ELSEVIER

Journal of Chromatography A, 926 (2001) 211–220

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Analysis of procyanidins in chocolate by reversed-phase high-performance liquid chromatography with electrospray ionisation mass spectrometric and tandem mass spectrometric detection

Jan Wollgast, Lea Pallaroni, Marie-Elisabeth Agazzi, Elke Anklam*

Joint Research Centre of the European Commission, Institute for Health and Consumer Protection, TP 260, I-21020 Ispra, Italy

Abstract

The analysis of procyanidins in crude chocolate extracts by reversed-phase high-performance liquid chromatography (HPLC) with electrospray ionisation mass spectrometry (MS) is described in this report. Catechin monomers and procyanidin oligomers (dimers to hexamers) were identified according to the mass of the single charged pseudomolecular ion ($[M-H]^-$). Identification was further confirmed by collision-induced dissociation MS–MS analysis, which in addition, permitted the identification of double charged pentameric, hexameric, and heptameric ions. This study demonstrates the capability of the combination of HPLC and modern ion trap mass analysers to significantly reduce sample preparation and analysis time in combination with high specificity and structural information for compound identification.

Keywords: Food analysis; Stereoisomer separation; Procyanidins; Catechins

1. Introduction

Polyphenols are naturally occurring compounds produced in the secondary metabolism of many plants, which are widespread in nature. They occur in a variety of food, such as fruits, vegetables, nuts, seeds, beverages and even some manufactured food, as a compound of the natural ingredients used [1]. Cocoa is particularly rich in polyphenols (6–8%, w/w, of the dry bean), the predominant polyphenols being the catechins (flavan-3-ols) and procyanidins as the oligomeric and polymeric catechins (Fig. 1) [2].

Physiological effects of cocoa polyphenols on the human organism, such as antioxidative, anti-atherogenic, anti-inflammatory and immune-modulating have been subjects of a number of both in vitro and in vivo studies [3–14]. We have recently presented a review discussing the possible contribution of polyphenols in chocolate to human health [15]. Since then, the research on putative health effects has focused on the effects of single procyanidin fractions demonstrating specific physiological effects for some of the higher oligomeric fraction whereas the lower appeared to have no or less activity [5,10–14].

Thus, due to their possible beneficial implications in human health, there is a growing scientific and commercial interest to determine not only the procyanidin content in total but also the content of specific oligomers in cocoa products as well as in other food products. The method commonly used is high-performance liquid chromatography (HPLC), although other techniques such as thin-layer chromato-

*Corresponding author. Tel.: +39-0332-785390; fax: +39-0332-785930.

E-mail address: elke.anklam@jrc.it (E. Anklam).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00994-3

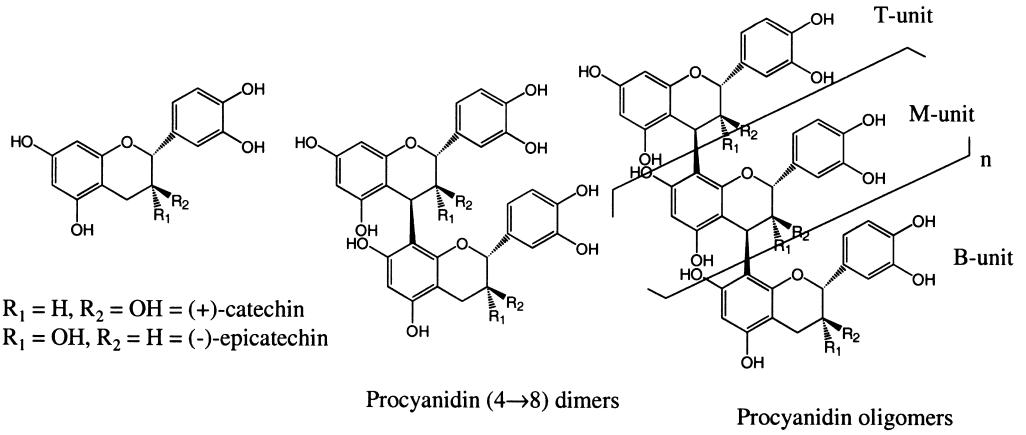

Fig. 1. Polyphenols typically found in chocolate.

graphy (TLC) [16] and capillary electrophoresis [17,18] have been used as well. The two monomeric flavan-3-ols catechin and epicatechin in chocolate and other cocoa products have been identified and quantified by reversed-phase (RP) HPLC with UV and fluorescence detection, respectively [19–22]. Although monomers and small oligomers may be separated from polymeric procyanidins by RP-HPLC, the latter appear as a broad unresolved peak in the UV or fluorescence detector towards the end of the chromatogram. Therefore, a method based on HPLC using a normal-phase silica column and a gradient of dichloromethane–methanol–formic acid–water mixtures has been developed to separate oligomeric and polymeric procyanidins on a molecular mass basis [23]. Procyanidin oligomers (dimers through pentamers) have been monitored at 280 nm, fractions collected over several runs and the procyanidins identified by thiolysis as described previously [16,24]. With the recent developments of interfacing and ionisation technologies for liquid chromatography, such as continuous flow fast atom bombardment (CF-FAB or flow FAB), thermospray (TSP), particle beam (PB), atmospheric pressure chemical ionisation (APCI), and electrospray ionisation (ESI), as well as advances in computer technologies, mass spectrometry (MS) coupled to HPLC has become a powerful tool in on-line detection and identification [25]. ESI as a soft ionisation technique producing only pseudomolecular ions with almost no fragmentation has become the technique of choice for the analysis of polyphenols by HPLC–MS. The modified normal-phase HPLC method [23] has been applied coupling the HPLC system to a quadrupole mass analyser with an ESI interface for the identification of procyanidins in chocolate and fresh cocoa beans, respectively [26]. Catechin monomers and procyanidin oligomers have been identified due to the mass-to-charge ratio (m/z) of their single or multiple charged pseudomolecular ions in the negative ion mode. However, neither the HPLC separation nor the MS allowed for the determination of stereoisomers, such as catechin and epicatechin both having the same retention time and mass-to-charge ratio of the pseudomolecular ion. In addition, the identification of compounds due only to their corresponding pseudomolecular ions can be difficult in the presence of interfering matrix compounds showing the same mass-to-charge ratios of their respective pseudomolecular ions. As a consequence, the sample preparation still required a solid-phase extraction (SPE) step to remove interfering sugars. Moreover, the analysis time of 70 min per sample is rather long and the method requires the use of toxic chlorinated solvents that are considered to be an ecological hazard [27].

The objective of the work described here was to develop a RP-HPLC–MS method capable of separating and tentatively identifying procyanidins in chocolate in a reasonably short analysis time and with a minimum of sample preparation. This was achieved by the use of a modern ion trap mass analyser that allows not only for the detection of pseudomolecular ions but also of specific fragments

of these ions due to collision-induced dissociation (CID) reactions with helium in the mass analyser. This study includes MS and MS–MS analysis of cocoa procyanidins with an ion trap and ESI in the negative ion mode as well as the investigation of the presence of multiple charged species by determination of the isotopic distribution of the signals.

2. Experimental

2.1. Chemicals and materials

Standards of (−)-epicatechin, (+)-catechin, and procyanidin B2 were purchased from Campoverde (Milan, Italy). Acetonitrile, *n*-hexane and acetone (Aldrich, Milan, Italy) were of HPLC grade. Water was purified in a Super-Q Plus, Millipore–Waters System (Millipore, Vimodrone Milan, Italy). Dark chocolate was purchased from a local supermarket.

2.2. Sample preparation

Approximately 10 g of chocolate was cut in small pieces, transferred into a 200-ml polytetrafluoroethylene (PTFE) beaker and liquid nitrogen added. The frozen samples were then ground in an analytical laboratory mill. One gram of the ground chocolate sample was defatted twice with 10 ml *n*-hexane for 5 min in an ultrasonic bath at 30°C and was subsequently centrifuged for 10 min at 3000 *g*. Catechins and procyanidins were extracted from the air-dried sample with 10 ml of a mixture of acetone–water–acetic acid (70:29.8:0.2, v/v/v) for 10 min at 30°C in the ultrasonic bath. The sample was filtered through a folded filter (595 1/2, Schleicher and Schuell, Milan, Italy) and the organic solvent was then removed by rotary evaporation under partial vacuum at 40°C. The remaining aqueous extract was filtered through a 45-μm PTFE filter (Waters SpA, Milan, Italy) and was ready for HPLC–MS separation without further clean up.

2.3. HPLC–MS equipment

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

2.4. HPLC–MS conditions

RP-HPLC analyses were performed using a 5-μm Supelcosil LC-18 column (250×4.6 mm; Supelco, Milan, Italy) at room temperature with a solvent system consisting of 0.2% acetic acid (A) and acetonitrile (B) under the following conditions: linear gradients from 6 to 25% B in A (0–18 min), 25 to 60% B in A (18–20 min), isocratic 60% B in A (20–25 min), 60 to 6% B in A (25–27 min), 6% B in A (27–30 min). The column was additionally re-equilibrated with 6% B in A for 5 min prior to each analysis. The flow-rate was set to 1 ml/min and the injection volume to 20 μl. The outlet of the HPLC system was split (3:1) to the ESI interface of the mass analyser. After identifying the retention times of the catechins and procyanidins in subsequent analyses the first 5 min of the LC eluent containing matrix compounds that were not retained by the column was diverted to waste, then the valve automatically switched over to the ESI source in order to avoid a quick decrease in sensitivity of MS analysis.

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

All MS and MS–MS analysis were carried out in the negative ion mode under the following optimised conditions: source voltage 3.9 kV, capillary voltage −31 V, capillary temperature 300°C, sheath gas (N_2) flow 80 arb (arbitrary units), auxiliary gas (N_2) flow 10 arb. Full scan MS spectra (m/z 250–2000) were first recorded during the chromatographic run and the pseudomolecular ions of each catechin and procyanidin identified. MS–MS spectra were recorded by isolating the pseudomolecular ion of interest in the ion trap followed by CID. The collision energy required in this process was set to 30% of the total available collision energy. High-resolution scans (~10 000 resolution compared to

~2000 in normal scan mode) were recorded in the range $\pm m/z$ 5 near the ESI generated single- or double-charged pseudomolecular ions of procyanidins for direct resolution and observation of carbon isotope distribution.

3. Results and discussion

The full scan negative ionisation mass spectrum of catechins and procyanidins derived from chocolate extraction is shown in Fig. 2. The monomeric (+)-catechin and (−)-epicatechin with a molecular mass of 290 show pseudomolecular ions ($[M–H]^-$) at m/z 289 (compound **I** in Fig. 2). It is well known that cocoa procyanidins consist of sub-units of the monomeric catechins (Fig. 1) with interflavanoid C–C linkages [15,22,25]. Thus, the subsequent oligomeric dimers, trimers, tetramers and so forth have molecular masses of 578 (290+288), 866 (578+288), 1154 (866+288) etc., and give pseudomolecular ions at m/z 577, 865, 1153, 1441 and 1729 for dimers through hexamers (compounds **II**–**VI**).

Fig. 3 shows the mass chromatograms of compounds **I**–**VI**. From these findings, the presence of monomeric catechins and dimeric through hexameric procyanidins in chocolate can already be suggested. Moreover, due to the separation power of RP-HPLC, the presence of several stereoisomers can be anticipated according to the number of peaks per mass chromatogram. As for example, there are two dimers (m/z 577) with retention times of 11.5 and 17.4 min, respectively. In addition, there seems to be present four isomeric trimers, four isomeric tetramers, as well as two isomeric hexamers.

In order to gain more confidence in identifying cocoa procyanidins, additional structural information was obtained by CID-MS–MS experiments on the detected pseudomolecular ions. The fragmentation pathways of dimeric and trimeric procyanidins and prodelphinidins are proposed to include mainly retro Diels–Alder reactions (RDA) and cleavage of the interflavanoid linkages, respectively [28]. These fragmentation pathways have been confirmed by direct infusion of an authentic standard of procyanidin B2 (epicatechin 4β→8 epicatechin) (data not shown).

In order to distinguish the flavanol units in proanthocyanidins (procyanidins and prodelphinidins) Porter developed a nomenclature [29] depending on the positions of the interflavanoid bonds. In accordance therewith, a T-unit (top) has only one interflavanoid linkage at C_4, the M-units (middle) have an additional linkage at C_6 or C_8, and the B-unit (base) has one interflavanoid bond at C_8 or C_6 (Fig. 1). The configuration of the bonds at C_4 is indicated by the αβ nomenclature.

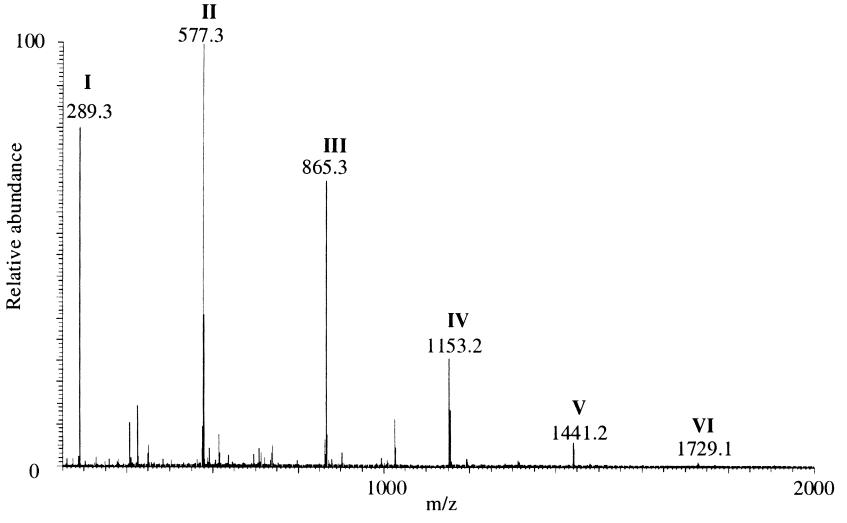

Fig. 2. Negative ion mass spectrum of chocolate extract. Compound **I**: catechin monomers, compounds **II–VI:** procyanidin oligomers (dimer through hexamer).

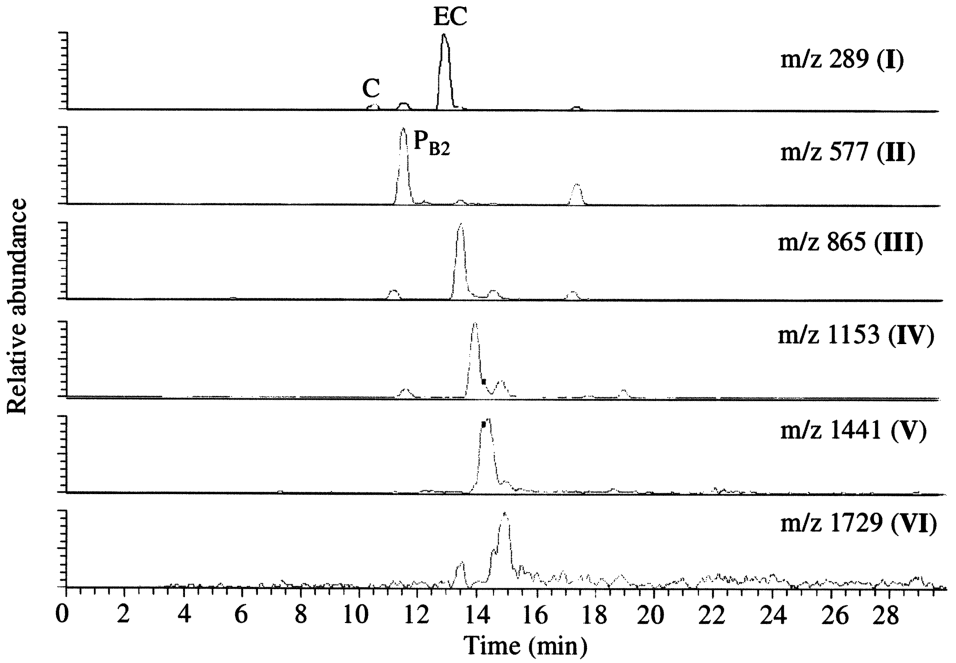

Fig. 3. Mass chromatograms of catechin monomers (m/z 289) and procyanidin oligomers (dimer through hexamers m/z 577 to 1729). C, (+)-catechin; EC, (−)-epicatechin, and P_{B2}, procyanidin B2 as identified by the retention times of authentic standards.

Fig. 4 shows the MS–MS spectrum of procyanidin tetramer. As expected by the suggested general scheme for proanthocyanidins [28] the loss of gallic acid ($C_6H_6O_3$, M_r 126) as well as losses due to RDA fission and interflavanoid cleavage are the predominant fragmentation pathways of the tetramer. The RDA fission in the C-ring of the flavanoid skeleton leads to the loss of a neutral $C_8H_8O_4$ fragment (M_r

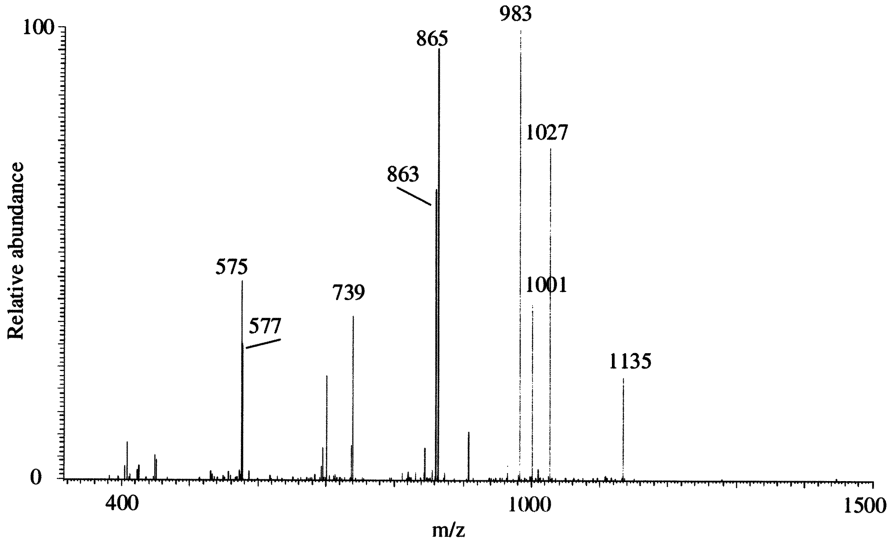

Fig. 4. CID-MS–MS spectrum of procyanidin tetramer ($[M-H]^-$ of m/z 1153).

168). Two pathways are possible for the cleavage of the interflavanoid linkage. One is based on the loss of neutral fragments containing the T-unit that leads to fragment ions containing the B-unit corresponding to already observed lower procyanidins, e.g. in the case of the tetramer a dimer $[M_B–H]^-$ at m/z 577 and a trimer $[M_B–H]^-$ at m/z 865, respectively. The other possibility is the loss of neutral fragments containing the B-unit leading to fragment ions containing the T-unit corresponding to lower procyanidins minus 2 H, e.g. here a dimeric ion $[M_T–H]^-$ at m/z 575 and a trimeric ion $[M_T–H]^-$ at m/z 863, respectively. These findings are summarised in Table 1 for the tetramer but they have also been confirmed for procyanidin dimers, trimers, pentamers, hexamers and heptamers.

It is known that multiple charged species like $[M–2H]^{2-}$ or $[M–3H]^{3-}$ are often observed with ESI [28] and this has already been reported for higher oligomeric cocoa procyanidins (from pentamer on) [26]. In order to investigate the existence of multiple charges, a special high-resolution mass scan has been applied. This experiment allowed for the determination of the carbon isotope distribution in the range near the ions of interest. In the case of a single charged ion the distance between two carbon isotope ions would be m/z 1, in the case of a double charged ion m/z 0.5 and so forth [25]. In addition, the relative distributions of the ions of the carbon ^{12}C and ^{13}C isotopes, respectively, can be calculated and compared to the measured intensities for further evidence of the presence of procyanidin oligomers [25]. Although the intensity was very low, the distance of approximately m/z 0.5 suggested the presence of double charged ions of pentamers at m/z 720, hexamers at m/z 864 and heptamers at m/z 1008. An example of the high-resolution spectrum of the double charged heptamer (m/z 1008) compared to the spectrum of a single charged trimer (m/z 865) is shown in Fig. 5. It is evident that due to the low intensity of a double charged ion of the procyanidn heptamer at m/z 1008 the relative abundance (RA) of the carbon isotopic distribution of ions at m/z 1008.4 (RA=80), m/z 1009.0 (RA=100), and m/z 1009.6 (RA=15), respectively, does not correspond well to the calculated carbon isotope distribution for a heptamer ($C_{105}H_{86}O_{42}$) due to interferences of other compounds from the matrix. Considering only the contribution of the isotopic distribution of ^{12}C and ^{13}C (and not the very low contribution of the isotopic distributions of ^{1}H and ^{2}H or ^{16}O and ^{17}O, respectively) the calculated distribution of the hepta-

Table 1
Main masses in the MS–MS spectrum of procyanidin tetramer (m/z 1153=$[M–H]^-$)

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

[a] Analogous to the suggestions of Friedrich et al. [28].

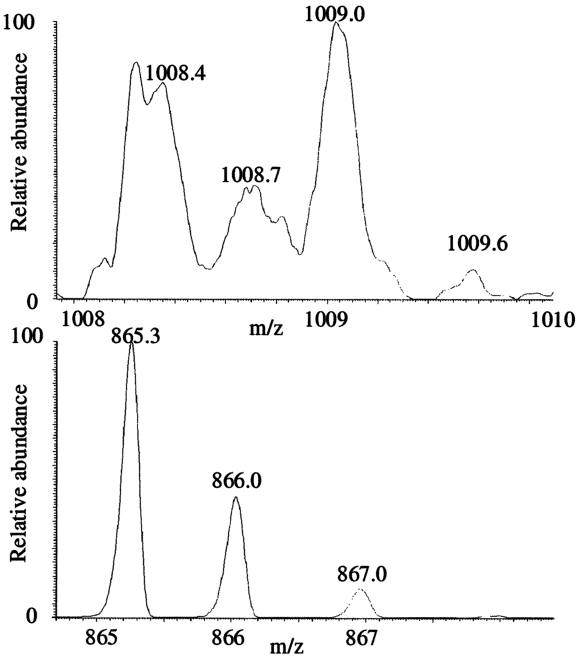

Fig. 5. Negative ion mass spectra (profile format) of double charged procyanidin heptamer ($[M-2H]^{2-}$ of m/z 1008) (top) and of single charged procyanidin trimer ($[M-H]^{-}$ of m/z 865) (bottom).

meric ions would be m/z 1008.3 (RA=85), m/z 1008.8 (RA=100), and m/z 1009.3 (RA=58). However, the approximate distance of m/z 0.5 suggests the presence of double charged heptameric ions and justifies further MS–MS experiments as discussed below. In contrast, for the trimer the calculated distributions of the carbon isotope ions would be m/z 865.2 (RA=100), m/z 866.2 (RA=50), and m/z 867.2 (RA=12) which corresponds very well to the measured relative intensities (Fig. 5).

In order to assure the presence of double charged ions of procyanidins CID-MS–MS experiments have been carried out on the ions at m/z 720, 864 and 1008, respectively. The spectra have been compared to the MS–MS spectra of the single charged pentamer and hexamer and in all three cases investigated for the fragmentation patterns due to RDA fission and breakage of interflavanoid linkages as discussed for the tetramer. Fig. 6a (bottom) represents the MS–MS spectrum of the single charged pentameric ion $[M-H]^{-}$ at m/z 1441, Fig. 6b (bottom) the MS–MS spectrum of the double charged pentameric ion $[M-2H]^{2-}$ at m/z 720. Both spectra show the typical fragmentation pattern of procyanidins as discussed before, although, in the MS–MS spectrum of the double charged ion there are other non-specific ions present (e.g. m/z 635, 644). However, this is probably due to matrix interferences as the intensity of the double charged pentameric ion is relatively low. In addition, the presence of a double charged pentameric ion is shown by the sum of the two mass chromatograms of m/z 863 and 865, Fig. 6b (top) showing a nearly identical chromatogram as for the single charged pentameric ion (Fig. 6a (top)). The presence of fragment ions at higher mass-to-charge ratios than the parent ion indicates double or multiple charges of the latter. Finally, in Fig. 7, the presence of a double charged heptameric ion at m/z 1008 is indicated by a MS–MS spectrum showing the typical fragmentation pattern of a procyanidin ion and a sum mass chromatogram of unique fragment ions at m/z 863 and 865, respectively.

4. Conclusion

RP-HPLC with ESI-MS detection in the negative ion mode allowed for an appropriate separation of catechins and procyanidins in chocolate in 20 min. The sample preparation only consisted of a two-stage extraction procedure (defatting of chocolate with hexane and extraction of polyphenols with acetone–water) and the total analysis time per sample including column re-equilibration was 35 min, half of the time required for a previous reported HPLC–MS method for cocoa procyanidins [26]. Catechin monomers and procyanidin oligomers were identified according to the mass of the single charged pseudomolecular ion ($[M-H]^{-}$). Identification was further confirmed by CID-MS–MS analysis, which in addition, permitted the identification of double charged pentameric, hexameric, and heptameric ions ($[M-2H]^{2-}$). The use of mass chromatograms of unique fragment ions subsequent to MS–MS is also promising for future quantitative analysis since the background noise can be significantly reduced thereby improving specificity and sensitivity of the analysis (Figs. 6 and 7).

Stereoisomers having the same molecular mass were separated well applying this RP-HPLC method.

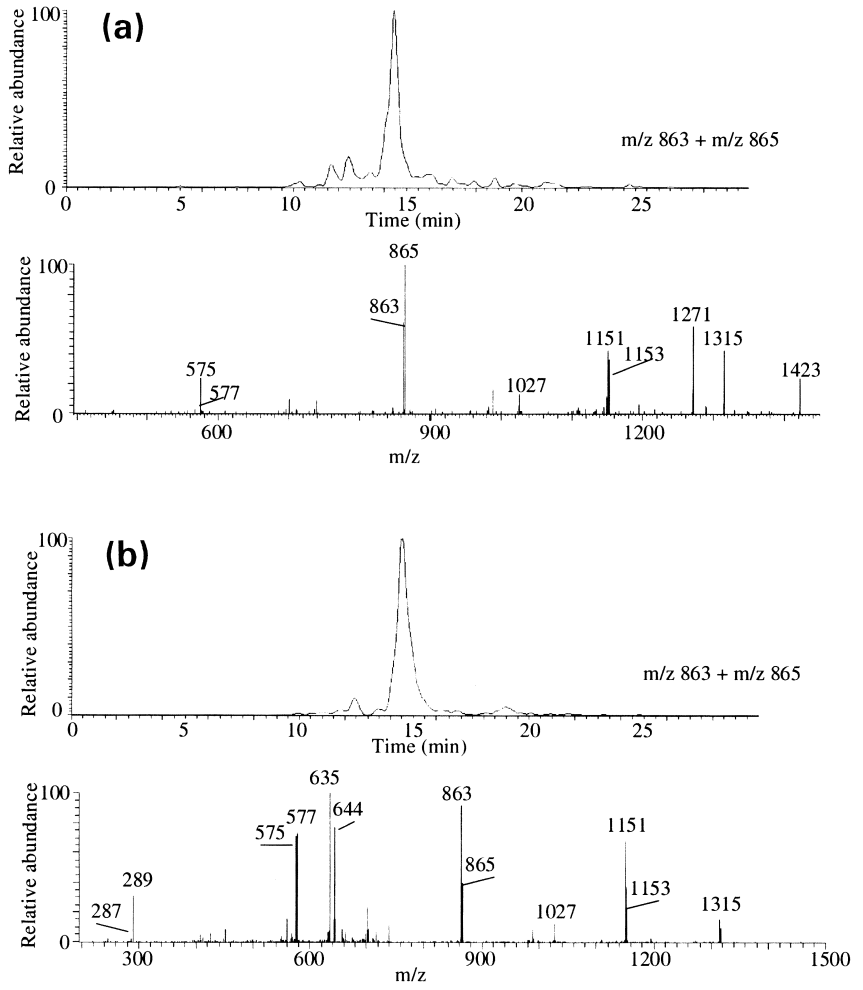

Fig. 6. (a) CID-MS–MS spectrum of single charged procyanidin pentamer ($[M-H]^-$ of m/z 1441) (bottom) and sum of mass chromatograms of two of the product ions ($[M-H]^-$ of m/z 863 and 865) (top), (b) CID-MS–MS spectrum of double charged procyanidin pentamer ($[M-H]^{2-}$ of m/z 720) (bottom) and sum of mass chromatograms of two of the product ions ($[M-H]^-$ of m/z 863 and 865) (top).

The major peaks in the mass chromatograms of the monomers and dimers were epicatechin and epicatechin 4→8 epicatechin (procyanidin B2), respectively as confirmed by comparing the retention times of authentic standards. Consequently, it could be assumed that the major peaks in the mass chromatograms of the trimer, tetramer, pentamer, and hexamer are also built up of epicatechin monomers. This assumption is supported by observations described previously [16] about the presence of almost exclusively epicatechin-based procyanidins in cocoa. However, it is then astonishing that the epicatechin dimer elutes before the epicatechin monomer, whereas the other epicatechin oligomers elute later in the order of increasing molecular mass.

It is evident that the monomeric composition of procyanidins cannot be deduced by comparing the retention times in RP-HPLC in the absence of procyanidin standards. Moreover, the position and stereochemistry of the interflavanoid linkage cannot be elucidated by means of mass spectrometry. Isolation of procyanidins and subsequent acid degradation in the presence of a nucleophilic agent, such as phloroglucinol or phenylmethanethiol [16,24] or the

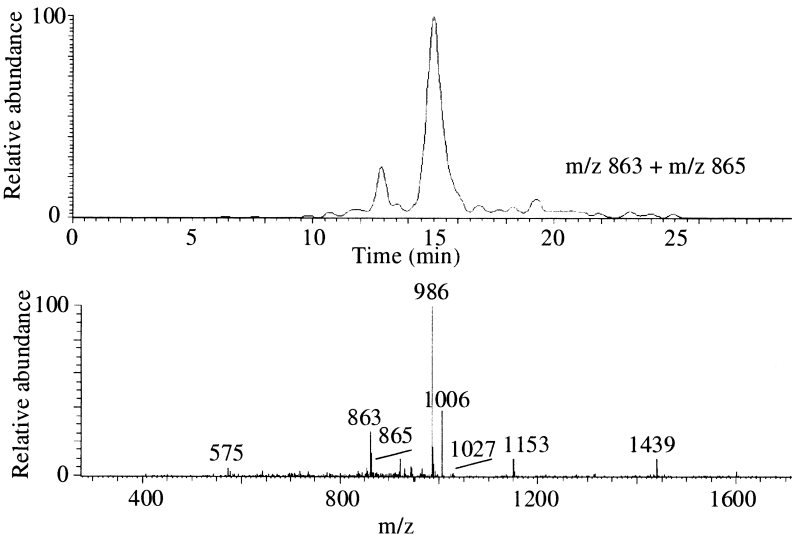

Fig. 7. CID-MS–MS spectrum of double charged procyanidin heptamer ($[M–H]^-$ of m/z 1008) (bottom) and sum of mass chromatograms of two of the product ions ($[M–H]^-$ of m/z 863 and 865) (top).

application of highly sophisticated techniques, such as nuclear magnetic resonance [16] and infrared spectroscopy would be necessary to confirm the full structure.

Acknowledgements

This work was supported in part by Kraft Foods International R&D, Munich, Germany.

References

[1] J. Wollgast, E. Anklam, Food Res. Int. 33 (2000) 423.
[2] A. Zumbe, BNF Nutr. Bull. 23 (1998) 94.
[3] K. Kondo, R. Hirano, A. Matsumoto, O. Igarashi, H. Itakura, Lancet 348 (1996) 1514.
[4] D. Rein, T.G. Paglieroni, T. Wun, D.A. Pearson, H.H. Schmitz, R. Gosselin, C.L. Keen, Am. J. Clin. Nutr. 72 (2000) 30.
[5] D. Rein, T.G. Paglieroni, D.A. Pearson, T. Wun, H.H. Schmitz, R. Gosselin, C.L. Keen, J. Nutr. (Suppl.) 130 (2000) 2120S.
[6] C. Sanbongi, N. Suzuki, T. Sakane, Cell. Immunol. 177 (1997) 129.
[7] D. Rein, S. Lotito, R.R. Holt, C.L. Keen, H.H. Schmitz, C.G. Fraga, J. Nutr. (Suppl.) 130 (2000) 2109S.
[8] J.F. Wang, D.D. Schramm, R.R. Holt, J.L. Ensunsa, C.G. Fraga, H.H. Schmitz, C.L. Keen, J. Nutr. (Suppl.) 130 (2000) 2115S.
[9] M. Richelle, I. Tavazzi, M. Enslen, E.A. Offord, Eur. J. Clin. Nutr. 53 (1999) 22.
[10] M. Karim, K. McCormick, C.T. Kappagoda, J. Nutr. (Suppl.) 130 (2000) 2105S.
[11] T.K. Mao, J.J. Powell, J. van de Water, C.L. Keen, H.H. Schmitz, M.E. Gershwin, Int. J. Immunother. XV (1999) 23.
[12] T.K. Mao, J. van de Water, C.L. Keen, H.H. Schmitz, M.E. Gershwin, J. Nutr. (Suppl.) 130 (2000) 2093S.
[13] T.K. Mao, J.J. Powell, J. van de Water, C.L. Keen, H.H. Schmitz, M.E. Gershwin, J. Med. Food 3 (2000) 107.
[14] G.E. Arteel, P. Schroeder, H. Sies, J. Nutr. (Suppl.) 130 (2000) 2100S.
[15] J. Wollgast, E. Anklam, Food Res. Int. 33 (2000) 449.
[16] L.J. Porter, Z. Ma, B.G. Chan, Phytochemistry 30 (1991) 1657.
[17] P.G. Pietta, P.L. Mauri, L. Zini, C. Gardana, J. Chromatogr. A 680 (1994) 175.
[18] P.J. Larger, A.D. Jones, C. Dacombe, J. Chromatogr. A 799 (1998) 309.
[19] H. Kim, P.G. Keeney, J. Food Sci. 49 (1994) 1090.
[20] J.A. Vinson, J. Proch, L. Zubik, J. Agric. Food Chem. 47 (1999) 4821.
[21] I.C.W. Arts, P.C.H. Hollman, D. Kromhout, Lancet 354 (1999) 488.
[22] I.C.W. Arts, B. van de Putte, P.C.H. Hollman, J. Agric. Food Chem. 48 (2000) 1752.
[23] J. Rigaud, M.T. Escribano-Bailon, C. Prieur, J.-M. Souquet, V. Cheynier, J. Chromatogr. A 654 (1993) 255.
[24] J. Rigaud, J. Perez-Ilzarbe, J.M. Ricardo da Silva, V. Cheynier, J. Chromatogr. 540 (1991) 401.

[25] R. Willoughby, E. Sheehan, S. Mitrovich, A Global View of LC/MS, Global View Publishing, Pittsburgh, PA, 1998.
[26] J.F. Hammerstone, S.A. Lazarus, A.E. Mitchell, R. Rucker, H.H. Schmitz, J. Agric. Food Chem. 47 (1999) 490.
[27] The 1987 Montreal Protocol on Substances that Deplete the Ozone Layer (http://www.unep.org/ozone/mont_t.htm).
[28] W. Friedrich, A. Eberhardt, R. Galensa, Eur. Food Res. Technol. 211 (2000) 56.
[29] L.J. Porter, in: J.B. Harborne (Ed.), The Flavanoids: Advances in Research Since 1980, Chapman and Hall, London, 1988, p. 21.