica gel [96,98], polyamide [96,99] or cellulose [100] and gel chromatography on Sephadex LH-20 [96,100,101]. A procedure for the separation of flavonoids from tea was described [98]: tea shoots were extracted with chloroform–benzene (1:1) to remove lipophilic substances and caffeine. Flavonoids and catechins were extracted with ethyl acetate. Catechins were removed from this extract by column chromatography on silica gel (washing with diethyl ether) and the residual flavonoids were desorbed with methanol. The eluent was analysed by TLC on polyamide and silica gel layers. Ten individual compounds (flavonol glycosides, flavones, flavanones and dihydroflavonols) were identified.

None of these methods allowed the determination of flavonols or flavonol glycosides. The first intensive quantitative study on flavonol glycosides from tea was performed by Bokuchava and Ulyanova [102]. Flavonol glycosides were extracted with methanol from unprocessed tea leaves, which had been previously treated with chloroform to remove caffeine. Ammonia solution was added to the methanolic extract (pH 10) to force the destruction of the bulk of catechins and tannins by oxidation. Oxidation products were separated from the solution by filtration and the filtrate was analysed by 2-D PC using 1-butanol–acetic acid–water (40:12:29) and 2% aqueous acetic acid as solvents. Slices of the chromatogram were sprayed with $AlCl_3$ (1% ethanolic) to detect flavonol glycosides. The compounds of interest were eluted from the paper using ethanol (50%, aqueous) and determined spectrophotometrically. Owing to the alkaline treatment, myricetin glycosides, which are very sensitive to oxidation, could not be determined by this method.

In the last few years, HPLC has been introduced as the method of choice for flavonol analysis of tea. Detection was carried out using a UV detector or, with some advantage, by DAD. Tsushida *et al.* [103] obtained flavonol aglycones by hydrolysing a tea extract with 1.2 *M* HCl for 30 min under reflux, followed by ethyl acetate extraction. They separated the three aglycones myricetin, quercetin and kaempferol by HPLC on RP-18 material using a phosphate buffer as eluent. Absorbance was monitored at 325 nm, which is not in accordance with the UV maxima of flavonol aglycones at *ca.* 270 and 370 nm. HPLC analysis of flavonol glycosides from tea was introduced by Biedrich *et al.* [104]. Flavonol glycosides were isolated from black tea by column chromatography on polyamide. An RP-HPLC method with gradient elution [solvents: (A) 2% aqueous acetic acid, (B) acetonitrile] was developed for separation. By means of the UV spectra obtained from DAD, several peaks could be identified as flavonol glycosides. The authors concentrated on the determination of rutin (quercetin 3-O-rhamnoglucoside), which could be identified by co-chromatography. McDowell *et al.* [105] used the RP-HPLC system and method of Bailey *et al.* [89] to study the contribution of flavonol glycosides to the colour of black tea liquors. From chromatograms of the entire tea brew they estimated that 48% of the absorption at 380 nm was caused by flavonol glycosides, 25% by TFs and the remainder by the so-called TRs (*cf.*, section 2.3.6). Liang *et al.* [85] developed an HPLC method to separate and determine several groups of tea constituents, including flavonols. The analytes were extracted from tea with ethanol (nonphenolic pigments had been removed previously with light petroleum). The extract was cleaned up using a Sep-Pak column and analysed by HPLC on a μBondapak fatty acid column using 55% aqueous methanol adjusted to pH 3.0 with phosphoric acid. The detection wavelength was 254 nm. Seven peaks appeared during the first 5 min, three of which were identified by co-chromatography (quercetin, myricetin and rutin). Although the separation was very poor, quantification was carried out. The amounts of, *e.g.*, myricetin (up to 1.67 mg/g) in Chinese green teas were much higher than reported by other workers. As no further confirmation of peak identity and purity were carried out, the results obtained by this method seem to be unreliable. Recently, Engelhardt and co-workers [106–108] published a method for the HPLC determination of flavonol glycosides. Analytes were extracted with methanol and aqueous methanol and cleaned up by column

chromatography on polyamide (washing with water, elution with methanol). HPLC separation of the eluate was done by two isocratic systems: 2% aqueous acetic acid–acetonitrile (85:15) and 2% aqueous acetic acid–1,4-dioxane–methanol (77:13:10) on an RP column (ODS-Hypersil). Chromatograms were monitored by DAD at 354 nm (Fig. 4). Fifteen flavonol glycosides were isolated by means of preparative HPLC and identified by various methods such as UV, ^{1}H and ^{13}C NMR, MS, hydrolysis and analytical HPLC and GLC. The method was applied to the determination of flavonol glycosides in 30 samples of black, green, oolong and instant teas [106].

2.3.3. Flavones and glycosides

In addition to flavonol glycosides, tea contains several flavone glycosides in lower amounts. The structure of the most important aglycone in tea, apigenin, is shown in Fig. 3. In contrast to flavonol glycosides, which have O-linked carbohydrate moieties, flavone glycosides in tea appear nearly exclusively as C-glycosides. Isolation and identification techniques are similar to those applied to flavonol glycosides. Moreover, C-glycosides cannot be hydrolysed under normal conditions. This causes some problems in the identification of the sugar moieties of tea flavone glycosides. However, it facilitates the separation of flavone glycosides from the bulk of flavonol glycosides.

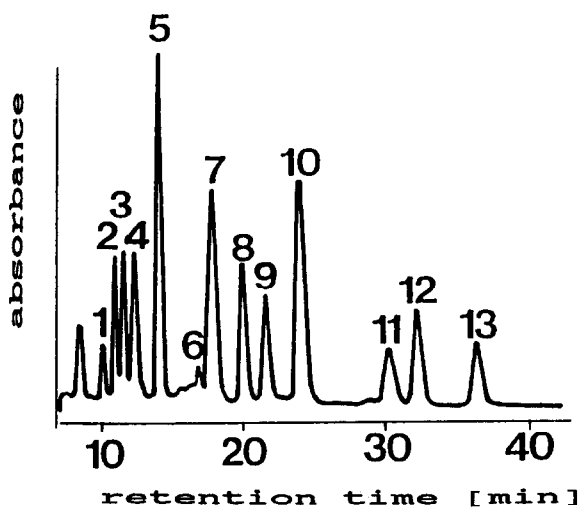

Fig. 4. HPLC separation of black tea flavonol glycosides (Sikkim Temi first flush). Chromatogram at 354 nm. 1 = Myricetin-3-O-rhamnoglucoside; 2 = myricetin-3-O-galactoside; 3 = myricetin-3-O-glucoside; 4 = quercetin-3-O-glucorhamnogalactoside; 5 = quercetin-3-O-rhamnodiglucoside; 6 = quercetin glycoside; 7 = quercetin-3-O-rhamnoglucoside and kaempferol-3-O-glucorhamnogalactoside; 8 = quercetin-3-O-galactoside; 9 = quercetin-3-O-glucoside; 10 = kaempferol-3-O-rhamnodiglucoside; 11 = kaempferol galactoside; 12 = kaempferol-3-O-rhamnoglucoside; 13 = kaempferol-3-O-glucoside. From ref. 108 with permission. Identification of peaks 4 and 7 according to ref. 106.

There are only a few papers on flavone glycoside analysis in tea. Some of them have been mentioned above (see Section 2.3.2). The first report on flavones in tea was published by Sakamoto [109]. He observed nineteen flavones in a green tea infusion. Separation was carried out by 2-D PC [solvents: (1) 1-butanol–acetic acid–water (4:1:2); (2) 2% aqueous acetic acid]. IR and UV spectra were recorded after elution of the spots. The identification of some of the compounds was described in a later paper [110]. Chauboud *et al.* [111] used a similar solvent system for the isolation of another flavone glycoside from tea. Apigenin di-C-glycosides obtained from tea by the method of Sakamoto [109] were separated using HPLC by Tsushida *et al.* [103]. The detection wavelength was set at 325 nm. Recently, Engelhardt *et al.* [112] worked on flavone glycosides from black tea. Flavone glycosides were extracted together with flavonol glycosides (see section 2.3.2) by methanol. The eluate obtained from column chromatography on polyamide was refluxed with HCl to hydrolyse the flavonol O-glycosides. The hydrolysate was cleaned up again using a polyamide SPE cartridge and analysed by HPLC (ODS-Hypersil, gradient elution with 2% acetic acid and acetonitrile, DAD). Fig. 5 shows the HPLC trace of the

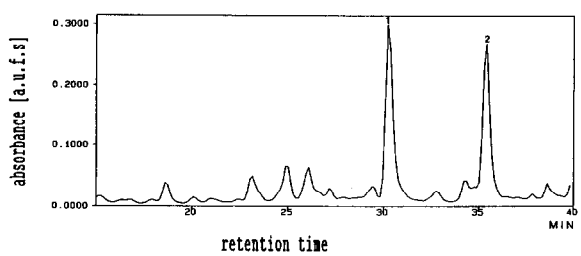

Fig. 5. HPLC trace of a hydrolysed tea brew (Sikkim Temi). Column, Hypersil-ODS (5-μm); solvents (A) 2% aqueous acetic acid, (B) acetonitrile, gradient 90% A, in 15 min to 85% A, 5 min isocratic, in 10 min to 70% A, 10 min isocratic; flow-rate, 1 ml/min; detection at 340 nm. 1 = Vitexin; 2 = isovitexin. From ref. 112, with permission.

flavone glycosides from a black tea sample monitored at 340 nm. The major drawback of acid hydrolysis is due to a Wessely–Moser rearrangement leading to isomerization of unsymmetrically substituted flavones (*e.g.*, vitexin and isovitexin). For that reason, quantitative data could only be presented for the sum of the amounts of those isomers.

2.3.4. Phenolic acids and esters

The major representatives of this group of tea constituents are gallic acid and its tea-specific ester with quinic acid (theogallin) and hydroxycinnamoyl acid/quinic acid esters (depsides).

2.3.4.1. Free gallic acid. Gallic acid (Fig. 6) is the most important phenolic acid in tea. The amount of gallic acid increases during the fermentation owing to its liberation from catechin gallates. Oshima and Nakabayashi [113] described a spectrophotometric method for its determination in tea using HCl–formaldehyde and alkaline phosphomolybdic acid.

Determination by means of HPLC is more specific than spectrophotometry. Sample clean-up does not seem to be essential, as most workers employed direct injection of a tea brew after filtration through a membrane filter. In general the separation is carried out on RP-18 columns. One of the first HPLC applications to tea analysis was that of Hoefler and Coggon [76], who separated a number of tea components including gallic acid using a μBondapak C_{18} column (10 μm) and a fixed-wavelength UV detector (254 nm). The mobile phase was 0.02 *M* citrate–phosphate buffer (pH 4.5).

More convenient is the use of non-salt-containing mobile phases as done by Ma and Wang [84]. The gradient elution solvents contained water–acetic acid–methanol (98:1:1) and DMF–water–acetic acid–methanol (50:48:1:1). They used a Micropak SP-C_{18} column (5 μm) and a UV detector set at 280 nm, which led to an increase of sensitivity. Gallic acid contents in brews obtained from Chinese tea samples ranged from 0.4 to 1.6 g/kg dry mass. These results correspond fairly well with Engelhardt's [114] results (0.9–5.5 g/kg dry mass). He also used direct injection of a tea brew on a Nucleosil RP-18 column with an isocratic system [2% aqueous acetic acid–acetonitrile (90:10)]. Recently, Bailey *et al.* [89] published investigations on non-volatile tea constituents. They detected several compounds in a black tea liquor, including gallic acid, using DAD. Separation was carried out on a 5-μm Hypersil-ODS column by a linear gradient of 2% aqueous acetic acid and acetonitrile. Only a single sample was analysed and no quantification was done.

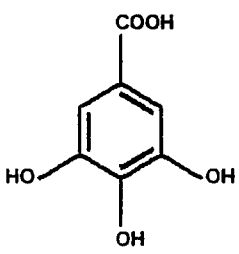

Fig. 6. Structure of gallic acid.

Kuhr and Engelhardt [87] developed an RP-HPLC method for the determination of gallic acid together with flavanols, theogallin and caffeine in tea employing solid-phase extraction on RP-18 cartridges for the clean-up procedure, as discussed in Section 2.3.1. Fifteen black tea and two each of green, oolong and instant tea samples were analysed. Gallic acid contents ranged between 0.6 and 6.2 g/kg dry mass in tea samples. They also studied different techniques for gallic acid extraction. The yield of a methanolic extraction was higher than that of a normal tea brew. They noticed a considerable increase in gallic acid during extraction when using the official German method (refluxing with water for 1 h), probably caused by depolymerization/decomposition of, *e.g.*, catechin gallates or TRs.

2.3.4.2. Theogallin. Although theogallin is a particularly interesting compound, as it specifically appears in tea, only few investigations have been published on this subject. Theogallin was isolated and characterized as galloylquinic acid by Roberts and Myers [115]. They used a Craig distribution system, cellulose acetate column chromatography and preparative PC for isolation. The purity of the isolate was monitored by 2-D PC [eluents: (1) butanol–aqueous acetic acid; (2) aqueous acetic acid]. The structure of theogallin (Fig. 7) was finally assigned to be 5-O-galloylquinic acid [116] by NMR analysis.

Bhatia and Ullah [117] observed a decrease in theogallin content during tea fermentation. They also used 2-D PC followed by photometric measure-

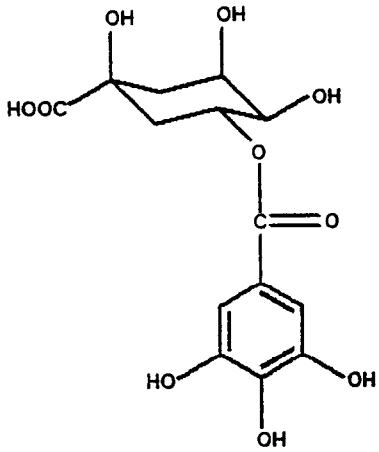

Fig. 7. Structure of theogallin.

ment of the spots at 275 nm. Unfortunately, the solvent composition used was not given. An HPLC method for determination of theogallin has been mentioned and described above [87] (see Section 2.3.1).

2.3.4.3. Chlorogenic acids and p-coumaroylquinic acids. Derivatives of hydroxycinnamic acids are widely distributed in the plant kingdom. Caffeoyl- and *p*-coumarylquinic acids (CQAs and CouQAs, respectively, see Fig. 8) have been described in tea. It should be noted that a different (non-IUPAC) nomenclature was used in the earlier literature.

Early investigations on hydroxycinnamic acid esters in tea were carried out by Roberts [118]. A black tea infusion was prepared and extracted with ethyl acetate. Analysis of this extract by means of 2-D PC led to the detection of three isomers each of

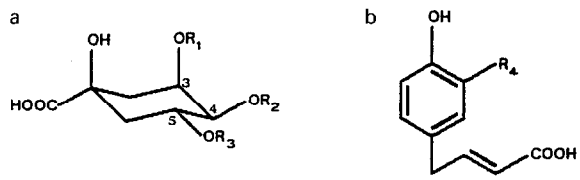

Fig. 8. Structures of *p*-coumaroyl- and caffeoylquinic acids. Numbering according to IUPAC rules. (a) R_1 = X (see b); R_2 = R_3 = H, 3-CouQA; R_1 = R_3 = H; R_2 = X, 4-CouQA; R_1 = R_2 = H; R_3 = X, 5-CouQA; R_1 = Y; R_2 = R_3 = H, 3-CQA (neo-chlorogenic acid); R_1 = R_3 = H; R_2 = Y (see b), 4-CQA (crypto-chlorogenic acid); R_1 = R_2 = H; R_3 = Y, 5-CQA (*n*-chlorogenic acid). X = *p*-coumaric acid (R_4 = H); Y = caffeic acid (R_4 = OH).

CQA and *p*-CouQA among several other phenolic compounds. In a later paper, Roberts and Williams [119] reported the UV spectra of 5- and 3-CQA measured after elution from the paper and that of CouQAs which were obtained by direct measurement on the paper.

Other techniques used for hydroxycinnamic acid isolation were preparative PC, TLC and column chromatography on cellulose, silica or polyamide phases. A method for the separation and determination of isomers of CQAs in tea was developed by a Chinese group [120]. The analytes were extracted by 20% aqueous acetic acid for 2 h, followed by membrane filtration. For separation they used a μBondapak C_{18} column, 20% aqueous acetic acid as mobile phase and a UV detector set at 324 nm. Peak identification of neo-, crypto-, *n*- and isochlorogenic acid was done by co-chromatography. Isochlorogenic acid means all possible isomers of dicaffeoylquinic acid. Unfortunately, peak identification was not confirmed by, *e.g.*, co-chromatography using an additional HPLC system. All four compounds mentioned above appeared in the first 3 min of the run. Contents in black tea were in the ranges 134–156 mg per 100 g (neo-CQA), 268–281 mg per 100 g (crypto-CQA), 67–99 mg per 100 g (*n*-CQA) and 99–109 mg per 100 g (iso-CQA).

Engelhardt *et al.* [121] obtained CQAs and CouQAs from a tea brew. Different clean-up procedures prior to HPLC analysis, such as ultrafiltration, polyamide column chromatography and SPE with RP-18 cartridges, were compared with direct injection of the tea brew. The SPE clean-up gave the best results. Owing to the high polarity of the CQAs and CouQAs, they can be eluted even with water, which makes this clean-up very simple. Fig. 9 shows an HPLC trace of a black tea brew treated in this way. Peak identity and purity were confirmed by UV spectra obtained with DAD. The appearance of mono-CQA and CouQAs in tea could be confirmed. However, this technique could not verify the identification of dicaffeoylquinic acids. Comparison of the results obtained from mono-CQA determinations in black tea with the results in ref. 120 shows a discrepancy which is higher than the natural range of contents that could be expected for different samples and the different extraction modes. In the Chinese work [120], the determination may have included some co-eluting compounds.

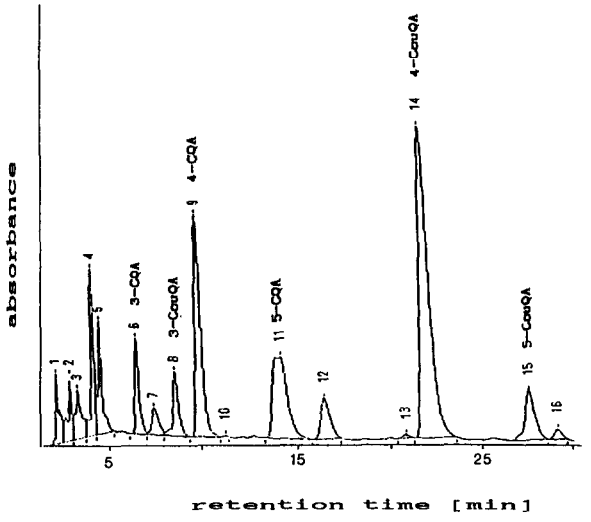

Fig. 9. HPLC separation of CQA and CouQA from a black tea brew, cleaned up by SPE. From ref. 121, with permission.

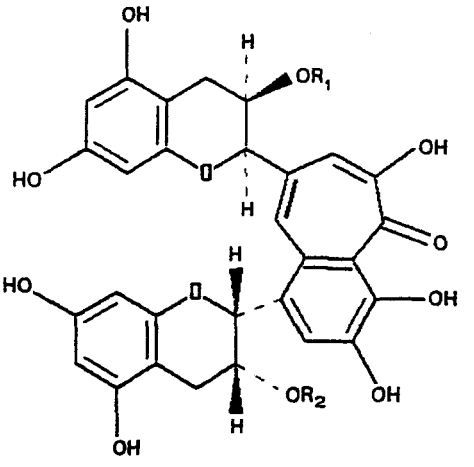

Fig. 10. Structures of the major theaflavins in tea. $R_1 = R_2 = H$, theaflavin; $R_1 = H$; R_2 = galloyl, theaflavin-3-gallate; R_1 = galloyl; $R_2 = H$, theaflavin-3'-gallate; $R_1 = R_2$ = galloyl, theaflavin-3,3'-digallate.

2.3.5. Theaflavins

During the fermentation stage of black tea processing, catechin or EC and their gallates are involved in an enzyme-catalysed oxidative reaction to form the so-called TFs [122]. These compounds, containing a benzotropolone group, are orange-red, constitute about 0.3–1.8% of black tea (dry mass base) and contribute significantly to the bright colour and brisk taste of tea brews [123]. Pure TFs in aqueous solution are normally very astringent but in tea the astringency is reduced owing to an interaction with bitter caffeine. Fig. 10 shows the formulae of the major TFs. Theaflavic acids are the oxidation products of an analogous reaction with the quinones of flavanols and gallic acid. Several attempts have been made to find correlations between TF contents, results of tea tasters and the quality of tea [124,125], but this is still controversial. After isolation by means of 2-D PC and gel chromatography the structures of the various TFs could be elucidated by MS, NMR and IR techniques [54,126–128].

The commonly used spectrophotometric method for the determination of total TFs in tea infusions was developed by Roberts and Smith [129,130] and modified by Ullah [131] for climatic conditions in subtropical countries. After extracting the TF from hot aqueous tea brews with ethyl acetate or IBMK and measuring the absorbance at 380 and 460 nm, respectively, the total TF amount was calculated based on mean molar absorptivities. However, later investigations showed that these values depend on the composition of the TF fraction. This easy and rapid method was mostly employed for investigating the influence of different processing parameters, *e.g.*, time and temperature of fermentation [117]. Hilton and Ellis [124] introduced another spectrophotometric method involving flavognost reagent (2-aminoethyl diphenylborate in ethanol), which forms a green complex with the *cis*-1,2-dihydroxybenzene ring associated with the TFs. In general, this rapid method gives reproducible results if the conditions of several critical stages [132–135] such as particle size of ground material, temperature of water used and infusion time are well defined and under control. The method has also often been applied to studies on the effects of different parameters on processing of tea and the resulting tea quality [125,136] and to investigations of the kinetics of tea infusion [134,135,137–139].

Other non-chromatographic methods for TF analysis were described by Fernando and Plambeck [140], who used voltammetric methods, and Hall *et al.* [141], who applied NIR spectrometry. However, these methods did not become common because the required analytical equipment is often not available. The limitation of all of these non-chromatographic methods arises from their inability to sep-

arate the mixture of TFs into its individual components, so that only values for the total amounts of TFs can be obtained.

The purpose of Collier and Mallows' work [142] was to develop a more specific and reproducible method. They reported a method for separating and determining the TF by temperature-programmed GLC of their trimethylsilyl ethers. Fractions suitable for analysis were prepared from whole tea solubles by extraction with ethyl acetate and chromatography on Sephadex LH-20. TF could be separated from the monogallates and the digallate in 25 min on columns packed with 3% OV-1 on Chromosorb W, but the two isomeric monogallates were not resolved. The results obtained by this GLC method were lower than those given by Roberts' spectrophotometric method. The authors explained this result by incomplete extraction of TRs from solutions in ethyl acetate in Roberts' method. Another possible reason will be discussed later.

By means of column chromatography on Sephadex LH-20 with gradient elution of aqueous acetone from 40 to 80%, Takeo [143] was able to separate TF, TF monogallates (the two isomers were not resolved) and TF digallate. The contents of three TFs were determined spectrophotometrically from the molar absorptivity of authentic TF at 460 nm.

An HPLC separation of TFs on RP material was published by Hoefler and Coggon [76] and led to remarkable improvements. The separation was performed on a μBondapak C_{18} (10-μm) column. The mobile phase was acetic acid–acetone–water and UV detection (365-nm filter) was used. With a slightly modified version of this method (380-nm detection wavelength, different eluent proportions) the changes undergone by tea polyphenols during storage of tea brews were investigated [144]. Robertson [78,79] and Robertson and Bendall [145] used a modification of Hoefler and Coggon's method for the HPLC analysis of TFs formed from flavanols in a model fermentation system under various conditions. Bajaj *et al.* [146] analysed the behaviour of TFs themselves in a model oxidation system with a similar method.

Wellum and Kirby [147] systematically examined the factors that could be important in TF analysis using HPLC: sample preparation, column temperature, the use of a ternary solvent system and solvent gradient programming. Finally, they prepared the sample by means of solid-phase extraction using Sep-Pak C_{18} cartridges with TF recoveries of 98–100%. Separation on Partisil ODS columns (5 or 10 μm) was performed at 80°C applying a gradient elution system with acetone–water–methanol. Nevertheless, the two isomeric monogallates remained unresolved. Later, Anan *et al.* [148] succeeded in separating them. They also used an RP column [ODS-120A, 10 μm (Toyo Soda), 250 × 4.6 mm I.D.], elevated temperature (57°C) and detection at 375 nm, but a mobile phase containing phosphoric acid in addition to water and acetone. Sample preparation consisted only of extracting black tea with 40% aqueous acetone for 30 min, adding water, centrifuging and filtering the supernatant. The recovery of TFs was between 91.1 and 96%.

An isocratic RP-HPLC method and the flavognost method were compared in determining the TFs [149]. The HPLC method is more time consuming owing to the need for preseparation by gel chromatography, but is able to separate the four TFs (see Fig. 11) and determine their relative amounts. In general, the results obtained by the flavognost method were considerably higher than the HPLC results.

The use of DAD in the HPLC analysis of black tea [89] revealed the presence of interfering substances (flavonol glycosides) in fractions used to determine TFs and TRs in the spectrophotometric analysis developed by Roberts and Smith [129]. The contribution of flavonol glycosides to the absor-

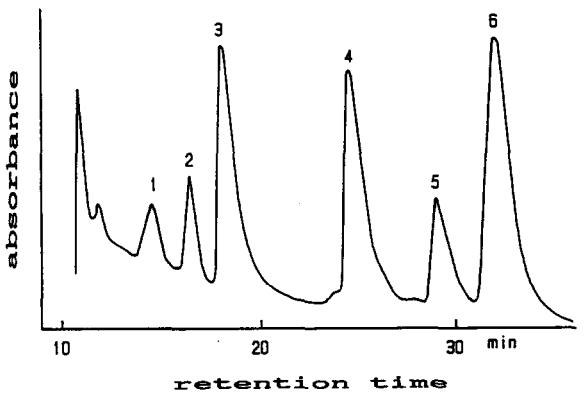

Fig. 11. HPLC separation (375 nm) of theaflavins in black tea. 1 = Isotheaflavin (tentative); 2 = epitheaflavic acid; 3 = TF; 4 = TF-3′-gallate; 5 = TF-3-gallate; 6 = TF-3,3′-digallate. From ref. 149, with permission.

bance at 380 nm is about 33%. Therefore, Roberts and Smith's method always led to an overestimation of TFs [105]. A recently published method took this fact into account. The authors used C_{18} sorbent cartridges and stepwise elution of TRs and TFs with acidified methanol for sample preparation and measured the absorbance of different fractions at one or more of the wavelength settings 380, 440 or 460 nm [150]. A comparison with HPLC, NIR and spectrophotometric methods using flavognost and aluminium chloride reagents and Roberts and Smith's method was also described. Nevertheless, in the analysis of TFs the peak identity should be verified with photodiode-array detection as described in recent publications [149,151] to avoid misleading results. Recently a comparative study of RP-HPLC of black tea phenolic pigments resulted in improved chromatograms concerning resolution and peak shape using a Hypersil ODS column (5 μm) with a citrate buffer [152]. The chelating agent citric acid reduced secondary retention through removal or masking of surface metals. Eight TFs and three theaflavic acids could be separated for the first time in one chromatogram monitored at 460 nm.

2.3.6. Thearubigins

Thearubigins (TRs) is the name originally assigned to a heterogeneous group of orange-brown, weakly acidic pigments formed by enzymic oxidative transformation of flavanols during the manufacture of black tea [122]. TRs have been claimed to be the most abundant polyphenolic fraction of black tea contributing significantly to taste, depth of colour and body of a tea brew and therefore influencing the quality. Roberts *et al.* [54] first attempted to separate this complex mixture of substances by means of 2-D PC and by liquid–liquid partitioning. They classified the TRs into three fractions: the SI-TRs extractable into ethyl acetate, the SII-TRs remaining in the aqueous phase and the SIa-TRs also in the aqueous phase but being more soluble in diethyl ether. Using successive extraction of tea liquor with ethyl acetate and *n*-butanol followed by fractional precipitation, Brown *et al.* [153] isolated five TR fractions, each being degradable into anthocyanidins, gallic acid or flavanols under different conditions of hydrolysis. For that reason TRs were assigned to be polymeric proanthocyanidins containing flavonoid residues. Cattell and Nursten [101,154] examined the relatively low-molecular-mass fraction SI soluble in ethyl acetate by means of gel chromatography on Sephadex LH-20 and 2-D PC. They obtained three subfractions, each having an M_r of about 1500. After degradation only small amounts of anthocyanidins were obtained and it was concluded that these TR fractions are pentameric flavanols with hydrolysable and non-hydrolysable links and benzotropolone units.

Several attempts have been made to separate the TRs by means of gel chromatography often in combination with 2-D PC [143,155–159]. However, many of these so-called "separations of TRs" were in fact separations of low-molecular-mass compounds accompanying the TRs, especially when whole tea brews were analysed. Many of these misleading results were obtained because of adsorption effects which disturbed the SEC. In other instances only poor separations could be achieved when analysing single TR fractions. Most promising was the application of the porous polymer packing material Toyopearl [157] for gel filtration, which showed a much higher resolving power than the frequently used Sephadex LH-20. However, chromatography of TRs is difficult, because they consist of compounds with a wide molecular mass range (700–40 000) [160] and strongly adsorb on active surfaces. Another method for isolating TR fractions was carried out by Wedzicha and Lo [161] using a multilayer coil planet centrifuge for counter-current chromatography. They obtained pure SII and a mixture of SI and SIa TR in a relatively short time (1–2 h).

The most frequently used method for the determination of TRs is that of Roberts and Smith [129] already mentioned in Section 2.3.5, measuring the absorbance of distinct solutions at 380 nm [42,44,117,130,159,162–168] or a modified version [124,131,143,169,170]. McDowell *et al.* [105] checked this method with HPLC and revealed that TRs contribute only about 23% to the absorption of the sample solution at 380 nm, whereas flavonol glycosides account for 72% and TFs for 5%. It is obvious that quantitative measurement based on absorbance at particular wavelengths can only result in approximate values for such an inhomogeneous group of compounds, especially when these substances could not be completely separated from others. A recently developed spectrophotometric

method measured absorbance at 380, 440 and 460 nm after sample preparation using C_{18} cartridges and stepwise elution with acidified methanol [150]. The selectivity of this method for separating TR and TFs was assessed by analysing the different fractions by means of HPLC with DAD.

A different way to elucidate the TR formation and structure is the method of Robertson [78,79] and Robertson and Bendall [145] using a model fermentation system. The products formed during *in vitro* enzymatic oxidation of flavanols under controlled conditions were analysed by HPLC. They used an RP-C_{18} column (Hypersil ODS, 5 μm) (20 cm × 0.4 cm I.D.) and a mobile phase consisting of 29% aqueous acetone containing 1% acetic acid. In the HPLC elution profile monitored at 375 nm they could identify the TFs among separated peaks and a number of compounds also appeared in green tea extract. The remainder was summarized under the name TRs. With a similar HPLC method Roberts *et al.* [144] carried out a study of changes undergone by TRs and TFs during storage of tea brews. From the HPLC profiles the increase in the TR fraction was determined.

To overcome the problems with the high affinity of TRs for stationary phases such as RP-C_{18} material, Wedzicha and Donovan [171] checked the potential applicability of normal-phase HPLC of derivatized SI-TR. After extraction from black tea infusions and inspection to be free from low-molecular-mass impurities, SI-TRs were converted into acetyl and methyl derivatives. The best separation of the more stable methylated SI-TRs by HPLC was obtained using a silica phase (Partisil 10) (25 cm × 0.46 cm I.D.) with a chloroform–methanol gradient, but no further identification was done.

The combination of HPLC with DAD provided additionally spectral information on each resolved component. Based on the method reported by Robertson and Bendall [145], Opie and co-workers [80,172] used the model fermentation system together with HPLC and DAD on 3-μm RP-18 particle packings to improve separation. They also replaced acetone with acetonitrile, which is advantegeous in obtaining spectra in the UV region. More than 40 pigments were separated from untreated black tea liquors and four of them were identified as TFs (see Fig. 12). Most of the other peaks showed absorption maxima between 390 and 410 nm, *i.e.*, in the yellow-pale orange region. Changes in TR profile during fermentation or substances produced by *in vitro* oxidation of a standard catechin mixture could be observed and compared by means of this method.

A comparative study of different types of RP materials (Hypersil-ODS, Hypersil octyl wide pore, Hamilton PRP-1) for the analysis of black tea pigments has recently been published [152]. The best resolution was obtained on a Hypersil-ODS column with a citrate buffer acting as a chelating agent for removing or masking surface metals. The pigments were classified by their chromatographic behaviour into three groups: pigments which ran close to the void volume of the HPLC columns because of size

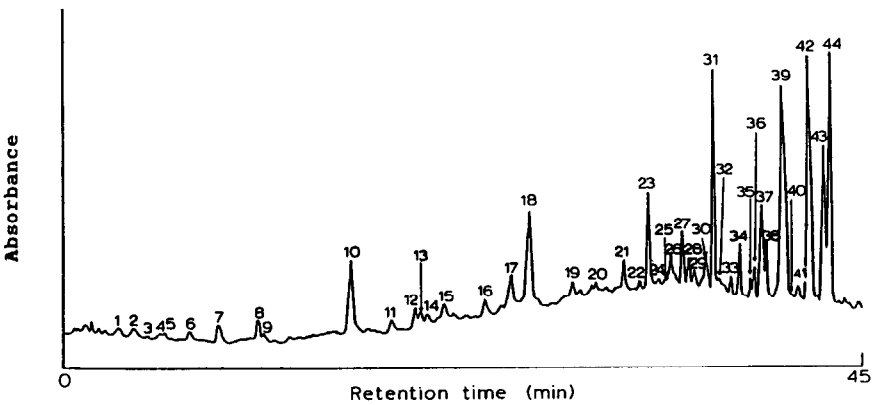

Fig. 12. HPLC analysis of a whole black tea liquor (450 nm). From ref. 80, with permission.

exclusion, resolved pigments and unresolved pigments. The resolved pigments were further classified by DAD UV–VIS spectra into TFs, TF acids and resolved TRs. The unresolved components, which were eluted as a broad, convex band on the wide-pore column and the Hamilton column (styrene–divinylbenzene copolymer), showed absorption acrosss the entire spectral region, indicating that they were brown pigments, possibly polymeric TRs.

It should be stressed that only chromatographic methods can improve our knowledge of TRs. Classical methods overestimate TR contents and so data have to be revised.

2.4. Miscellaneous

2.4.1. Non-phenolic pigments

There are two major groups of non-phenolic pigments which contribute to the colour of tea leaves, chlorophylls and its degradation products (pheophytins, pheophorbides) and carotenoids. Most of the papers on non-phenolic pigments deal with both groups. Therefore, we shall discuss them together in this section. Until recently, only PC and TLC had been applied to non-phenolic pigments of tea. Subsequently HPLC has also been introduced into this field.

Vachnadze *et al.* [60,173] used TLC for the separation of chlorophyll from a crude extract of tea catechins (solvent: ethanol). This extract was monitored for chlorophyll by means of TLC on a silica gel layer which had been impregnated with formamide. Light petroleum–formic acid (10:1) was employed as a solvent. Five chlorophyll spots were observed under UV radiation. A method for the determination of chlorophylls and accompanying pigments such as carotenoids from tea by means of TLC–spectrophotometry was established by Dzhindzholiya and Revishvili [174]. Tea was macerated with acetone, $CaCO_3$ and Na_2SO_4. The mixture was filtered and separated by TLC on a Silufol layer with light petroleum–ethanol (16:1) as the mobile phase. Determination by means of spectrophotometry was carried out after elution of the carotene, chlorophyll and carotenoide spots with light petroleum, diethyl ether and ethanol, respectively. Dev Choudhury and Bajaj [175] reported a solvent system for the separation of chlorophylls, their derivatives (pheophorbides and pheophytins) and carotenoids in tea. A Chinese researcher [61] developed a rapid method for the determination of chlorophylls and carotenoids by 2-D TLC. He employed acetone as a solvent extraction. The compounds were separated on polyamide using ethanol–water (1:1.1) in the first and toluene–ethanol–water (2:6.8:1) in the second direction. Detection was done by spectrofluorimetry. In addition to several catechins, chlorophyll *a* and *b*, xanthophyll and β-carotene were detected and identified. Komiya *et al.* [176] described a number of methods to determine tea constituents, including chlorophyll, by means of spectrophotometry. The chlorophyll concentration was very low in all samples.

TLC analysis of unsaponifiable matter from various seed oils including tea seed was performed by Nasirullah and Kapur [177]. The $CHCl_3$ solution of the hydrolysed oil was separated with benzene–ethanol (94:6). β-Carotene and several other compounds were identified. A rapid method for the extraction and TLC separation of tea leaf carotenoids was described by Tirimanna and Wickremasinghe [178]. Monohydroxyxanthophylls and carotenes could be separated on silica gel and polyhydroxyxanthophylls and epoxides on infusorial earth plates impregnated with vegetable oil. Nine of the fourteen carotenoids found could be identified.

Two HPLC methods for the separation of non-phenolic pigments in tea have been published. Kitada *et al.* [179] described various methods for the determination of several non-phenolic tea constituents (L-ascorbic acid, tocopherol, carotene and chlorophyll) in Japanese green, oolong and black tea by HPLC. Carotenes and chlorophylls were separated on Zorbax-ODS and detected with a UV–VIS detector. Sencha (Japanese green tea) contained higher amounts of carotenoids and chlorophyll than oolong and black tea. These compounds were not detected in tea brews prepared by the usual method. Recently, Taylor and McDowell [180] published a method for the rapid classification of the non-phenolic pigments of tea leaf. Fresh green tea leaf was extracted with methanol as described [181]. Separation was performed on a Hypersil-ODS column (5 μm, 250 × 4.6 mm I.D.) with gradient elution using acetonitrile–water (90:10) as solvent A and ethyl acetate as solvent B (1.5 ml/min, 0–20 min 100% A to 50% B, 20–30 min 50% B). The HPLC

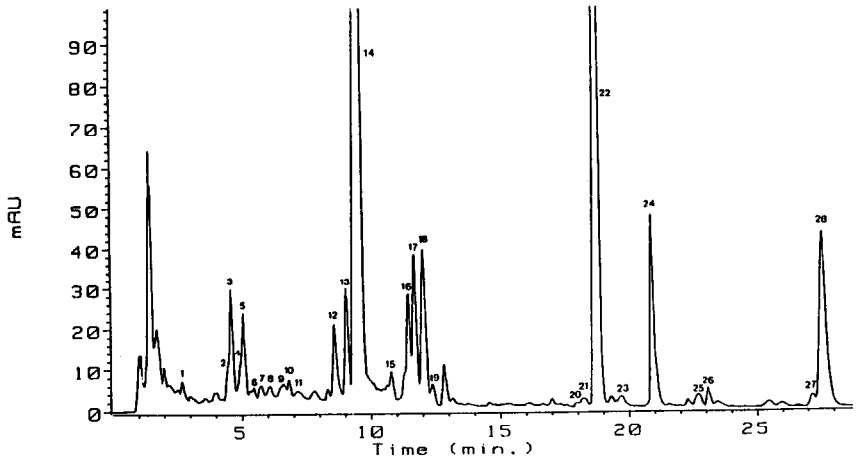

Fig. 13. HPLC elution profile of carotenoids and chlorophylls prepared from fresh leaf extracts of tea monitored at 450 nm. Identification of the main peaks: 3 and 5 = neochrome and flavoxanthin; 12 and 13 = luteoxanthin and flavoxanthin; 14 = lutein; 16 and 17 = mono isomers of lutein or lutein epoxide; 18 = isomer of lutein or lutein epoxide; 22 = chlorophyll *b*; 24 = chlorophyll *a*; 28 = *β*-carotene. From ref. 180, with permission.

trace recorded at 450 nm is shown in Fig. 13. Detection was carried out by DAD. The spectroscopic data of all 28 pigments obtained were given.

2.4.2. Amino acids

The following amino acids have been identified in tea leaves by PC or liquid chromatography [5]: aspartic acid, glutamic acid, glycine, serine, glutamine, tyrosine, threonine, alanine, valine, leucine, isoleucine, phenylalanine, lysine, arginine, histidine, tryptophan, aspargine and proline. In addition to these usual amino acids, tea contains a unique substance called theanine (5-N-ethylglutamine), which usually accounts for more than 50% of the free amino acid fraction of tea leaf and about 1% of the total dry weight. Fig. 14 shows the structure of theanine. Theanine is not found in tea proteins [182], but takes part in the biosynthesis of polyphenols. Correlations between content of amino acids and theanine and tea quality are discussed controversially in the literature. On one hand, green tea quality should depend to a large extent on amino acid level, especially that of theanine [8], and the highest quality black teas were found to possess the lowest amount of theanine [183], whereas on the other hand a high content of free amino acids is said not to be neither characteristic of green or black tea and no correlation between theanine and quality could be found [182]. Many of the amino acids in tea are involved in aroma formation. During black tea manufacture aldehydes are produced by flavanol quinone oxidation and through Strecker degradation of amino acids. Co and Sanderson [184] confirmed this mechanism in detail using a model tea fermentation system with radiotracer techniques and headspace GLC.

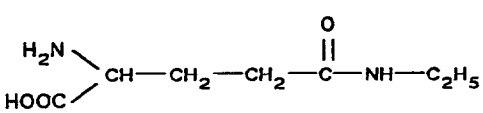

Fig. 14. Structure of theanine.

Early investigations on amino acids in tea using 2-D PC and the ninhydrin method partly after sample clean-up through a cation-exchange column were carried out by several groups [185–188]. They intended to examine the changes undergone by free amino acids, either as individual compounds or in total, during each processing step in the manufacture of black tea. In 1978 Chakraborty *et al.* [189] used this method to determine free amino acids in different parts of tea shoots and their effect on quality.

Theanine, aspartic and glutamic acid in green tea have been analysed by means of capillary tube isotachophoresis; however, these compounds could not be separated in a single run [190]. Especially for

theanine a GLC method after conversion into its TMS derivative was reported in 1974 [191]. The mass spectra were also discussed, but no method for tea sample preparation was given. Another very time-consuming method for determination of theanine in tea used a combination of TLC (4 h for one run!) and densitometry after a complex sample clean-up procedure [183,192]: extraction of tea with hot water, precipitation of polyphenolic and coloured substances with lead acetate, centrifugation, removal of excess lead by passing hydrogen sulphide through the solution and filtration.

Neumann and Montag [193] developed a method for the separation and determination of theanine in an automatic amino acid analyser together with twenty other amino acids. After purification of the aqueous tea extract by means of a strongly acidic cation exchanger, amino acids were separated with a special elution buffer system in about 160 min using ninhydrin reagent for detection (see Fig. 15). This method can be also employed for the separation of amino acids after hydrolysis.

Free amino acids and S-methylmethionine (anti-ulcer agent) in green tea extracts were simultaneously determined with an HPLC–amino acid analyzer and lithium citrate buffers which could also separate theanine and γ-aminobutyric acid from others. Analysis was carried out on a high-resolution column within 180 min. Conventional columns and sodium citrate buffers did not succeed in separating the amino acids satisfactorily [194]. Amino

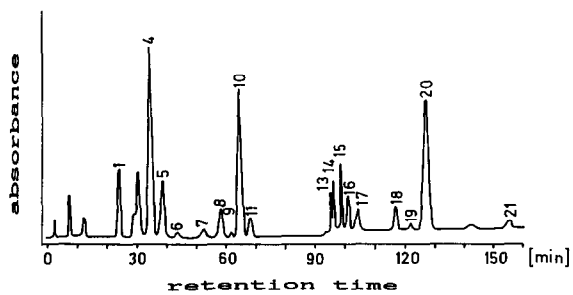

Fig. 15. HPLC separation of amino acids from a Taiwan oolong tea extract. 1 = Asparagic acid; 2 = threonine; 3 = serine; 4 = theanine; 5 = glutamic acid; 6 = proline; 7 = glycine; 8 = alanine; 9 = cysteine; 10 = homocitrullin (internal standard); 11 = valine; 12 = methionine; 13 = isoleucine; 14 = leucine; 15 = 4-aminobutyric acid; 16 = tyrosine; 17 = phenylalanine; 18 = lysine; 19 = histidine; 20 = NH_3; 21 = arginine. From ref. 193, with permission.

acid analysers are still applied [67,194,195], but have mostly been replaced with other more convenient methods such as HPLC with fluorescence detection or special RP-HPLC techniques [196–198].

After precolumn derivatization with dansyl chloride, sensitive determination of amino acids in green tea on a LiChrosorb RP-18 column was possible in about 32 min with a linear gradient of acetonitrile [198]. Zhu [196] determined theanine by HPLC with a RP-alkylphenyl C_{18} column, aqueous phosphoric acid as the mobile phase and detection at 240 nm. Analysis of amino acids by HPLC with fluorescence detection has become more common in the last few years. Hirose and Tamada [199] and Chang *et al.* [200] separated amino acids on ion-exchange columns, monitoring the fluorescence after reaction with *o*-phthalaldehyde in the presence of β-mercaptoethanol. The fluorimetric detection involved excitation at 350 nm and emission at 450 nm [200]. Other workers chose slightly different wavelength pairs: 338/425 [85], 360/500 [66], 340/455 [201], 335/450 [202] or 365/435 nm [203] for theanine alone and RP columns [66,201–203]. Using this sensitive method, a set of up to eighteen amino acids could be determined in about 90 min [85].

2.4.3. Metal speciation analysis

Research by chromatographic means has also been carried out in the field of metal speciation. The reason for these activities is the suggestion that tea polyphenols lower the absorption of Fe, Zn and Ca in the gut [204]. Disler *et al.* [205] showed in animal studies (rats) that the tannins in the tea are responsible for inhibiting the absorption of food Fe and medicinal Fe, probably by forming non-absorbable complexes with the Fe within the intestinal lumen. Brown *et al.* [206] also used rats for an animal study, and stated that beverages (coffee, black tea, cocoa and bush tea) reduced the bioavailability of Fe (whole-body retention of ^{59}Fe) by 31–45%. Individual polyphenols (EC and chlorogenic, gallic, caffeic and tannic acids) were less inhibitory than the beverages, reducing Fe bioavailability by 2–30%, while caffeic acid and tannic acid were the most inhibitory. Other workers (*e.g.*, ref. 207) stated that the addition of 100 mg of ascorbic acid or 200 g of milk completely counteracted the inhibitory effect of tea on Fe absorption. As Fe is one of the elements with a worldwide marginal status

[208], *in vivo* and *in vitro* studies have been undertaken. The binding of Cu, Zn and Fe to organic complexing compounds, tea and other beverages has been investigated [209–211]. The organically complexed metals were extracted using ethyl acetate and than analysed by RP-HPLC with UV–VIS detection and other more specific devices. The total amount of individual metals and their distribution in tea were determined by coupling HPLC and AAS. The results showed that polyphenolic compounds —especially the flavonoids— are important for the binding of the metals.

A radio-thin-layer method has also been used to detect the binding sites of Fe and Ni in tea [211]. To one tea sample ^{63}Ni or ^{59}Fe was added, followed by extraction with ethyl acetate and analysis by TLC on silica gel [with ethyl acetate or ethyl acetate–methyl ethyl ketone–formic acid–water (50:30:10:10)] or on RP-18 [methanol–water (70:30)], with radiometric detection. It turned out that the added metal ions are bound to the same compounds as Fe and Ni naturally occurring in tea and that strongly polar phenols play a major role in binding of Ni and Fe. The chromatographic behaviour of flavonoid standards and of flavonoids isolated from tea was analysed by HPLC (with electrochemical detection) and AAS. The metals (such as Cu, Fe and Al) are complexed by flavonoids containing vicinal OH bonds [212].

The high contents of aluminium in tea are also of interest. The level in tea made from twelve commercial brands was 3.9 mg/l (range 2.7–4.9 mg/l) [213], or from 2.2 to 4.5 mg/l (thirteen different tea infusions) as determined by AAS [214]. From an animal study [215] it was concluded that the Al in tea was very poorly absorbed but that tea, either in the form of an infusion or as tea leaves, had an adverse effect on Fe status.

2.4.4. Vitamins

Black tea contains only small amounts of some vitamins [8]. As the nutritional benefits of tea are not mainly due to vitamin contents, only a few methods have been published. The relatively high amounts of ascorbic acid in fresh tea leaves diminishes during fermentation. However, some determinations of vitamins by means of chromatographic methods have been published. In most instances green teas were analysed. Liang *et al.* [85] used HPLC for the detection of ascorbic acid. They found 2 mg/g in two green tea samples.

Tsushida and co-workers [216,217] also detected ascorbic acid in green tea by HPLC after extraction with 2% metaphosphoric acid by steeping for 10 min. Dehydroascorbic acid in the extract was chromatographed after reduction with H_2S. A modification of the indophenol titration method has been published [218]. Interfering colours could be removed by contacting with bovine skin powder. Ohtsuki *et al.* [194] described the determination of S-methylmethionine, which they called vitamin U, together with free amino acids (see Section 2.4.2) on a high-resolution column, MCI Gel CK-10U (150 mm × 4.6 mm I.D.). S-Methylmethionine was identified as dimethyl sulphide in the column eluates by GLC with flame photometric detection. Kitada *et al.* [179] used HPLC with an electrochemical detection system for the determination of ascorbic acid and HPLC with fluorescence detection for tocopherol in different teas. All the compounds examined were much more common in sencha than semi-fermented (oolong) and fermented (black) teas. The average contents of ascorbic acid and α-tocopherol in sencha were 167 and 13.5 mg per 100 g, respectively.

The riboflavin contents of green tea have been determined by Anan *et al.* [219] using HPLC. Tea was extracted with dilute HCl in boiling water for 30 min. The extract was mixed with an enzyme preparation. The HPLC system consisted of an ODS column and water–acetonitrile–acetic acid (88.5:11:0.5) as the mobile phase. A fluorescence detection system (excitation at 360 nm and emission at 500 nm) was used.

3. CONCLUSIONS AND FURTHER RESEARCH NEEDS

One of the major challenges in tea analysis is the improvement of our knowledge of the thearubigins, which is only possible by combining chromatographic and spectroscopic analysis with a suitable clean-up. Another problem, which is less on the analytical side, is to set up a suitable concept for volatile flavour compounds. In the field of metal speciation and bioavailability there is need for studies in which both the *in vivo* and the *in vitro* aspects are covered. Moreover, depending on the different composition and contents of polyphenolic com-

pounds, the effect of different teas on bioavailability needs to be checked.

Interdisciplinary research (chemical, biochemical and medical) is also needed for the improvement of our knowledge of the positive health effects of tea polyphenols. A chemical measure for tea quality has not yet been set up.

4. ABBREVIATIONS

Ac	Acetyl
AAS	Atomic absorption spectrometry
BSA	Bistrimethylsilylacetamide
CouQA	*p*-Coumaroylquinic acid
CQA	Caffeoylquinic acid
2-D	Two-dimensional
DAD	(Photo)diode-array detection
DMF	Dimethylformamide
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FI	Flavour index
GLC	Gas–liquid chromatography
HPLC	High-performance liquid chromatography
IBMK	Isobutyl methyl ketone
IR	Infrared spectrometry
IUPAC	International Union of Pure and Applied Chemistry
MS	Mass spectrometry
NIR	Near-infrared spectrometry
NMR	Nuclear magnetic resonance spectrometry
PC	Paper chromatography
PVPP	Polyvinylpyrrolidone
RP	Reversed-phase
SDE	Simultaneous distillation and extraction
SEC	Size-exclusion chromatography
SPE	Solid-phase extraction
THF	Tetrahydrofuran
TF	Theaflavin
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TR	Thearubigin
UV	Ultraviolet
VIS	Visible

REFERENCES

1 B. Merzenich and A. Imfeld, *Tee*, Edition Dia, St. Gallen, Cologne, 1986.
2 U. H. Engelhardt, A. Finger, B. Herzig and S. Kuhr, in W. Baltes, T. Eklund, R. Fenwick, W. Pfannhauser, A. Ruiter and H. P. Thier (Editors), *Strategies for Food Quality Control and Analytical Methods in Europe, Proceedings of EURO FOOD CHEM VI, Hamburg, September 22–26, 1991*, Behrs Verlag, Hamburg, 1991, p. 647.
3 M. A. Bokuchava and N. I. Skobeleva, *CRC Crit. Rev. Food Sci. Nutr.*, 12 (1980) 302.
4 M. A. Bokuchava and N. I. Skobeleva, *Adv. Food Res.*, 17 (1969) 215.
5 R. L. Wickremasinghe, *Adv. Food Res.*, 24 (1978) 229.
6 K. C. Willson and M. N. Clifford (Editors), *Tea —Cultivation and Consumption*, Chapman and Hall, London, 1992.
7 P. K. Mahanta, in H. F. Linskens and J. F. Jackson (Editors), *Modern Methods of Plant Analysis —New Series*, Vol. 8, Springer, Berlin, 1988, p. 221.
8 N. Graham, in G. A. Spiller (Editor), *The Methylxanthine Beverages and Foods*, Alan R. Liss, New York, 1984, p. 30.
9 J. E. James, *Caffeine and Health*, Academic Press, London, 1991.
10 T. Suzuki and G. R. Waller, in H. F. Linskens and J. F. Jackson (Editors), *Modern Methods of Plant Analysis —New Series*, Vol. 8, Springer, Berlin, 1988, p. 184.
11 Bundesgesundheitsamt, *Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG*, Beuth Verlag, Berlin, 1987, 47.00/6.
12 *Schweizerisches Lebensmittelbuch*, Eidgenössische Druck- und Materialzentrale, Bern, 1971, p. 57.
13 Z. Y. Dzneladze and E. V. Zhordaniya, *Subtrop. Kult.*, (1985) 73.
14 U. Juergens and R. Riessner, *Dtsch. Lebensm.-Rundsch.*, 76 (1980) 39.
15 M. Dulitzky, E. De la Teja and H. F. Lewis, *J. Chromatogr.*, 317 (1984) 403.
16 *ISO/TC34/SC8-Report Interlab Test*, ISO, London, 1989.
17 K. Kuwano and T. Mitamura, *Nippon Nogei Kagaku Kaishi*, 60 (1986) 115; *C.A.*, 104 (1986) 184951f.
18 H. G. Maier, personal communication, 1992.
19 J. Van Duijn and G. H. D. Van der Stegen, *J. Chromatogr.*, 179 (1979) 199.
20 A. Kunugi, T. Aoki and S. Kunugi, *Shokuhin Eiseigaku Zasshi*, 29 (1988) 136; *C.A.*, 109 (1988) 169060z.
21 M. Nishizawa, T. Chonan, I. Sekijo and T. Sugii, *Hokkaidoritsu Eisei Kenkyushoho*, (1982) 7.
22 T. Kazi, *C. R. 11th Colloq. Sci. Int. Cafe*, (1985) 227.
23 M. F. Vergnes and J. Alary, *Talanta*, 33 (1986) 997.
24 R. S. Ramakrishna, M. Dias, S. Palamakumbura and M. Jeganathan, *Can. J. Chem.*, 65 (1987) 947.
25 F. J. Muhtadi, S. S. El-Hawary and M. S. Hifnawy, *J. Liq. Chromatogr.*, 13 (1990) 1013.
26 B. Guo and H. Wan, *J. Chromatogr.*, 505 (1990) 435.
27 J. Chen, *Sepu*, 5 (1987) 367; *C.A.*, 108 (1988) 73903e.
28 P. Schreier, in H. F. Linskens and J. F. Jackson (Editors), *Modern Methods of Plant Analysis —New Series*, Vol. 8, Springer, Berlin, 1988, p. 296.
29 I. Flament, *Food Rev. Int.*, 5 (1989) 317.

30 M. A. Bokuchava and N. I. Skobeleva, in I. D. Morton and A. J. McLeod (Editors), *Food Flavours, Part B, the Flavour of Beverages*, Elsevier, Amsterdam, 1986, p. 49.
31 G. W. Sanderson, in F. Drawert (Editor), *Geruchs- und Geschmacksstoffe*, Verlag H. Carl, Nürnberg, 1975, p. 65.
32 J. Th. Heins, H. Maarse, M. C. ten Noever de Brauw and C. Weurman, *J. Gas Chromatogr.*, 4 (1966) 395.
33 D. Reymond, F. Mueggler-Chavan, R. Viani, L. Vuataz and R. Egli, *J. Gas Chromatogr.*, 4 (1966) 28.
34 R. L. Wickremasinghe, E. L. Wick and T. Yamanishi, *J. Chromatogr.*, 79 (1973) 75.
35 O. G. Vitzthum and P. Werkhoff, in G. Charambolous (Editor), *Analysis of Foods and Beverages —Headspace Techniques*, Academic Press, London, New York, 1978, p. 115.
36 T. Yamanishi, A. Kobayashi, H. Sato, A. Ohmura and H. Nakamura, *Agric. Biol. Chem.*, 29 (1965) 1016.
37 T. Yamanishi, Y. Kita, K. Watanabe and Y. Nakatani, *Agric. Biol. Chem.*, 36 (1972) 1153.
38 M. A. Gianturco, R. E. Biggers and B. H. Ridling, *J. Agric. Food Chem.*, 22 (1974) 758.
39 F. Mueggler-Chavan, R. Viani, J. Bricout, D. Reymond and R. Egli, *Helv. Chim. Acta*, 49 (1966) 1763.
40 H. A. Bondarovich, A. S. Giammerino, J. A. Renner, F. W. Shephard, A. J. Stingler and M. A. Gianturco, *J. Agric. Food Chem.*, 15 (1967) 36.
41 T. Takeo and P. K. Mahanta, *J. Sci. Food Agric.*, 34 (1983) 307.
42 P. O. Owuor, C. O. Othieno, J. M. Robinson and D. M. Baker, *J. Sci. Food Agric.*, 55 (1991) 241.
43 P. O. Owuor, C. O. Othieno, J. M. Robinson and D. M. Baker, *J. Sci. Food Agric.*, 55 (1991) 1.
44 P. O. Owuor, S. O. Obaga and C. O. Othieno, *J. Sci. Food Agric.*, 50 (1990) 9.
45 P. O. Owuor, T. Takeo, H. Horita, T. Tsuhida and T. Morai, *J. Sci. Food Agric.*, 40 (1987) 341.
46 P. Schreier and W. Mick, *Chem. Mikrobiol. Technol. Lebensm.*, 8 (1984) 97.
47 W. Mick and P. Schreier, *J. Agric. Food Chem.*, 32 (1984) 924.
48 W. Mick, E. M. Götz and P. Schreier, *Lebensm.-Wiss. Technol.*, 17 (1984) 104.
49 P. Schreier and W. Mick, *Z. Lebensm.-Unters.-Forsch.*, 179 (1984) 113.
50 P. Werkhoff, W. Bretschneider, M. Güntert, R. Hopp and H. Surberg, *Z. Lebensm.-Unters.-Forsch.*, 192 (1991) 111.
51 H. Kinugasa and T. Takeo, *Agric. Biol. Chem.*, 54 (1990) 2537.
52 S. Cheng and C. Ho, *Food Rev. Int.*, 4 (1988) 353.
53 N. Kinae, M. Yamashita, S. Esaki and S. Kamiya, in P. A. Finot, H. U. Aeschbacher, R. F. Hurrell and R. Liardon (Editors), *The Maillard Reaction in Food Processing, Human Nutrition and Physiology*, Birkhäuser, Basle, 1990, p. 221.
54 E. A. H. Roberts, R. A. Cartwright and M. Oldschool, *J. Sci. Food Agric.*, 8 (1957) 72.
55 I. S. Bhatia and M. R. Ullah, *J. Sci. Food Agric.*, 19 (1968) 535.
56 G. I. Forrest and D. S. Bendall, *Biochem. J.*, 113 (1969) 741.
57 P. J. Hilton and R. Palmer-Jones, *J. Sci. Food Agric.*, 24 (1973) 813.
58 P. J. Hilton, R. Palmer-Jones and R. T. Ellis, *J. Sci. Food Agric.*, 24 (1973) 819.
59 J. B. Cloughley, *J. Sci. Food Agric.*, 32 (1981) 1213.
60 V. Y. Vachnadze, D. A. Kipiani and K. S. Mudzhiri, *Zh. Biol. Khim.*, 1970 (1969); *C.A.*, 74 (1971) 136144k.
61 T. Ting, *Chung-Kuo Nung Yeh Hua Hsueh Hui Chih*, 19 (1981) 170; *C.A.*, 97 (1982) 214337s.
62 A. R. Pierce, H. N. Graham, S. Glassner, H. Madlin and J. G. Gonzalez, *Anal. Chem.*, 41 (1969) 298.
63 P. D. Collier and R. Mallows, *J. Chromatogr.*, 57 (1971) 29.
64 T. Nagata, *Chagyo Gijutsu Kenkyu*, (1981) 6; *C.A.*, 97 (1982) 37559t.
65 N. M. Zaprometov and N. V. Stankova, *Bull. Liaison Groupe Polyphenols*, (1980) 306; *C.A.*, 94 (1981) 117028x.
66 T. Nagata and S. Sakai, *Ikushugaku Zasshi*, 34 (1984) 459.
67 T. Nagata, *Jpn. Agric. Res. Q.*, 19 (1986) 276.
68 R. Saijo, *Agric. Biol. Chem.*, 46 (1982) 1969.
69 G. I. Nonaka, O. Kawahara and I. Nishioka, *Chem. Pharm. Bull.*, 31 (1983) 3906.
70 T. Takeo and K. Oosawa, *Chagyo Shikenjo*, 12 (1976) 125; *Food Science and Technology Abstracts*, 10 (8) (1978) H1093.
71 F. Yayabe, H. Kinugasa and T. Takeo, *Nippon Nogei Kagaku Kaishi*, 63 (1989) 845; *C.A.*, 111 (1989) 6208f.
72 F. Hashimoto, G. Nonaka and I. Nishioka, *Chem. Pharm. Bull.*, 37 (1989) 77.
73 M. Nakagawa, *Chagyo Gijutsu Kenkyu*, 58 (1980) 38; *C.A.*, 94 (1981) 119538n.
74 F. Yoshibe, I. Mukai, C. Takeo, H. Oosu, H. Kato, A. Tanaka and K. Shibata, *Jpn. Kokai Tokkyo Koho*, JP 02 311 474 (1990); *C.A.*, 114 (1991) 140123q.
75 M. H. Lee, S. C. Chen and B. W. Min, *J. Chin. Agric. Chem. Soc.*, 27 (1989) 82; *Food Science and Technology Abstracts*, 22(2) (1990) H0052.
76 A. C. Hoefler and P. Coggon, *J. Chromatogr.*, 129 (1976) 460.
77 R. Saijo, *Chagyo Gijutsu Kukuyu*, 61 (1981) 28.
78 A. Robertson, *Phytochemistry*, 22 (1983) 889.
79 A. Robertson, *Phytochemistry*, 22 (1983) 897.
80 S. C. Opie, A. Robertson and M. N. Clifford, *J. Sci. Food Agric.*, 50 (1990) 547.
81 S. Hirose and S. Tamada, *Chagyo Kenkyu Hokoku*, 50 (1979) 51; *C.A.*, 93 (1980) 44073a.
82 T. Matsuzaki and Y. Hara, *Nippon Nogei Kagaku Kaishi*, 59 (1985) 129; *Food Science and Technology Abstracts*, 19 (5) (1990) T0021.
83 Mitsui Norin Co. Ltd., *Jpn. Kokai Tokkyo Koho*, JP 60 013 780 (1985); *C.A.*, 103 (1985) 157635d.
84 Q. Ma and X. Wang, *Fenxi Ceshi Tongbao*, 7 (1988) 25; *C.A.*, 109 (1988) 53296b.
85 Y. R. Liang, Z. S. Liu, Y. R. Xu and Y. L. Hu, *J. Sci. Food Agric.*, 53 (1990) 541.
86 S. Terada, Y. Maeda, T. Masui, Suzuki and K. Ina, *Nippon Shokuhin Kogyo Gakkaishi*, 34 (1987) 20.
87 S. Kuhr and U. H. Engelhardt, *Z. Lebensm.-Unters.-Forsch.*, 192 (1991) 526.
88 Bundesgesundheitsamt, *Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG*, Beuth Verlag, 1985, 47.00/4.
89 R. G. Bailey, I. McDowell and H. E. Nursten, *J. Sci. Food Agric.*, 52 (1990) 509.
90 D. Treutter, *J. Chromatogr.*, 467 (1989) 185.
91 Y. Oshima and T. Nakabayashi, *J. Agric. Chem. Jpn.*, 26 (1953) 754.

92 Y. Oshima and T. Nakabayashi, *J. Agrid. Chem. Jpn.*, 26 (1953) 274.
93 E. A. H. Roberts, R. A. Cartwright and D. J. Wood, *J. Sci. Food Agric.*, 7 (1956) 738.
94 E. A. H. Roberts, W. Wight and D. J. Wood, *New Phytol.*, 57 (1958) 211.
95 F. Imperato, *Chem. Ind. (London)*, 3 (1980) 388.
96 I. D. Chkhikvishvili, V. A. Kurkin and M. N. Zaprometov, *Prikl. Biokhim. Mikrobiol.*, 22 (1986) 410.
97 I. D. Chkhikvishvili and M. N. Zaprometov, *Subtrop. Kul't.*, 4 (1986) 73.
98 I. D. Chkhikvishvili, V. A. Kurkin and M. N. Zaprometov, *Khim. Prir. Soedin.*, 5 (1984) 661.
99 D. A. Surmanidze, V. D. Shcherbukhin and T. O. Revishvili, *Biotekhnologiya*, 4 (1988) 491.
100 R. R. Dzhindzholiya, M. R. Pruidze and R. G. Dadiani, *Prikl. Biokhim. Mikrobiol.*, 15 (1979) 782.
101 D. J. Cattell and H. E. Nursten, *Phytochemistry*, 15 (1976) 1967.
102 M. A. Bokuchava and M. S. Ulyanova, *Fenol'nye Soedin. Ikh Biol. Funkts., Mater. Vses. Simp.*, 1 (1966) 224.
103 T. Tsushida, T. Matsuura, S. Ohta and T. Murai, *Chagyo Gijutsu Kenkyu*, 69 (1986) 45.
104 P. Biedrich, U. H. Engelhardt and B. Herzig, *Z. Lebensm.-Unters.-Forsch.*, 189 (1989) 149.
105 I. McDowell, R. G. Bailey and G. Howard, *J. Sci. Food Agric.*, 53 (1990) 411.
106 U. H. Engelhardt, A. Finger, B. Herzig and S. Kuhr, *Dtsch. Lebensm.-Rundsch.*, 88 (1992) 69.
107 A. Finger, U. H. Engelhardt and V. Wray, *Phytochemistry*, 30 (1991) 2057.
108 A. Finger, U. H. Engelhardt and V. Wray, *J. Sci. Food Agric.*, 55 (1991) 313.
109 Y. Sakamoto, *Agric. Biol. Chem.*, 31 (1967) 1029.
110 Y. Sakamoto, *Agric. Biol. Chem.*, 34 (1970) 919.
111 A. Chaboud, J. Raynaud, L. Debourcieu and J. Reynaud, *Pharmazie*, 41 (1986) 745.
112 U. H. Engelhardt, A. Finger, B. Herzig and S. Kuhr, *Tagungsband der 11. Königssteiner Chromatographietage*, GIT, Darmstadt, 1991, p. 118.
113 Y. Oshima and T. Nakabayashi, *J. Agric. Chem. Jpn.*, 26 (1953) 377.
114 U. H. Engelhardt, in H. Porkert (Editor), *Tagungsband der 10. Königssteiner Chromatographietage*, Millipore, Eschborn, 1989, p. 449.
115 E. A. H. Roberts and M. Myers, *J. Sci. Food Agric.*, 9 (1958) 701.
116 G. V. Stagg and D. Swaine, *Phytochemistry*, 10 (1971) 1671.
117 I. S. Bhatia and R. Ullah, *J. Sci. Food Agric.*, 16 (1965) 408.
118 E. A. H. Roberts, *J. Sci. Food Agric.*, 9 (1958) 212.
119 E. A. H. Roberts and D. M. Williams, *J. Sci. Food Agric.*, 9 (1958) 217.
120 M.-J. Li, Q.-K. Chen and H. F. Wang, *Tea Sci. Res. J.*, (1983) 106.
121 U. H. Engelhardt, A. Finger and B. Herzig, *Lebensmittelchem. Gerichtl. Chem.*, 43 (1989) 57.
122 E. A. H. Roberts, *J. Sci. Food Agric.*, 9 (1958) 381.
123 T. Yamanishi, in R. L. Roussef (Editor), *Bitterness in Foods and Beverages*, Elsevier, Amsterdam, 1990, Ch. 9, p. 161.
124 P. J. Hilton and R. T. Ellis, *J. Sci. Food Agric.*, 23 (1972) 227.
125 P. O. Owuor, S. G. Reeves and J. K. Wanyoko, *J. Sci. Food Agric.*, 37 (1986) 507.
126 Y. Takino, A. Ferretti, V. Flanagan, M. Gianturco and M. Vogel, *Tetrahedron Lett.*, 45 (1965) 4019.
127 D. T. Coxon, A. Holmes, W. D. Ollis and V. C. Vora, *Tetrahedron Lett.*, 60 (1970) 5237.
128 P. D. Collier, T. Bryce, R. Mallows, P. E. Thomas, D. J. Frost, O. Korver and C. K. Wilkins, *Tetrahedron*, 29 (1973) 125.
129 E. A. H. Roberts and R. F. Smith, *Analyst (London)*, 86 (1961) 94.
130 E. A. H. Roberts and R. F. Smith, *J. Sci. Food Agric.*, 14 (1963) 689.
131 M. R. Ullah, *Curr. Sci.*, 41 (1972) 422.
132 A. Robertson and M. N. Hall, *Food Chem.*, 34 (1989) 57.
133 I. McDowell, S. G. Reeves, P. O. Owuor and F. Gone, *Tea*, 6, No. 2 (1985) 25.
134 W. Spiro and W. E. Price, *Analyst (London)*, 111 (1986) 331.
135 S. G. Reeves and F. Gone, *Tea*, 5(1) (1984) 28.
136 P. O. Owuor and F. O. Gone, *Tea*, 9(2) (1988) 81.
137 M. Spiro, W. E. Price, W. M. Miller and M. Arami, *Food Chem.*, 25 (1987) 117.
138 M. Spiro and W. E. Price, *Food Chem.*, 24 (1987) 51.
139 M. Spiro and S. Siddique, *J. Sci. Food Agric.*, 32 (1981) 1135.
140 A. R. Fernando and J. A. Plambeck, *Analyst (London)*, 113 (1988) 479.
141 M. N. Hall, A. Robertson and C. N. G. Scotter, *Food Chem.*, 27 (1988) 61.
142 P. D. Collier and R. Mallows, *J. Chromatogr.*, 57 (1971) 19.
143 T. Takeo, *Jpn. Agric. Res. Q.*, 8 (1974) 159.
144 G. R. Roberts, R. S. S. Fernando and A. Ekanayake, *J. Food Sci. Technol. (India)*, 18 (1981) 118.
145 A. Robertson and D. S. Bendall, *Phytochemistry*, 22 (1983) 883.
146 K. L. Bajaj, T. Anan, T. Tsushida and K. Ikegaya, *Agric. Biol. Chem.*, 51 (1987) 1767.
147 D. A. Wellum and W. Kirby, *J. Chromatogr.*, 206 (1981) 400.
148 T. Anan, H. Takayanagi and K. Ikegaya, *Nippon Shokuhin Kogyo Gakkaishi*, 35 (1988) 487.
149 B. Steinhaus and U. H. Engelhardt, *Z. Lebensm.-Unters.-Forsch.*, 188 (1989) 509.
150 D. L. Whitehead and C. M. Temple, *J. Sci. Food Agric.*, 58 (1992) 149.
151 I. McDowell, J. Feakes and C. Gay, *J. Sci. Food Agric.*, 55 (1991) 627.
152 R. G. Bailey and H. E. Nursten, *J. Chromatogr.*, 542 (1991) 115.
153 A. G. Brown, W. B. Eyton, A. Holmes and D. Ollis, *Phytochemistry*, 8 (1969) 2333.
154 D. J. Cattell and H. E. Nursten, *Phytochemistry*, 16 (1977) 1269.
155 D. J. Millin, D. Swaine and P. L. Dix, *J. Sci. Food Agric.*, 20 (1969) 296.
156 T. Takeo and K. Oosawa, *J. Food Sci. Technol.*, 19 (1972) 406.
157 T. Ozawa, *Agric. Biol Chem.*, 46 (1982) 1079.
158 R. R. Dzhindhzoliya and M. F. Saipova, *Subtrop. Kul't.*, (1988) 40; *C.A.*, 111 (1989) 56145d.

159 M. Hazarika, S. K. Chakravarty and P. K. Mahanta, *J. Sci. Food Agric.*, 35 (1984) 1208.
160 D. J. Millin and D. W. Rustidge, *Process Biochem.*, (1967) 9.
161 B. L. Wedzicha and M. F. Lo, *J. Chromatogr.*, 505 (1990) 357.
162 D. J. Wood and E. A. H. Roberts, *J. Sci. Food Agric.*, 15 (1964) 19.
163 E. A. H. Roberts, *J. Sci. Food Agric.*, 14 (1963) 700.
164 S. Nagalakshmi and R. Seshadri, *Food Ind. Rev.*, 5 (1986) 13.
165 A. K. Biswas, A. K. Biswas and R. Sarkar, *J. Sci. Food Agric.*, 22 (1971) 196.
166 D. J. Millin and D. Swaine, *J. Sci. Food Agric.*, 32 (1981) 905.
167 M. Spiro and S. Siddique, *J. Sci. Food Agric.*, 32 (1981) 1027.
168 R. F. Smith, *J. Sci. Food Agric.*, 19 (1968) 530.
169 M. R. Ullah, *Two Bud*, 33 (1986) 46.
170 M. R. Ullah, N. Gogoi and D. Baruah, *J. Sci. Food Agric.*, 35 (1984) 1142.
171 B. L. Wedzicha and T. J. Donovan, *J. Chromatogr.*, 478 (1989) 217.
172 S. Opie, A. Robertson and H. Davies, *Tech. Memo. Campden Food Preserv. Res. Assoc.*, 477 (1988) 1.
173 V. Y. Vachnadze, D. A. Kipiani and K. S. Mudzhiri, *Tr. Inst. Farmakokhim., Akad. Nauk Gruz. SSR*, No. 11 (1969) 20.
174 R. R. Dzhindzholiya and T. O. Revishvili, *Subtrop. Kul't.*, (1977) 75.
175 M. N. Dev Choudhury and K. L. Bajaj, *Chem. Anal. (Warsaw)*, 24 (1979) 703.
176 M. Komiya, S. Kawabata and K. Yagasaki, *Reports of the Central Customs Laboratory (Kanzei Chuo Bunsekishoho)*, 27 (1987) 97.
177 Nasirullah and O. P. Kapur, *J. Oil Technol. Assoc. India*, 17 (1985) 37; *C.A.*, 104 (1985) 131906p.
178 A. S. L. Tirimanna and R. L. Wickremasinghe, *Qual. Plant. Mater. Veg.*, 20 (1971) 341.
179 Y. Kitada, K. Tamase, M. Sasaki, Y. Yamazoe, Y. Maeda, M. Yamamoto and T. Yonetani, *J. Jpn. Soc. Food Sci. Technol. (Nippon Shokuhin Kogyo Gakkaishi)*, 36 (1989) 927.
180 S. J. Taylor and I. J. McDowell, *J. Sci. Food Agric.*, 57 (1991) 287.
181 K. Eskins and H. J. Dutton, *Anal. Chem.*, 51 (1979) 1885.
182 K. Neumann and A. Montag, *Dtsch. Lebensm.-Rundsch.*, 79 (1983) 160.
183 W. Feldheim, P. Yongvanit and H. Cummings, *J. Sci. Food Agric.*, 37 (1986) 527.
184 H. Co and G. W. Sanderson, *J. Food Sci.*, 35 (1970) 160.
185 G. R. Roberts and G. W. Sanderson, *J. Sci. Food Agric.*, 17 (1966) 182.
186 R. L. Wickremasinghe, *J. Nat. Sci. Counc. Sri Lanka*, 7, No. 1 (1979) 5.
187 I. S. Bhatia and S. B. Deb, *J. Sci. Food Agric.*, 16 (1965) 759.
188 R. L. Wickremasinghe and T. Swain, *J. Sci. Food Agric.*, 16 (1965) 57.
189 S. Chakraborty, R. A. K. Srivastava and M. N. Dev Choudhury, *Two Bud*, 25 (1978) 17.
190 Y. Shiogai, T. Yagi and Akiyama, *Bunseki Kagaku*, 26 (1977) 701.
191 O. Vitzthum and P. Werkhoff, *J. Chromatogr.*, 95 (1974) 39.
192 P. Yongvanit, *Kaffee Tee Markt*, 36 (1986) 11.
193 K. Neumann and A. Montag, *Dtsch. Lebensm.-Rundsch.*, 78 (1982) 172.
194 K. Ohtsuki, M. Kawabata, H. Kokura and K. Taguchi, *Agric. Biol. Chem.*, 51 (1987) 2479.
195 B. Li and H. Hu, *Fenxi Ceshi Tongbao*, 8 (1989) 51; *C.A.*, 112 (1990) 117439x.
196 H. Zhu, *Shengwu Huaxue Yu Shengwu Wuli Jinzhan*, (1986) 65; *C.A.*, 107 (1987) 5834n.
197 C. Li, *Shipin Yu Fajiao Gongye*, (1988) 54; *C.A.*, 109 (1988) 36694n.
198 K. Ohtsuki, M. Kawabata, K. Taguchi and H. Kokura, *Kyoto-furitsu Daigaku Gakujutsu Hokoku, Rigaku, Seikatsu Kagaku*, (1979) 39; *C.A.*, 93 (1980) 112426v.
199 S. Hirose and S. Tamada, *Chagyo Kenkyu Hokoku*, (1979) 61; *C.A.*, 91 (1979) 191483w.
200 J. Chang, Z. Song and Z. Guo, *Sepu*, 7 (1989) 106; *C.A.*, 111 (1989) 38068b.
201 H. Takayanagi, T. Anan and K. Ikegaya, *Chagyo Kenkyu Hokoku*, (1989) 29; *C.A.*, 111 (1989) 152265p.
202 T. Tsushida and T. Takeo, *Chagyo Gijutsu Kenkyu*, (1983) 29; *C.A.*, 100 (1984) 119420k.
203 N. Hamada and H. Murakita, *Shimadzu Hyoron*, 45 (1988) 183; *C.A.*, 110 (1989) 152881r.
204 P. B. Disler, S. R. Lynch, J. D. Torrance, M. H. Sayers, T. H. Bothwell and R. W. Charlton, *S. Afr. J. Med. Sci.*, 40 (1975) 109.
205 P. B. Disler, S. R. Lynch, R. W. Charlton, J. D. Torrance, T. H. Bothwell, R. B. Walker and F. Mayet, *Gut*, 16 (1975) 193.
206 R. Brown, A. Klein and R. F. Hurrell, *Spec. Publ. R. Soc. Chem.*, 72 (1989) 152.
207 P. Christian and S. Seshadri, *J. Sci. Food Agric.*, 49 (1989) 431.
208 R. D. Baynes and T. H. Bothwell, *Annu. Rev. Nutr.*, 10 (1990) 133.
209 G. Weber and G. Schwedt, *Fresenius' Z. Anal. Chem.*, 316 (1983) 594.
210 G. Weber and G. Schwedt, *Z. Lebensm.-Unters.-Forsch.*, 178 (1984) 110.
211 G. Weber and G. Schwedt, *J. Chromatogr.*, 285 (1984) 380.
212 G. Weber, *Chromatographia*, 26 (1988) 133.
213 S. J. Fairweather-Tait, G. R. Moore and S. E. J. Fatemi, *Nature (London)*, 330 (1987) 213.
214 M. J. Baxter, J. A. Burrell and R. C. Massey, *Food Addit. Contam.*, 7 (1990) 101.
215 S. J. Fairweather-Tait, Z. Piper, S. J. A. Fatemi and G. R. Moore, *Br. J. Nutr.*, 65 (1991) 61.
216 T. Tsushida and T. Takeo, *Chagyo Gijutsu Kenkyu*, (1980) 34; *C.A.*, 94 (1981) 82314h.
217 T. Tsushida and F. Fukazawa, *Chagyo Gijutsu Kenkyu*, (1980) 29.
218 K. Yagasaki and T. Kato, *Kanzei Chuo Bunsekishoho*, 25 (1985) 103.
219 T. Anan, H. Takayanagi and K. Ikegaya, *Nippon Shokuhin Kogyo Gakkaishi*, 35 (1988) 396.