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IDENTIFICATION AND QUANTITATION OF DIKETOPIPERAZINES BY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY, USING A MOVING BELT INTERFACE

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SUMMARY

High-performance liquid chromatography in combination with mass spectrometry using a moving belt interface was applied to rapid profiling of diketopiperazines in cocoa powder. On-line high resolution measurements were used for the identification of Cyclo(-Pro-Leu), Cyclo(-Pro-Gly), Cyclo(-Pro-Phe), Cyclo(-Val-Phe), Cyclo(-Gly-Phe), Cyclo(-Ala-Val), Cyclo(-Ala-Gly), Cyclo(-Ala-Phe), Cyclo(-Asn-Pro) and Cyclo(-Ala-Pro) in cocoa powder extracts. Evidence for the presence of pyrrolidone and a diketopiperazine Cyclo(-Ala-Pro) with a double bond in the pyrrolidine ring has been obtained.

INTRODUCTION

Diketopiperazines (DKPs) —cyclic dipeptides— have been studied by a large number of research groups. An interesting aspect of this class of compounds is the rôle they play in the bitter principle of various products. This has been observed for instance in roasted malts for brewing[1], corn steep water[2], liquid cultures of *Penicillium italicum* isolated from oranges[3] and roasted cocoa[4,5]. The role of non-volatile compounds in the bitter principle of cocoa is understood and for a long time the two main purine bases of cocoa, theobromine and caffeine, were thought to be responsible. However, Pickenhagen *et al.*[4,5] considered organoleptic findings and analytical results and came to the conclusion that the differences in cocoa bitterness observed cannot be ascribed only to the presence of the purine bases. Organoleptic tests revealed that a persistent metallic bitterness is experienced on the back of the tongue some time after ingestion of theobromine, whereas the cocoa bitterness is perceived directly in the whole of the mouth and disappears quickly. This typical flavour attribute of cocoa was found to be formed after roasting and could be correlated with the formation of DKPs during this process. Although the DKPs are themselves quite bitter the specific cocoa bitterness is obtained only in the presence of both theobro-

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mine and DKPs in the molar ratio of approximately 2:1. Complexation between DKPs and theobromine was observed[4,5] in aqueous mixtures, altering the organoleptic properties of the individual compounds but yielding the specific cocoa bitterness. The formation of DKPs is known to occur upon thermal decomposition of proteins. For roasting cocoa beans this was confirmed by treating small peptides under similar conditions[4].

For the analysis of DKPs a thin-layer chromatographic method[4–6] has been described, as have gas chromatographic methods without[7,8] and with derivatization with trimethylsilyl[8,9] or trifluoroacetyl groups[10,11]. In most applications, mass spectrometry (MS) is used in either the off- or on-line mode. However, there is still a need for a rapid method to profile and to quantify DKPs, preferably without a derivatization step. High-performance liquid chromatography (HPLC) offers good separation of DKPs, however their detection is difficult. Its combination with mass spectrometry offers high sensitivity and selectivity without the need to derivatize the DKPs. Rapid on-line analysis using electron impact (EI) or chemical ionization (CI) is a standard feature of a LC–MS system based on a moving belt system, as shown for instance in the analysis of the pungent principles of pepper and capsicum oleoresins[12].

In general EI is chosen for identification purposes because, in this mode, fragmentation provides structural information. CI is selected to provide molecular weight information and sensitive quantitation because the low level of fragmentation is an advantage in this respect.

A moving belt interface can be used in combination with a magnetic sector instrument[13] which offers the possibility of on-line high resolution measurements for identification purposes or high resolution in combination with multiple ion detection for high selectivity and sensitivity. This was demonstrated for drugs at low picogram levels in serum[14]. The combination of LC and low/high resolution MS is of interest in this respect because this combination will also provide high selectivity and structural information when analyzing complex matrices. Moreover, with MS, structure fragments specific to a certain class of compounds can be detected, which can be used for profiling purposes.

In the present study an LC–MS method based on a moving belt interface has been evaluated for the analysis of DKPs in extracts of roasted cocoa beans.

EXPERIMENTAL

Sample work-up

Powdered roasted cocoa beans were first defatted with pentane. A 5-g amount of the defatted powder in water was heated under reflux with stirring. After cooling, the particles still present were removed by centrifugation at 10 000 rpm. A first clean-up of the aqueous solution was performed by chromatography on an XAD-4 column (70 mm × 20 mm I.D.). After washing the column with water, the adsorbed material was eluted with 50 ml of methanol. The methanol solution was stored over sodium sulphate overnight, then concentrated to approximately 3 ml. Chloroform (24 ml) was added and this solution was subjected to chromatography on a silica gel column (50 mm × 13 mm I.D.). The diketopiperazine fraction was eluted with 25 ml of chloroform–methanol (8:1). This extract was concentrated to 5 ml and is used for HPLC–MS analysis.

HPLC–MS analysis

A Finnigan MAT moving belt interface was coupled to a Finnigan MAT 8230 double-focusing mass spectrometer equipped with an EI/CI ion source. Measurements were performed under low resolution (R = 1000) scanning conditions and under high (R = 7000) resolution conditions using multiple ion detection and high voltage scanning. Direct probe EI measurements were performed on a Varian MAT 711 mass spectrometer at a resolution of 1000. The LC system comprised a Waters M6000 pump, a Waters U6K injector and a Polygosil 5 Si 60 column (250 mm × 4.6 mm). Chloroform–methanol (8:1) was used as the mobile phase at a flow-rate of 0.8 ml/min.

Other typical LC–MS conditions were: ion-source temperature 200°C; electron impact electron energy 70 eV; chemical ionization with ammonia as reagent gas at a pressure of 0.5–1.0 Torr; belt speed 4 cm/s; solvent evaporator temperature 100°C. The DKP standards were kindly provided by Dr. W. Pickenhagen (Firminich SA, Geneva, Switzerland).

RESULTS

DKP standards

Standards of eight diketopiperazines (DKPs) were analyzed by direct probe EI-MS. The eight most intense masses, the molecular ions and their intensities are given in Table I. All DKPs show a molecular ion in their spectrum, although a rather low intensity is observed for Cyclo(-Pro-Leu) and Cyclo(-Ala-Gly). The EI spectra contain structure-specific fragments and allow a straightforward interpretation. For instance, the presence of phenylalanine in DKPs is easily recognized, because an intense peak at m/z = 91 (tropylium ion) is formed as well as the ions $[M-91]^+$ and $[M-91-CO]^+$. These three types of ions result in the following peak intensities:

TABLE I

DIRECT PROBE ELECTRON-IMPACT SPECTRA (70 eV) OF SOME DIKETOPIPERAZINES

Listed are the eight most intense masses, the molecular ions (in italics) and their intensities (%) in parentheses.

Cyclo(-Pro-Leu)	70 (100),	154 (96.9),	41 (32.6),	86 (28)	
$C_{11}H_{18}N_2O_2$	43 (26.9),	55 (15.0),	69 (14.6),	42 (13.7),	*210* (0.2)
Cyclo(-Pro-Gly)	111 (100),	83 (85.8),	*154* (82.8),	41 (77.4)	
$C_7H_{10}N_2O_2$	70 (70.8),	69 (54.6),	42 (50.3),	55 (46.2)	
Cyclo(-Pro-Phe)	125 (100),	*244* (45.1),	70 (40.5),	91 (38.4)	
$C_{14}H_{16}N_2O_2$	153 (36.7),	120 (11.2),	92 (9.2),	41 (8.7)	
Cyclo(-Val-Phe)	91 (100.0),	127 (56.6),	*246* (34.3),	85 (30.8)	
$C_{14}H_{18}N_2O_2$	72 (22.9),	99 (19.8),	92 (17.3),	55 (16.5)	
Cyclo(-Gly-Phe)	91 (100.0),	*204* (20.6),	85 (14.8),	65 (12.8)	
$C_{11}H_{12}N_2O_2$	92 (12.1),	39 (7.5),	51 (5.1),	77 (4.7)	
Cyclo(-Ala-Val)	128 (100.0),	72 (41.3),	113 (39.0),	99 (24.0)	
$C_8H_{14}N_2O_2$	43 (21.0),	55 (20.0),	41 (19.8),	56 (17.8),	170 (0.2)
Cyclo(-Ala-Gly)	85 (100.0),	*128* (43.9),	57 (29.4),	56 (19.3)	
$C_5H_8N_2O_2$	43 (15.3),	41 (13.4),	45 (7.5),	72 (6.4)	
Cyclo(-Ala-Phe)	91 (100.0),	127 (25.5),	99 (21.8),	*218* (15.0)	
$C_{12}H_{14}N_2O_2$	92 (14.2),	65 (12.0),	39 (8.2),	71 (6.2)	

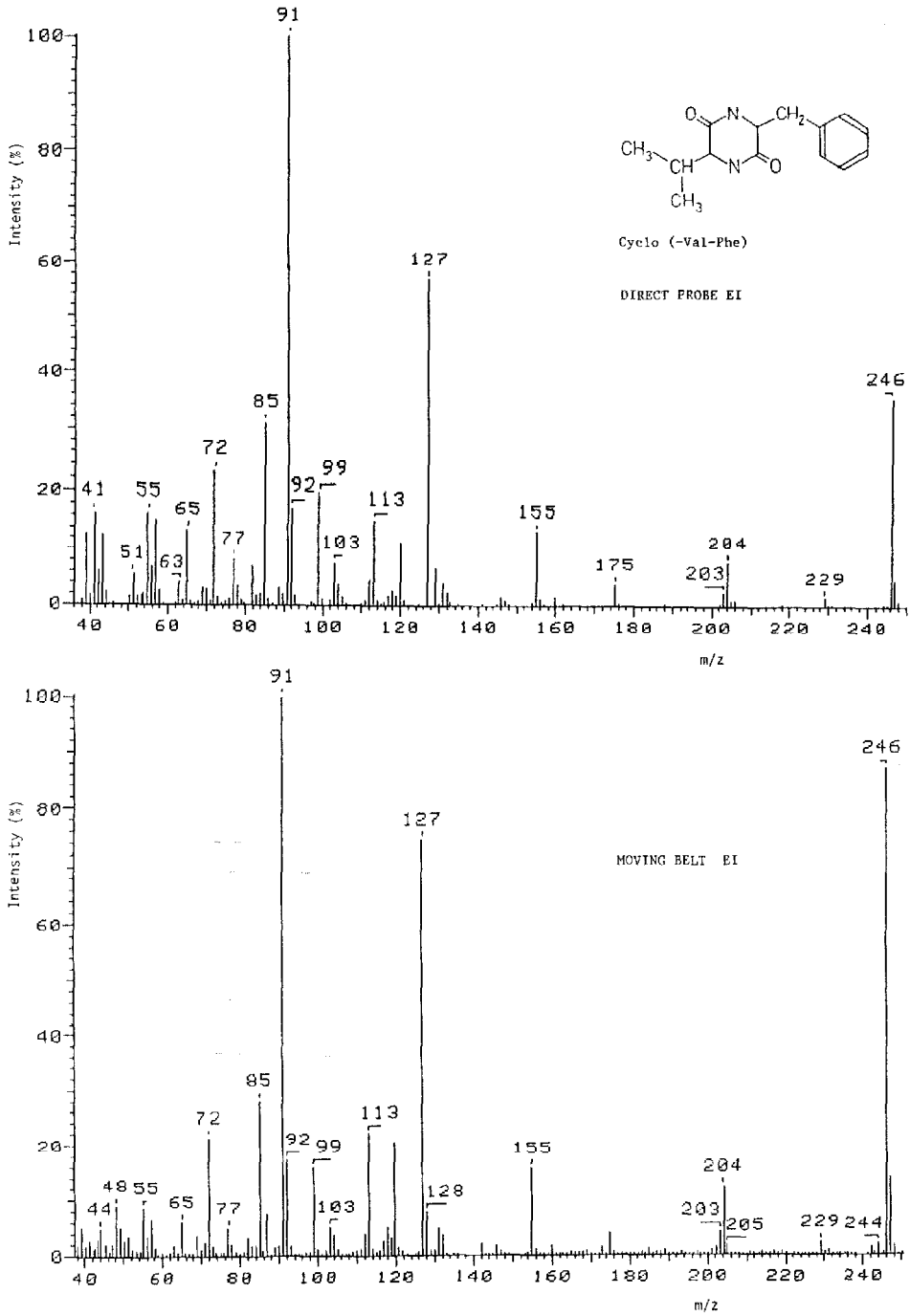

Fig. 1. Electron-impact spectra of Cyclo(-Val-Phe) obtained by direct probe inlet and by LC–MS using a moving belt interface.

Cyclo(-Pro-Phe) m/z = 91 (38.4), 153 (36.7) and 125 (100%); Cyclo(-Val-Phe) m/z = 91 (100), 155 (13.1) and 127 (56.6%); Cyclo(-Gly-Phe) m/z = 91 (100), 113 (3.6) and 85 (14.8%); Cyclo(-Ala-Phe) m/z = 91 (100), 127 (25.5) and 99 (21.8%). The DKPs containing proline, show a prominent peak at m/z = 70 (C_4H_8N): Cyclo(-Pro-Leu) 100, Cyclo(-Pro-Gly) 70.8 and Cyclo(-Pro-Phe) 40.5%.

In Fig. 1 two EI spectra of Cyclo(-Val-Phe) are compared which have been measured with a direct insertion probe inlet system and with LC–MS using a moving belt interface. Both spectra show the same fragment ions, but a clearly higher molecular ion intensity is observed in the spectrum obtained with the moving belt. This is often the case and is a direct result of the construction of the moving belt interface entering the ion source and of flash evaporation of the sample inside the source. This is reflected in a desorption characteristic, known under CI conditions as the direct CI (DCI) effect. In addition to the characteristic fragmentation of this DKP resulting from the presence of phenylalanine as mentioned above, valine characteristics are also found such as the ions $[M-42]^+$, $[M-43]^+$ and the latter fragmentation followed by loss of CO yielding $[M-43-28]^+$ at m/z = 175.

In the present case, EI is a powerful technique for identification, but for profiling DKPs and their subsequent quantitation, CI is preferred. Typical ammonia CI (DCI) for Cyclo(-Val-Phe) results in the formation of $[M+H]^+$ and $[M+NH_4]^+$ (see Fig. 2), providing direct molecular weight information on the compound investigated.

The performance of the total LC–MS system was tested with a mixture of eight DKPs. The total ion current profile obtained is given in Fig. 3. The separation of the DKPs is sufficient to allow profiling using the selectivity of the mass spectrometer,

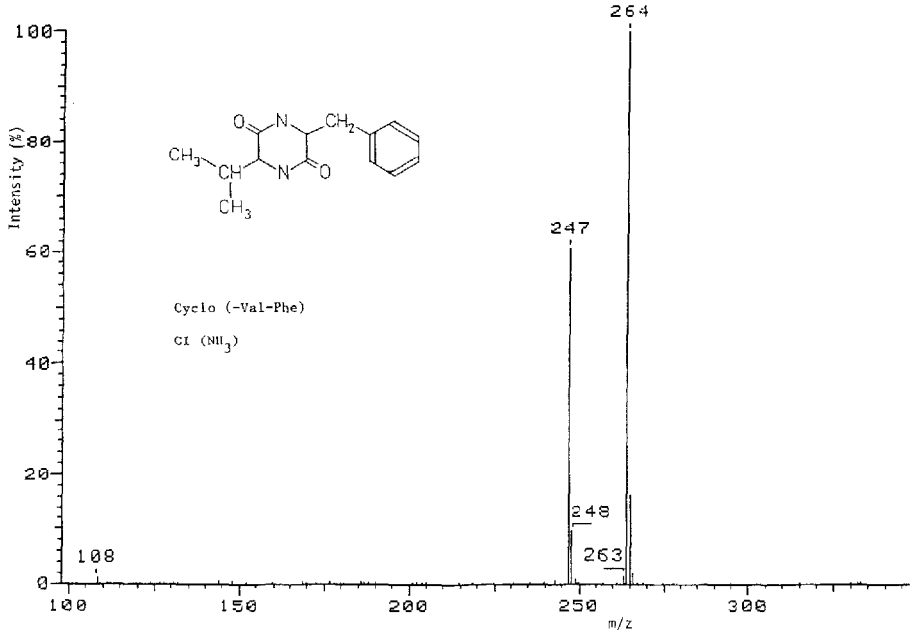

Fig. 2. Ammonia chemical ionization spectrum of Cyclo(-Val-Phe) obtained by LC–MS.

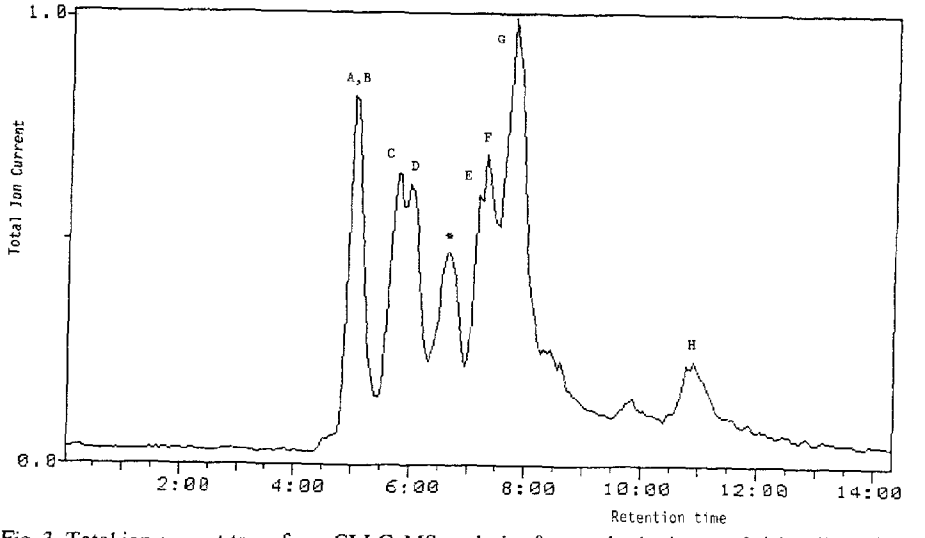

Fig. 3. Total ion current trace from CI LC–MS analysis of a standard mixture of eight diketopiperazines. A = cyclo(-Pro-Leu); B = Cyclo(-Pro-Phe); C = Cyclo(-Val-Phe); D = Cyclo(-Ala-Pro); E = Cyclo(-Ala-Val); F = Cyclo(-Gly-Phe); G = Cyclo(-Pro-Gly); H = Cyclo(-Ala-Gly); * = impurity.

as shown in Fig. 4 in which the reconstructed multiple ion detection traces are given for the various DKPs, summing the intensities of $[M+H]^+$ and $[M+NH_4]^+$ for each individual DKP. An impurity was found as indicated in Fig. 3. An attempt to identify this impurity was made when it appeared that this compound was also present in the analyzed cocoa extracts. Multiple ion detection showed that two ions at m/z = 86 and m/z = 103 are related to this chromatographic peak. High resolution (10000) measurements provided the elemental composition of the peak at m/z = 103 as $C_4H_{11}N_2O$ —the $[M+NH_4]^+$ of C_4H_7NO— while m/z = 86 is the protonated molecule of C_4H_7NO. This elemental composition suggests the impurity is pyrrolidone, which is likely to be present in the cocoa extracts after several purification steps since its structure shows a similarity with the basic structure of DKPs.

Another interesting observation for the standard mixture is the presence of two minor compounds with a molecular weight of 166, with $[M+H]^+$ and $[M+NH_4]^+$ at m/z 167 and 184, eluting just after Cyclo(-Ala-Pro) (see Fig. 5). Investigation of the individual standards showed these compounds originate from the Cyclo(-Ala-Pro) standard. High resolution gave $C_8H_{11}N_2O_2$ as the elemental composition of the protonated compound. This points to compounds with an additional double bond in the pyrrolidine ring of Cyclo(-Ala-Pro). DKPs with a double bond in the pyrrolidine ring have been described as being formed by *Penicillium italicum* isolated from oranges[3].

The DKPs from the standard mixture are eluted from the silica gel column in the order given in Table II, in which the intensities of the peaks due to $[M+H]^+$ and $[M+NH_4]^+$ are also listed. In the order of increasing retention time, the proton affinity of the DKPs appears to decrease more or less regularly, as is seen from the ratio of the protonated and ammoniated molecules. For example, ratios $[M+NH_4]^+/[M+H]^+$ of 0.15, 0.19, 0.67 and 1.8 are found for the DKPs containing

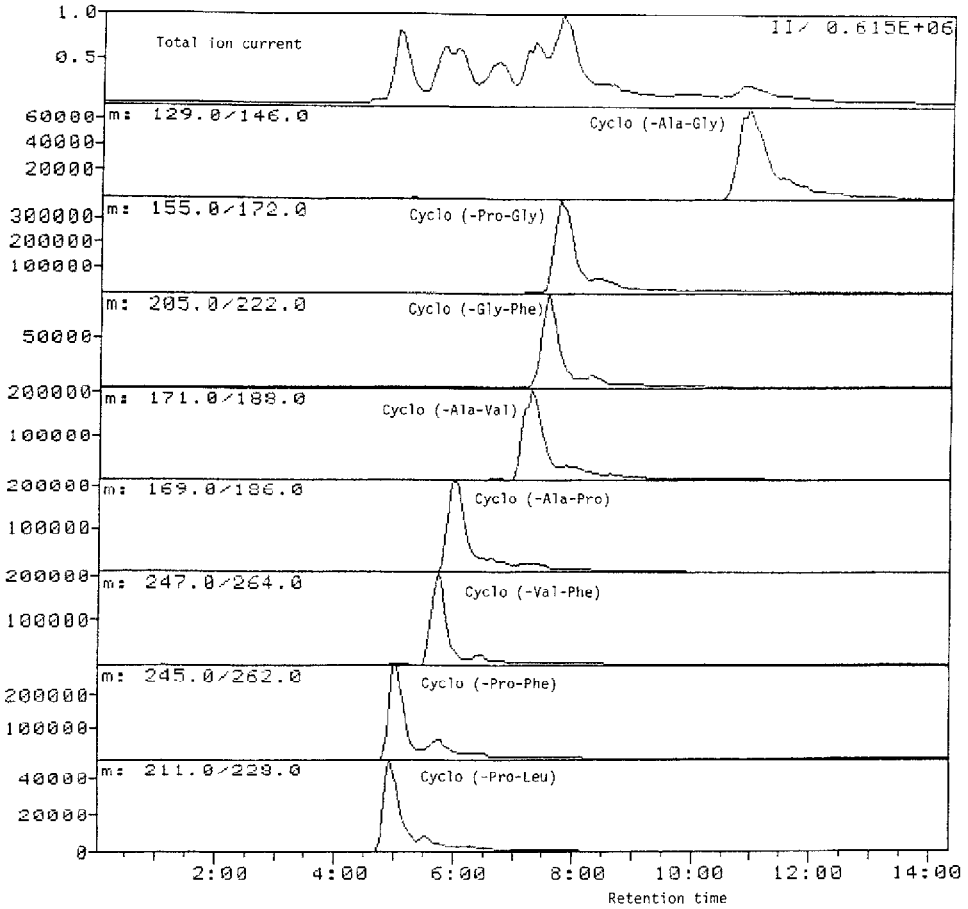

Fig. 4. Multiple ion detection of eight diketopiperazines by CI LC–MS. Each trace represents the sum of the peak intensities of the protonated and ammoniated molecules.

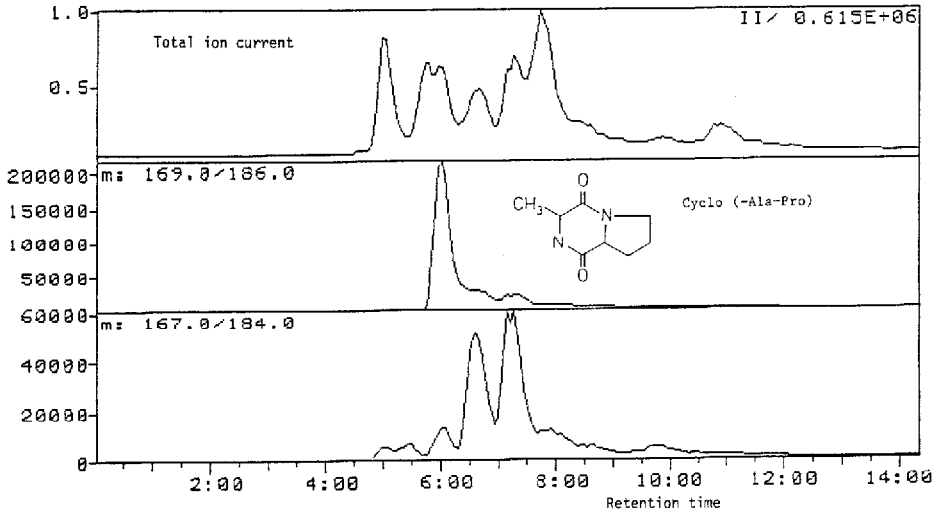

Fig. 5. CI LC–MS total ion current trace and multiple ion detection traces for Cyclo(-Ala-Pro) and two unknown impurities with a molecular weight of m/z = 166.

TABLE II

CHEMICAL IONIZATION (AMMONIA) LC–MS SPECTRA OF SOME DIKETOPIPERAZINES

Intensities (%) of the protonated and ammoniated peaks are listed.

	$[M+H]^+$	$[M+NH_4]^+$
Cyclo(-Pro-Leu)	100	15.1
Cyclo(-Pro-Phe)	100	18.7
Cyclo(-Val-Phe)	61.3	100.0
Cyclo(-Ala-Pro)	100	66.6
Cyclo(-Ala-Val)	39.2	100.0
Cyclo(-Gly-Phe)	12.6	100.0
Cyclo(-Pro-Gly)	54.3	100.0
Cyclo(-Ala-Gly)	13.3	100.0

proline with leucine, phenylalanine, alanine and glycine, respectively. For the DKP series containing phenylalanine in combination with proline, valine and glycine, these ratios are 0.19, 1.6 and 7.9, respectively. The shorter the alkyl chain length (leucine, alanine, glycine for instance) the smaller are the electron-donating properties of this side chain, which means a decrease in the proton affinity. Apparently, the interaction of the DKPs having shorter alkyl chains with the silica gel surface is stronger, resulting in a longer retention time.

Cocoa powder extracts

The analysis of a typical cocoa extract under the same conditions as for the standard mixture is given in Fig. 6. Two dominant peaks are present, one being caffeine and the other theobromine. Despite the several purification steps, the high content of these two purine bases dominates the total ion current (TIC) profile. The high concentration of theobromine gives rise to memory effects on the belt for this compound. Comparison of the TIC profile with a profile obtained by adding the

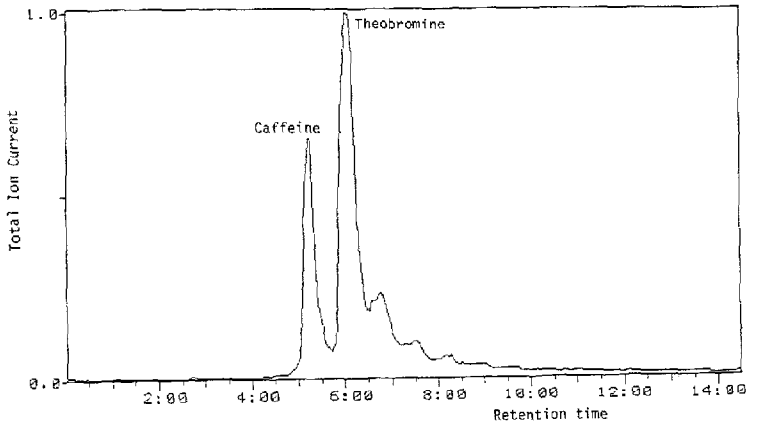

Fig. 6. CI LC–MS total ion current trace of a typical cocoa powder extract.

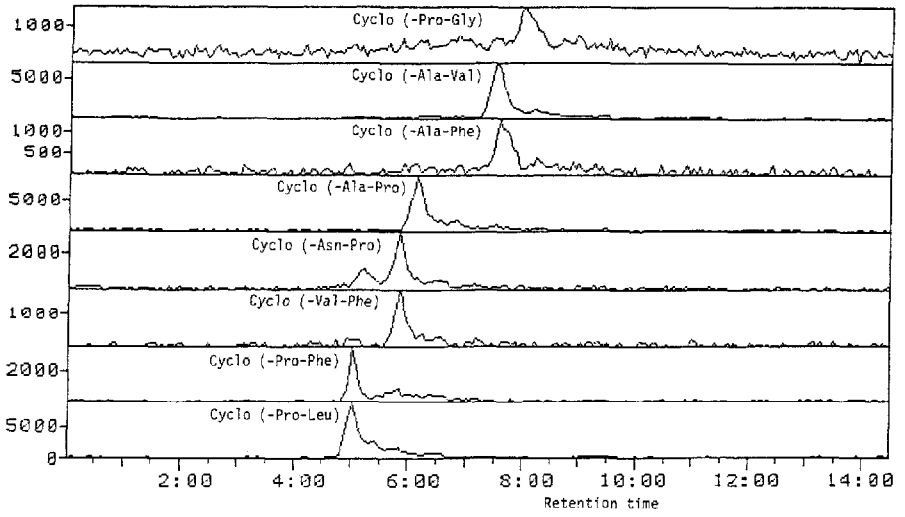

Fig. 7. Multiple ion traces of some DKPs found in a cocoa extract. Each trace represents the sum of the intensities of the peaks from the protonated and ammoniated molecules.

protonated and ammoniated molecule peaks of caffeine and theobromine (195/212 and 181/198) shows hardly any differences. However, from reconstructed multiple ion traces, eleven DKPs could be detected: Cyclo(-Pro-Leu), Cyclo(-Pro-Phe), Cyclo(-Val-Phe), Cyclo(-Ala-Pro), Cyclo(-Ala-Phe), Cyclo(-Ala-Val), Cyclo(-Gly-Phe),

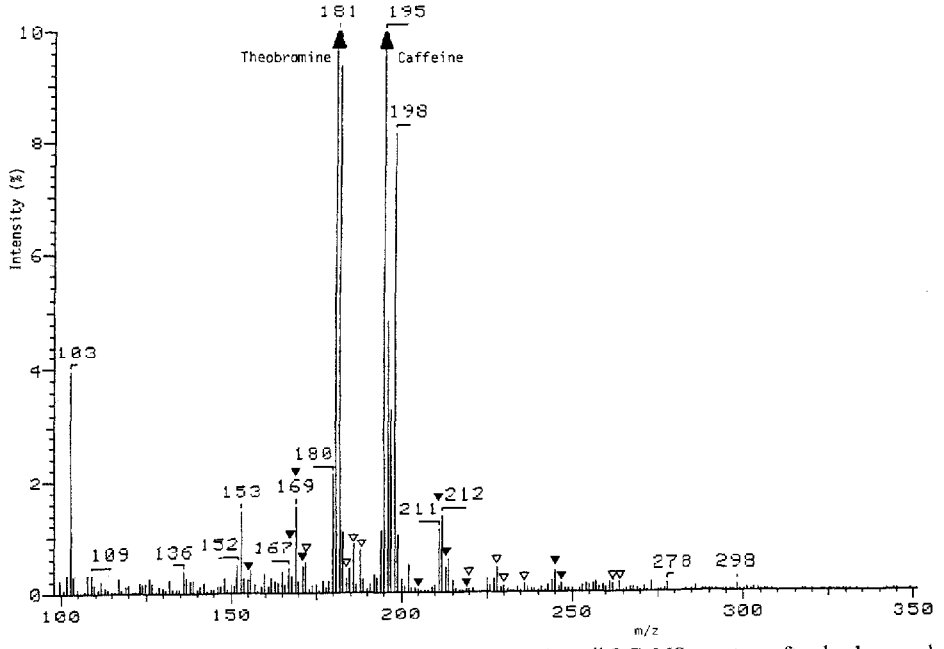

Fig. 8. Averaged cocoa extract profile obtained by summing all LC–MS spectra, after background subtraction. For the DKPs both the protonated (▼) and ammoniated (▽) peaks are indicated.

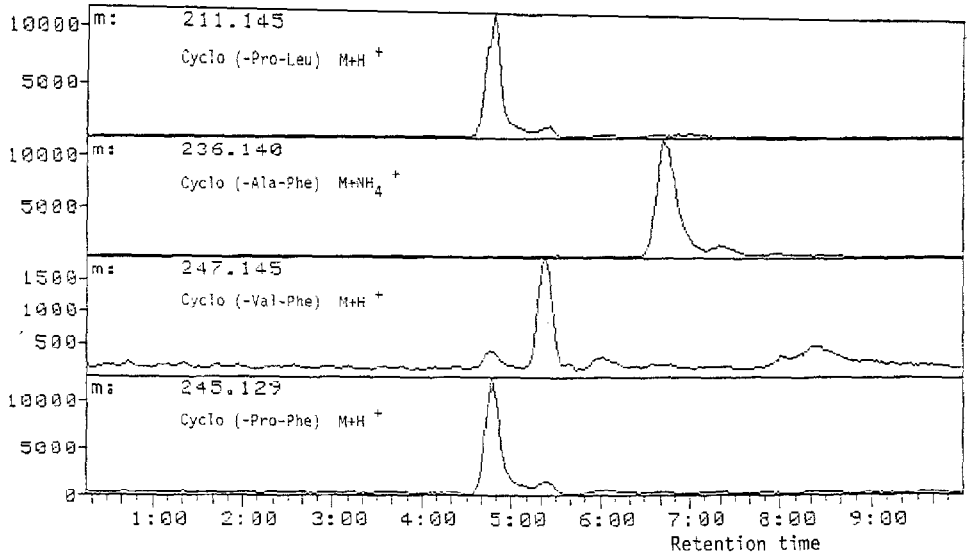

Fig. 9. High resolution CI LC–MS detection of four diketopiperazines.

Cyclo(-Ala-Gly), Cyclo(-Asn-Pro), Cyclo(-Pro-Gly) and Cyclo(-Ala-dehydroPro). For this purpose, the ion traces were based on the sum of the intensities of the protonated and ammoniated peaks (see Fig. 7). The small peak preceding Cyclo-(-Asn-Pro) is the ^{13}C isotope of the $[M + NH_4]^+$ peak of caffeine.

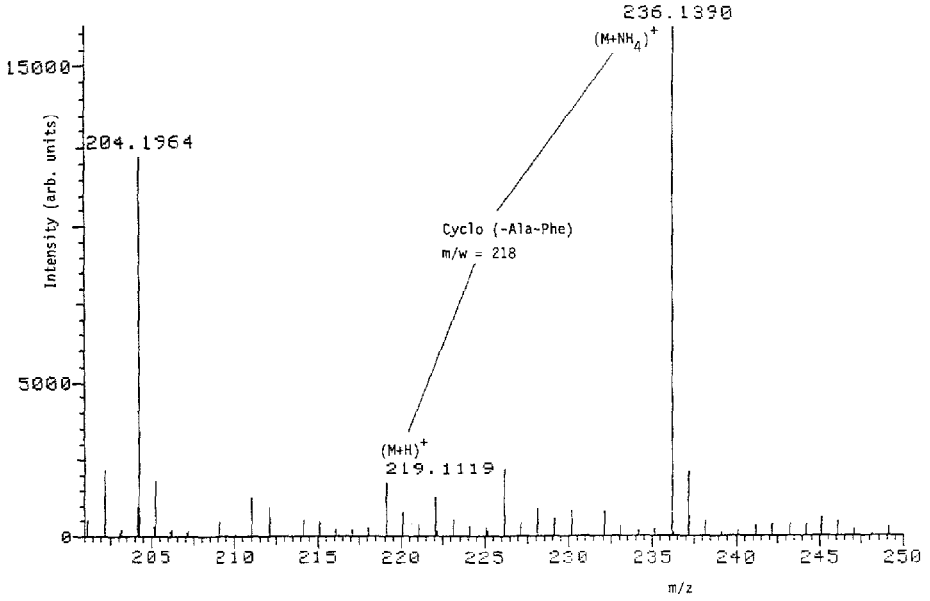

Fig. 10. High resolution data obtained by high voltage scanning over the mass range 210–250 during the elution of Cyclo(-Ala-Phe).

By adding all spectra in the retention time window from 4.5 to 9.0 min, after background subtraction using a spectrum outside this window, an averaged profile is obtained (see Fig. 8). Theobromine and caffeine are off-scale, and below 2% the first peaks which can be attributed to DKPs are visible. The DKPs found by LC–MS reconstructed multiple ion detection are marked in this averaged spectrum. The high intensity of the peak at $m/z = 103$ is striking. As mentioned for the standard mixture, this peak is probably due to pyrrolidone. The peaks at m/z 198 and 212 are due to the ammoniated molecules of theobromine and caffeine, while a component with a molecular weight of 135 is also found as indicated by the presence of peaks at m/z 136, $[M+H]^+$, and m/z 153, $[M+NH_4]^+$. This compound is eluted 17 s after theobromine but so far no attempt has been made to identify it.

In this way several cocoa extracts obtained after different roasting processes were screened for the presence of DKPs and quantitated using the standard mixture by comparing peak areas. When interferences were found, high resolution multiple ion detection was used as shown in Fig. 9 for Cyclo(-Pro-Phe), Cyclo(-Val-Phe), Cyclo(-Ala-Phe) and Cyclo(-Pro-Leu). Identification was based on exact mass measurements, carried out by on-line high voltage scanning at a resolution of 7000. Reference compounds such as methyl decanoate were used for calibrating the mass range 200–250. As an example, such an experiment to identify Cyclo(-Ala-Pro) by measuring the protonated and ammoniated molecules with an accuracy better than 1.5 millimass units is shown in Fig. 10.

CONCLUSIONS

LC–MS is a suitable method to identify DKPs and other compounds present in cocoa extracts. Quantitation can be done by using standard mixtures for calibration and applying chemical ionization. High resolution is a powerful tool to reduce the background and to enable measurement of the elemental compositions of unknowns.

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