Identification of Proline-Based Diketopiperazines in Roasted Coffee

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Five proline-based diketopiperazines were identified in water extracts of roasted coffee proteins and roasted coffee itself. These are cyclo(pro-ile), cyclo(pro-leu), cyclo(pro-phe), cyclo(pro-pro), and cyclo(pro-val). The isolation included gel chromatography and solvent ($CHCl_3$) extraction; in the case of roasted coffee brews, polyamide column chromatography was also used. The identification was achieved by LC-ESI-MS and -MS/MS by comparison of the retention time and the fragmentation pattern with reference compounds. As a second method GC-EI-MS was used. By both methods the presence of diketopiperazines in roasted coffee was unambiguously verified.

Keywords: *Roasted coffee; green coffee water-soluble proteins; bitterness; diketopiperazines; LC-ESI-MS; GC-EI-MS*

INTRODUCTION

Bitterness is an important attribute of roasted coffee quality. However, the compounds responsible for the perceived bitterness in roasted coffee are still unknown. Caffeine is the only characterized bitter compound, and it accounts for 10−30% of the total bitterness (Voilley et al., 1977; Chen, 1979). Other green coffee constituents such as trigonelline, chlorogenic acids, or quinic acid are not important for the bitterness of roasted coffee (Belitz, 1975). It has rather been suggested that the bitter compounds of roasted coffee are formed during the roasting process (Belitz, 1975). Claims have been made that nitrogen-containing heterocyclic compounds contribute to the perceived bitterness of coffee beverages. In model experiments with proline and carbohydrates under roasting conditions, bispyrrolidino- and pyrrolidinohexose reductones are formed, which are intensively bitter (threshold values = 0.02−0.04 mmol/L), but their presence in coffee is still elusive (Pabst et al., 1988). Green coffee contains ∼10% proteins (Macrae, 1985) divided into a water-soluble albumin fraction and a water-insoluble fraction, each contributing 50%. During the roasting process the total amount of amino acids in coffee beans is reduced by 20−40%; the content of reactive amino acids such as lysine is strongly decreasing, whereas other amino acids such as proline or phenylalanine remain nearly unchanged. Model experiments were carried out by roasting proteins from different plants or animals (Jugel et al., 1976). All aqueous extracts showed a strong bitter taste at concentrations between 0.00005 and 0.008%, which is the same order of magnitude as chininhydrochloride (0.001%). It was therefore concluded that proteins are in general important precursors for bitter compounds in roasted and fermented food.

Diketopiperazines are cyclic dipeptides formed from two amino acids by cyclodehydration. They were identified in several fermented and thermally treated foods and in some of them contributed to the perceived bitterness. The bitterness of cacao is due to the formation of diketopiperazines during the cacao production. A synergistic effect between theobromine and the diketopiperazines in cacao was also observed (Pickenhagen et al., 1975). The formation of diketopiperazines from nonbitter linear peptides or proteins was proposed instead of a stepwise cyclization from free amino acids (Rizzi, 1989). Diketopiperazines were also identified in roasted malt (Sakamura et al., 1978), in aged sake (Takahashi et al., 1974), in cereal grains (Dansi et al., 1970), and in other foods. Seven proline-based diketopiperazines have been identified in beer (Gautschi and Schmid, 1997). The flavor characteristics of these diketopiperazines were described as bitter, mouth coating, and astringent in concentrations from 10 to 50 ppm.

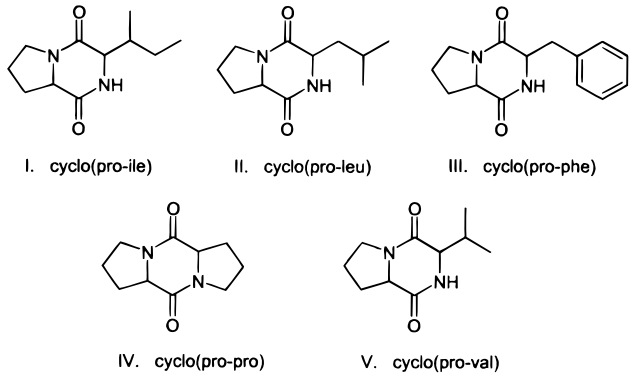

Figure 1. Structures of the diketopiperazines identified in roasted coffee proteins and roasted coffee.

In this paper we report the identification of five proline-based diketopiperazines in roasted coffee proteins, which are cyclo(pro-ile), cyclo(pro-leu), cyclo(pro-phe), cyclo(pro-pro), and cyclo(pro-val) (see Figure 1), and their identification in roasted coffee itself for the first time.

MATERIALS AND METHODS

Water. The water used throught this study was of HPLC grade.

Coffee. The green coffee (washed Brazil Arabica) was frozen in liquid nitrogen and ground to a particle size <0.5 mm. The ground coffee and all other samples were stored at −18 °C.

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The roasted coffee used was a commercial sample purchased from a local retailer.

Cold Water Extract. Ten grams of ground green coffee was extracted with 100 mL of cold water by sonication for 15 min. The mixture was centrifuged (13500 rpm, 20 min), filtered, and freeze-dried.

Precipitation with Acetone. Three grams of this freeze-dried extract was redissolved in 100 mL of water (supersonic bath, 10 min), and acetone (100 mL) was added. The suspension was centrifuged (13500 rpm, 20 min). The precipitate was diluted with water and freeze-dried.

Cleanup of the Acetone Precipitate. One hundred milligrams of the freeze-dried acetone precipitate was suspended in 50 mL of water, extracted for 15 min in a supersonic bath, and finally stirred for 2 h. The solution was centrifuged (13500 rpm, 30 min), filtered through a 0.45 μm membrane filter, and freeze-dried.

Gel Chromatography of the Cleaned Acetone Precipitate. Fifty milligrams of the cleaned acetone precipitate was dissolved in 4 mL of water. A 1.5 × 60 mm Sephadex G10 column was used for separation. The eluent was water, and 5 mL fractions were collected. Eluting peaks were monitored using a photometer at 254 nm.

Hot Water Extract. Forty milligrams of roasted and ground coffee was extracted with hot water (1000 mL), filtered, and freeze-dried.

Methanol Extract. One gram of the hot water extract was extracted with methanol (50 mL, HPLC grade). The solution was stirred for 2 h, filtered, and freeze-dried.

Polyamide Column Chromatography. Two hundred and fifty milligrams of the methanol extract was dissolved in water (2 mL). A polyamide column (10 × 1.5 cm i.d.) was conditioned with water (250 mL). The sample was added, eluted with water (250 mL), and freeze-dried.

Chloroform Extract. Five hundred milligrams of the water fraction of the polyamide column was extracted with chloroform (40 mL), stirred for 1 h, and filtered. The solvent was removed, water was added, and the mixture was freeze-dried.

Bradford Method. The method for protein quantification was carried out as described by Darbre (1986). Bovine serum albumin (BSA) was used as calibrant.

Biuret Method. The method was carried out as described by Darbre (1986). Calibrant was also BSA.

Gel Electrophoresis. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was performed (16% acrylamide) at a constant current of 50 mA (Janson and Rydén, 1989). Staining of the proteins was obtained with Coomassie brilliant blue. All fractions (cold water extract, acetone precipitate, cleanup of the acetone precipitate and the isolated proteins) and a color marker (wide molecular weight range = 6500−205000) (Sigma-Aldrich, Saint Louis, MO) were simultaneously analyzed.

Model Roasting. All roasting experiments were carried out in a self-constructed roasting apparatus, which is basically a GC oven. The substances to be roasted were mixed in a double-neck flask with 10 g of sea sand. The oven temperature applied was 290 °C (corresponding to a sample temperature of 220−240 °C), and the flask was inserted into the oven and flushed with nitrogen. After 3 min, the flask was cooled to room temperature, and the contents were extracted with 30 mL of boiling water, filtered, and freeze-dried.

Gel Chromatography of Roasted Coffee Proteins. Fifty milligrams of the roasted coffee proteins was redissolved in 3 mL of water, membrane filtered (0.45 μm), and separated on a Sephadex G10 column (see above). The eluent was water, and fractions of 25 mL were collected and freeze-dried.

RP-HPLC. Analytical RP-HPLC was carried out on a Nucleosil 100-5 C18 HD 250 × 4 mm column with a precolumn of Nucleosil 100-5 C18 HD 11 × 4 mm (Macherey-Nagel, Düren, Germany). The eluents were water (A) and ethanol (B), with isocratic elution (5% B) for 5 min, followed by a linear gradient to 50% B in 45 min and reset. Flow rate was 0.5 mL/min, detection wavelength was 220 nm, and injection volume was 20 μL. The sample concentration was ~1 mg/mL in 5% ethanol.

LC-ESI-MS. A Bruker Esquire mass spectrometer was used in the positive ESI mode. Settings were as follows: scan range, m/z 50−1000; nebulizer pressure, 50 psi; dry gas (N_2) flow, 10 L/min; dry temperature, 365 °C; capillary, −2500 V; endplate, −2000 V; capillary exit, 120 V; skimmer I, 40 V; skimmer II, 10 V; cut-off (trap drive), m/z 55.

For HPLC conditions see above.

Mass Spectroscopic Data for Compounds I—V. Cyclo(pro-ile) (**I**): ESI-MS, m/z 211; ESI-MS/MS of 211 (%), m/z 211 (1.1, $[M + H]^+$), 194 (6.5), 183 (100), 166 (21.0), 155 (14.5), 138 (37.6), 128 (4.0), 114 (6.8), 96 (1.9), 86 (7.0), 70 (64).

Cyclo(pro-leu) (**II**): ESI-MS, m/z 211; ESI-MS/MS of 211 (%), m/z 211 (1.1, $[M + H]^+$), 194 (37.4), 183 (100), 166 (20.7), 155 (14.7), 138 (37.0), 128 (3.0), 114 (6.2), 96 (1.4), 86 (6.0), 70 (62.7).

Cyclo(pro-phe) (**III**): ESI-MS, m/z 245; ESI-MS/MS of 245 (%), m/z 245 (0.1, $[M + H]^+$), 228 (3.6), 217 (87.9), 200 (6.3), 172 (32.9), 154 (7.8), 131 (10.2), 120 (100), 103 (6.9).

Cyclo(pro-pro) (**IV**): ESI-MS, m/z 195; ESI-MS/MS of 195 (%), m/z 195 (0.1, $[M + H]^+$), 187 (4.1), 151 (3.0), 98 (14.8), 70 (100).

Cyclo(pro-val) (**V**): ESI-MS, m/z 197; ESI-MS/MS of 197 (%), m/z 197 (0.2, $[M + H]^+$), 187 (18.7), 180 (27.4), 169 (100), 152 (8.6), 141 (34.3), 124 (31.3), 70 (46.7).

GC-EI-MS. The instrument was a Hewlett-Packard GC 5890 (series II) with an MSD 5972: splitless injction (200 °C); DB-5 MS (60 m × 0.25 mm i.d., 0.25 μm); carrier, He (1.1 mL min^{-1}); temperature program, 200 °C, 25 min isothermal, ramped to 300 °C in 10 min. MS settings: source temperature, 167 °C, 1.5 scans/s, 70 eV. Alternatively a Carlo Erba HRGC 5160 Mega GC with a DB-5 MS (60 m × 0.25 mm i.d., 0.25 μm) linked to a Finnigan MAT ITDS (EI-mode, 70 eV, trap temperature of 220 °C, transfer line temperature of 270 °C) was used. Temperature program: isothermal at 180 °C for 30 min, ramped at 10 °C/min to 300 °C.

Mass Spectroscopic Data for Compounds I − V. Cyclo(pro-ile) (**I**): EI-MS, m/z (%) 154 (95), 125 (22), 96 (4), 86 (11), 70 (100), 55 (17), 43 (10), 41 (5).

Cyclo(pro-leu) (**II**): EI-MS, m/z (%) 154 (90), 125 (12), 124 (10), 96 (7), 86 (18), 70 (100), 55 (14), 43 (19), 41 (14).

Cyclo(pro-phe) (**III**): EI-MS, m/z (%) 244 (30, M^+), 153 (48), 125 (100), 91 (54), 70 (70), 43 (10), 41 (28), 28 (27).

Cyclo(pro-pro) (**IV**): EI-MS, m/z (%) 194 (22, M^+), 166 (7), 138 (6), 124 (6), 110 (14), 96 (15), 70 (100), 55 (9), 42 (17).

Cyclo(pro-val) (**V**): EI-MS, m/z (%) 196 (4, M^+), 154 (85), 125 (30), 72 (39), 70 (100), 43 (20).

Synthesis of Diketopiperazines. Cyclo(pro-leu) was purchased from Bachem Biochemica GmbH (Heidelberg, Germany). All other diketopiperazines were synthesized in the institute (Ginz et al., 2000). Chemical synthesis was basically carried out as described by Pickenhagen et al. (1975). Alternatively, the corresponding amino acids were heated with sea sand at 290 °C for 2 min. After cooling, the mixture was dissolved in water, extracted with $CHCl_3$, and purified by preparative RP-HPLC (Nucleosil 100-7 C-18 250 × 21 mm) (Macherey-Nagel) with a water/ethanol gradient [isocratic elution (20% B) for 5 min, followed by a linear gradient to 80% B in 45 min and reset] at 5 mL/min and detection at 220 nm. In the case of chemical synthesis only one geometric isomer was observed (*cis*), whereas the thermal treatment yielded both forms. The isolated diketopiperazines were chemically characterized by ESI-MS, ^1H NMR, and ^{13}C NMR (data not shown).

Sensory Analysis. The sensory analyses were all carried out by a panel of six to eight participants. The panelists were trained to perceive the bitter taste according to an official method (BGVV, 1997).

RESULTS AND DISCUSSION

Identification of Diketopiperazines in Roasted Coffee Proteins. It is established that water extracts of roasted proteins in general have a strong bitter taste (Jugel et al., 1976). To find out if this is also true for coffee proteins and to gain further information about the structure of the compounds that are responsible for

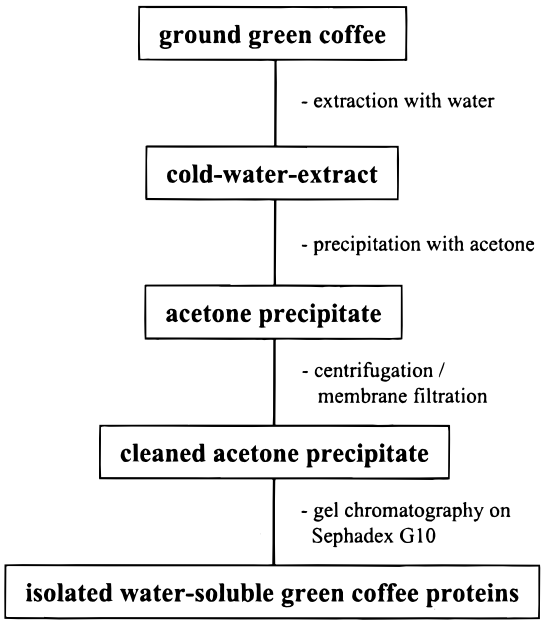

Figure 2. Isolation of water soluble proteins from green coffee.

this bitterness, the water-soluble green coffee proteins were isolated from the green coffee matrix. Three points were important for the isolation procedure: (i) it should involve as little chemical and thermal treatment as possible; (ii) the taste of the isolated proteins should remain unaffected; and (iii) toxic chemicals should be avoided. Dialysis was considered for the isolation in principle as described by Amorim and Josephson (1975). However, the time required (6 days) and the risk of contamination with microorganisms are disadvantageous. Another approach employed a dialysis for 24 h, but problems with the denaturation of the proteins were observed (Arnold, 1995). In this study a precipitation of the water-soluble green coffee proteins by acetone was used. This procedure is fast, and the risk of chemical changes or contamination is minimized. After a cleaning step with membrane filtration and centrifugation, the proteins were separated from the remaining nonproteins by gel chromatography (Figure 2). To determine whether the separation used was successful or not, the protein content of the four fractions was checked by both the Bradford and biuret methods (Darbre, 1986). The protein content increased from 21% in the cold water extract to 99% in the final fraction. To find out whether the water-soluble green coffee proteins have changed in composition during the isolation procedure, the fractions were finally checked by gel electrophoresis. The pattern was the same for all four fractions; only a small protein loss between 6500 and 14500 g/mol was observed (data not shown).

The isolated green coffee water-soluble proteins were roasted under nitrogen in a self-constructed model roasting apparatus, and hot water extracts were prepared. These hot water extracts of the roasted proteins were tasted by a panel trained for bitterness. All panelists judged the extracts as being intensely bitter. As expected, coffee proteins are able to form bitter substances under roasting conditions as do other plant and animal proteins (Jugel et al., 1976). In an RP-HPLC screening the chromatogram of the roasted proteins showed a variety of signals (data not shown). Therefore, a further separation on Sephadex G10 was carried out, which yielded eight fractions. The fractions were ana-

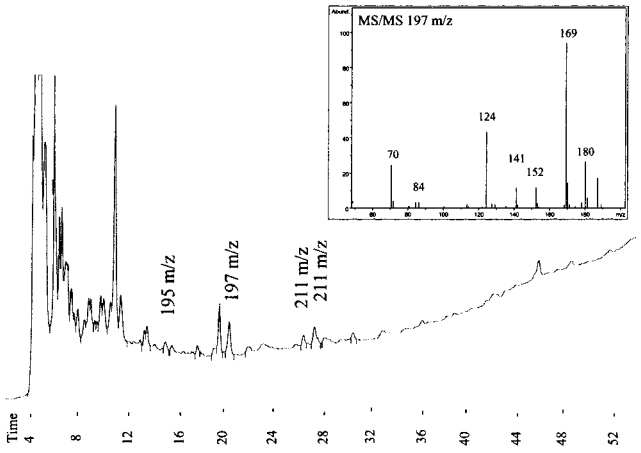

Figure 3. RP-HPLC separation of fraction III (Sephadex G10), monitored at 220 nm, and the ESI-MS/MS spectrum of cyclo(pro-val).

lyzed by LC-ESI-MS. In fraction III a group of four compounds with a characteristic fragmentation pattern and an intensive *m/z* 70 fragment was present. Another compound of that type was detected in fraction IV. In Figure 3 the chromatogram of fraction III (monitored at 220 nm) is given including the MS/MS spectrum of *m/z* 197 at a retention time of 20.4 min. The same fragmentation pattern (two losses of *m/z* −28 and two losses of *m/z* −17 from the $[M + H]^+$ ion) were also observed for the two signals of *m/z* 211 at retention times of 26.5 and 27.2 min and for the signal of *m/z* 245 in fraction IV with a retention time of 30.1 min. In an analysis of all 20 proteinaceous amino acids by ESI-MS/MS, no amino acid with an *m/z* 70 was found except proline, and therefore it was concluded that the compounds in question are likely to contain proline.

It is established in the literature that so-called diketopiperazines are present in various thermally treated foods (see above). Therefore, it was suggested that the compounds are cyclo(pro-ile), cyclo(pro-leu), cyclo(pro-phe), cyclo(pro-pro), and cyclo(pro-val). From an analysis of the five reference compounds by LC-ESI-MS, the retention times and mass spectra were found to be similar to those found in fractions III and IV. Furthermore, the MS/MS spectra were identical in terms of both fragmentation pattern and intensity, which confirmed the presence of these five proline-based diketopiperazines in roasted coffee proteins.

GC-EI-MS has been used as an alternative method for the identification of diketopiperazines. Seven proline-based diketopiperazines were identified in beer using GC-EI-MS (Gautschi and Schmid, 1997). In the original method a separation of cyclo(pro-leu) and cyclo(pro-pro) was not achieved, but their separation was essential in our case. To obtain further analytical proof, a modification of this method allowing the separation of all five diketopiperazines was developed. To separate the compounds of interest from the more polar compounds, the fractions of the gel chromatographic separation were extracted with $CHCl_3$. In Figure 4 a GC-EI-MS chromatogram of the cyclo(pro-leu) reference and of the chloroform extract of fraction III of the Sephadex G10 fractionation at the same retention time is shown. The retention time and the mass spectra of all five diketopiperazines coincided with the EI-MS data of the reference compounds and from the literature (Gautschi and Schmid, 1997). There were other signals with similar spectra present in the roasted coffee protein

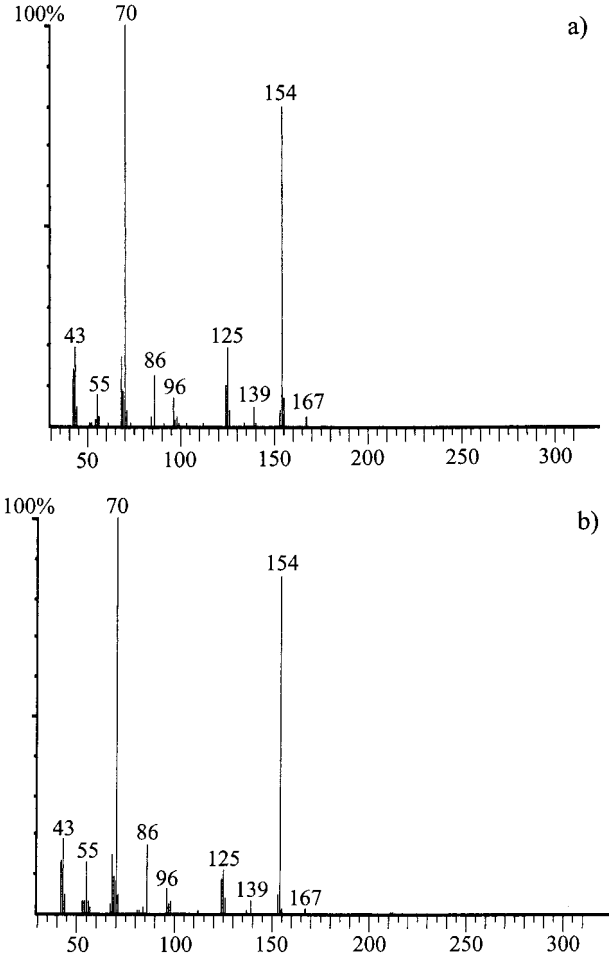

Figure 4. GC-EI-MS of cyclo(pro-leu): (a) reference compound; (b) $CHCl_3$ extract of fraction III of the gel chromatographic separation.

extracts, probably due to the formation of *cis*/*trans* isomers (see discussion in the coffee section).

Identification of Diketopiperazines from Roasted Coffee. After the identification of proline-based diketopiperazines (DKPs) from the protein fraction, coffee itself was analyzed for the presence of these compounds. In the bean environment a variety of chemical changes, such as Maillard type reactions, may compete with DKP formation or even prevent it.

Chen (1979) used organic solvent extraction and chromatographic methods to identify the bitterness of roasted coffee. On the basis of this method a cleanup was performed as given in Figure 5. All fractions were analyzed by LC-ESI-MS with the same method used for the identification of diketopiperazines in the roasted coffee proteins (see above). In fractions III and IV of the Sephadex G10 separation the signals of the five diketopiperazines were detected at the expected retention times. Cyclo(pro-ile), cylco(pro-leu), cyclo(pro-pro), and cyclo(pro-val) were identified in fraction III, and cyclo(pro-phe) was identified in fraction IV. The ESI-MS/MS spectra obtained were similar to those from the roasted coffee proteins and to the reference spectra of the five diketopiperazines. To verify the identification with a second independent analytical method, a chloroform extract of the water fraction of the polyamide column was analyzed by the same GC-EI-MS method as described above. In this case an additional separation with gel chromatography was not necessary. In Figure 6 the total ion count (TIC) of the chloroform extract is given.

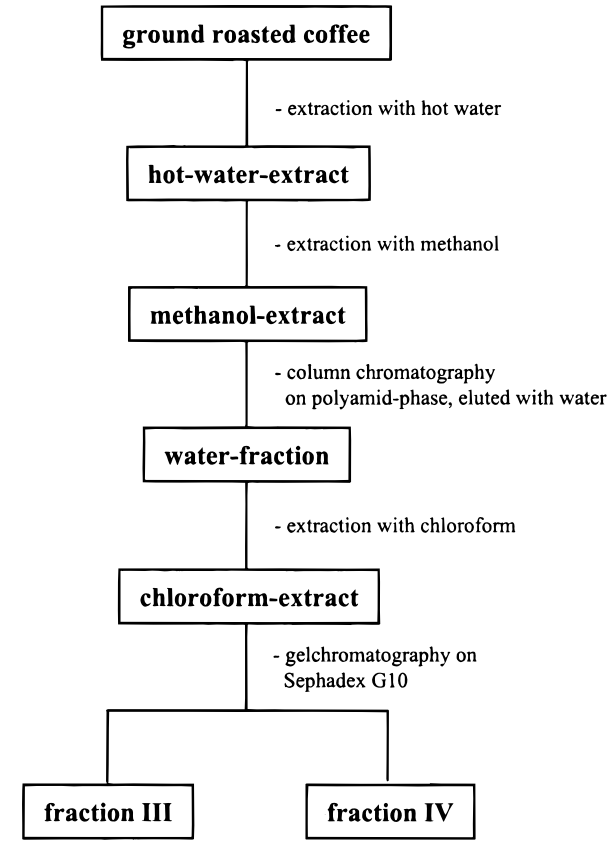

Figure 5. Cleanup for roasted coffee.

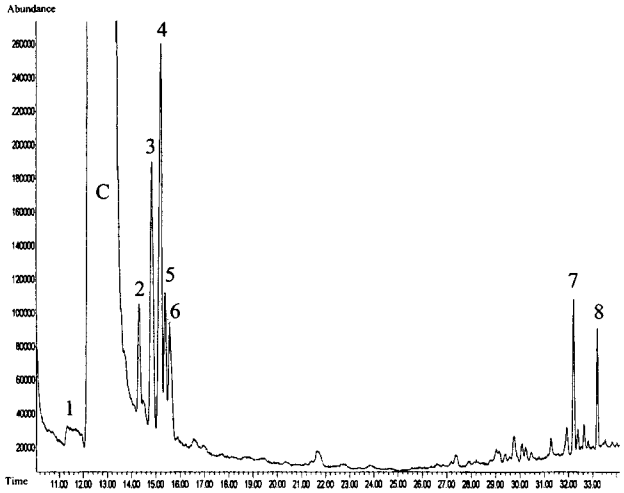

Figure 6. GC separation of diketopiperazines isolated from roasted coffee (MS detection, TIC shown): C, caffeine; 1, cyclo(pro-val); 2 and 5, cyclo(pro-ile); 3 and 4, cyclo(pro-leu); 6, cyclo(pro-pro); 7 and 8, cyclo(pro-phe).

By comparison with the reference compounds peaks 1, 2, 3, 6, and 7 were identified as cyclo(pro-val), cyclo(pro-ile), cyclo(pro-leu), cyclo(pro-pro), and cyclo(pro-phe), respectively. Thereby, the HPLC-ESI-MS results were confirmed and the presence of the five prolin-based DKPs in roasted coffee was proven. Further analyses of the other signals of the chloroform extract indicated the presence of both isomeric forms of each diketopiperazine. The cyclodehydration to a double-ring system or in the case of cylco(pro-pro) to a three-ring system leads to two isomeric forms. In the chloroform extract the EI-MS of peak 4 (Figure 6) is similar to the EI-MS of the identified cyclo(pro-leu) of peak 3. The fragmenta-

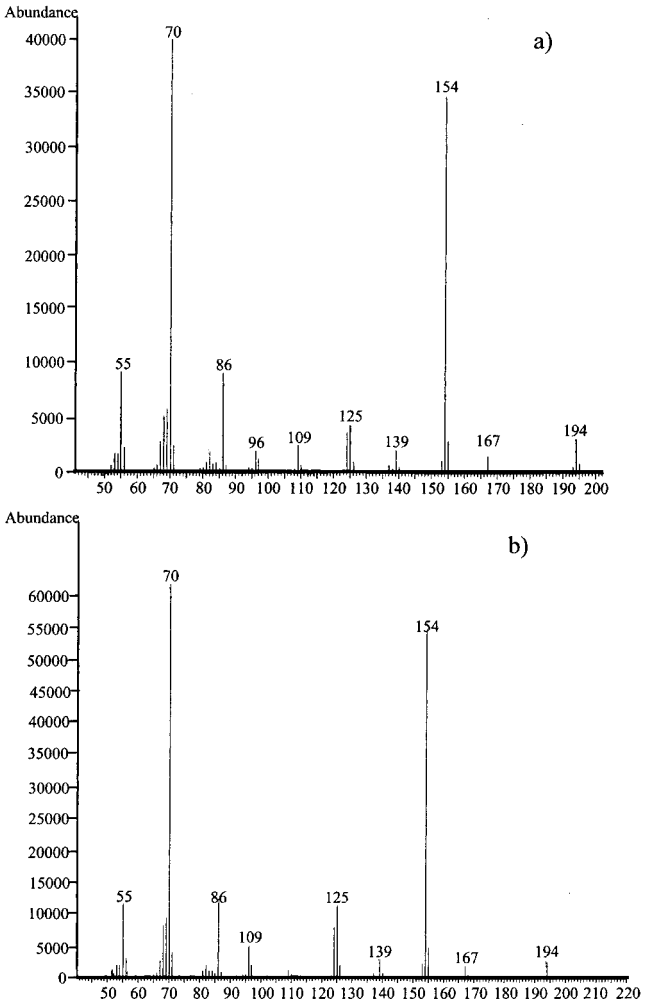

Figure 7. EI spectrum of cyclo(pro-leu) at retention times of 14.8 and 15.2 min given in Figure 6.

tion pattern and the fragment intensity of both EI-MS spectra are identical (Figure 7). This leads to the conclusion that both the *cis* and the *trans* forms of cyclo(pro-leu) are formed during the roasting process. The same effect was true for cyclo(pro-ile) (peaks 2 and 5, Figure 6) and cyclo(pro-phe) (peaks 7 and 8, Figure 6). In the case of cyclo(pro-pro) only one signal (peak 6, Figure 6) was detected, probably because the isomers are not separated under the applied conditions. In the case of cyclo(pro-val) (peak 1, Figure 6) the peak shape is kind of strange; however, a closer inspection led to the conclusion that peak 1 actually consists of two peaks.

As the educts of the chemical synthesis were optical pure compounds (L-amino acids), it can be assumed that only the *cis* compounds are formed. In the case of the preparation of the standards by thermal treatments, an isomerization is most likely, and, consequently, in those standards two peaks with identical mass spectra were observed. By comparison with the retention times it can be stated that under the GC conditions applied in the case of the phenylalanine-containing DKPs, the *trans* DKPs elute before the *cis* DKPs, whereas in the case of the proline-based DKPs the reverse order was detected.

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