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ORIGINAL ARTICLE

Determination and stereochemistry of proteinogenic and non-proteinogenic amino acids in Saudi Arabian date fruits

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Abstract Whereas an abundance of literature is available on the occurrence of common proteinogenic amino acids (AAs) in edible fruits of the date palm (*Phoenix dactylifera* L.), recent reports on non-proteinogenic (non-coded) AAs and amino components are scarce. With emphasis on these components we have analyzed total hydrolysates of twelve cultivars of date fruits using automated ion-exchange chromatography, HPLC employing a fluorescent aminoquinolyl label, and GC–MS of total hydrolysates using the chiral stationary phases Chirasil®-L-Val and Lipodex® E. Besides common proteinogenic AAs, relatively large amounts of the following non-proteinogenic amino acids were detected: (2*S*,5*R*)-5-hydroxypipecolic acid (1.4–4.0 g/kg dry matter, DM), 1-aminocyclopropane-1-carboxylic acid (1.3–2.6 g/kg DM), γ-amino-*n*-butyric acid (0.5–1.2 g/kg DM), (2*S*,4*R*)-4-hydroxyproline (130–230 mg/kg DM), L-pipecolic acid (40–140 mg/kg DM), and 2-aminoethanol (40–160 mg/kg DM) as well as low or trace amounts (<70 mg/kg DM) of L-ornithine, 5-hydroxylysine, β-alanine, and in some samples (<20 mg/kg DM) of (*S*)-β-amino*iso*butyric acid and (<10 mg/kg DM) L-*allo*-isoleucine. In one date fruit, traces of α-aminoadipic acid could be determined. Enantiomeric analysis of 6 M DCl/D_2O hydrolysates of AAs using chiral capillary gas chromatography–mass spectrometry revealed the presence of very low amounts of D-Ala, D-Asp, D-Glu, D-Ser and D-Phe (1.2–0.4 %, relative to the corresponding L-enantiomers), besides traces (0.2–1 %) of other D-AAs. The possible relevance of non-proteinogenic amino acids in date fruits is briefly addressed.

Keywords *Phoenix dactylifera* · (2*S*,5*R*)-5-hydroxypipecolic acid · 1-aminocyclopropane-1-carboxylic acid · Non-coded amino acids · D-amino acids · Plant amino acids · Ion-exchange chromatography · GC–MS · Nutritional relevance

Abbreviations

GC–MS	Gas chromatography mass spectrometry
M	Molecular mass (weight)
HPLC or LC	High performance liquid chromatography
SIM	Selected ion monitoring
AQC	6-Aminoquinoyl-carbamyl-*N*-hydroxysuccinimidyl carbamate
AMQ	6-Aminoquinoline
*i*TRAQ™	Isobaric tag for relative and absolute quantitation
MSTFA	*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide
DCl/D_2O	Deuterium chloride in deuterium oxide
TFAA	Trifluoroacetic acid anhydride
TFA	Trifluoroacetyl
Me	Methyl
Et	Ethyl

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Chirasil®-L-Val	Dimethylpolysiloxane functionalized with L-Val-*tert.*butylamide
Lipodex® E	(3-Butyl-2,6-pentyl)-γ-cyclodextrine

Common proteinogenic amino acids (AAs) are abbreviated according to three-letter code and are of the L-configuration; non-proteinogenic amino acids are abbreviated as follows:

α-aminoadipic acid	α-Aaa or a-AAA
β-Ala or b-Ala	β-Alanine
Aba or a-AB	α-Amino-*n*-butyric acid
β-Aba or β-Aib	β-Amino*iso*butyric acid
Acc	1-Aminocyclopropane-1-carboxylic acid
Cit	Citrulline
Cys	Cystine
Eta	Ethanolamine
GABA	γ-Amino-*n*-butyric acid
Hyl or Hy-Lys	5-Hydroxylysine
Hyp	(2*S*,4*R*)-4-hydroxyproline (*trans*-4-hydroxy-L-proline)
Nle	Norleucine (internal standard)
Orn	Ornithine
Pip	Pipecolic acid
Pip(OH)	(2*S*,5*R*)-5-hydroxypipecolic acid (*trans*-5-hydroxypipecolic acid)

Amino compounds being part of reference standards but not detected in hydrolysates:

Carn	Carnosine
Csystat	Cystathionine
1-M-His	1-Methylhistidine
3-M-His	3-Methylhistidine
P-Ser	*O*-Phosporyl-L-serine
P-Eta	Phosphoethanolamine
Sar	Sarcosine
Tau	Taurine

Introduction

The common name, date fruit, stands for the edible, sweet fruit of cultivars of the date palm, *Phoenix dactylifera* L., which is cultivated for millennia in the Middle East. Although Saudi Arabia is famous for its richness of oil, it is by far less known that the kingdom is also an important producer of date fruits, accumulating to about 1,008,000 metric tons in 2011, thus being second largest producer in Arab countries after Egypt with 1.373.467 metric tons (AOAD 2012). Besides the commercial value, date fruits are of highest social and ethnological acceptance in the whole Arab society. No occasion is celebrated without eating dates, and it is common custom to break the day-long Ramadan fast with dates.

The nutritional value and large caloric energy of about 300 kcal/100 g of dates result from the large quantities of sugars, comprising about 51 % of glucose and fructose, 3 % crude fibre, and 2–3 % protein. In addition, polyphenols, vitamins, mineral salts and trace elements are present. The low content of about 0.1–0.4 % fat in the fruits and absence of cholesterol are noteworthy. The high sugar content and low moisture of about 24 % of sun-dried fruits make dates an excellent staple food that can be stored without microbial spoilage for several months even at ambient temperature. For a comprehensive overview of components at various stages of ripening numerous date fruit cultivars, see Al-Shahib and Marshall (2003).

Of about 400 cultivars and local varieties of date palms cultured worldwide, about 40 named cultivars are grown and marketed in Saudi Arabia. The chemical composition, sensorial properties, and nutritional value depend on the respective cultivar as well as the stage of ripening, time of harvesting and processing. During ripening the colour of the fruits changes from green to stages of yellow and brown and even dark brown or almost black on drying and processing. The development of the fruit is classified into four stages of ripening using the Arabic terms *Kimri*, *Khalal*, *Rutab*, and *Tamr*. '*Khalal*' refers to the mature, fully coloured fruits (Fayadh and Al-Showiman 1990). Date fruits are also classified into soft, semi-dry, and dry fruit according to cultivar and treatment after harvesting. Names of cultivars and their growth conditions as well as stages of ripening and processing have to be considered when comparing chemical analyses of date fruits.

A fair number of reports on date fruits in general deals with the determination and quantitation of free and protein-bound essential and non-essential proteinogenic amino acids (El-Sohaimy and Hafez 2010; Bouaziz et al. 2008; Al-Farsi et al. 2005; Ishurd et al. 2004; Al-Shahib and Marshall 2003; Al-Hooti et al. 1997; Booij et al. 1993; Al-Aswad 1971; Auda et al. 1976; Al-Rawi et al. 1967). However, except for paper chromatographic approaches, in particular of Rinderknecht (1959) and Grobbelaar et al. (1955), data on non-proteinogenic amino acids and special amino compounds in date fruits are scarce.

In order to scrutinize and extend the aforementioned pioneering reports, we analyzed twelve popular cultivars of Saudi Arabian date fruits using contemporary analytical techniques with emphasis on the presence and stereochemistry of non-proteinogenic amino acids.

Brief history of reports on minor amino acids in date fruits

Following a report on the excretion of an unknown amino acid in human urine as a result of consumption of date fruits by Gartler and Dobzhansky (1954), Grobbelaar et al. (1955) analyzed date fruits for ninhydrin-positive amino acids using two-dimensional paper chromatography. The authors report on the detection of non-protein Pip(OH), Pip, $\Delta^{4,5}$-dehydropipecolic acid (baikiain), β-Ala, homoserine, GABA and, possibly, Hyl.

Using preparative IEC, Piez et al. (1956) focussed on cyclic imino acids in a total hydrolyates of date fruit pericarp and isolated Pip(OH) (1.8 g/kg), Pro (0.7 g/kg), Hyp (0.13 g/kg), and trace amounts of Pip (5 mg/kg); baikiain was not detected. Notably, these authors reported that Pip(OH) and Pip were found only in the free state in date fruits, whereas Hyp was found only in the bound form, and Pro occurred both in the free and bound states.

With the aim of determining the stereochemistry of 5-hydroxypipecolic acid in dates, Witkop and Foltz (1956) isolated Pip(OH) from 70 % ethanolic extracts using preparative cation-exchange chromatography and obtained 980 mg of crystalline Pip(OH) per kg of date fruits. The stereochemistry was determined as *trans*-5-hydroxy-L-pipecolic acid (corresponding to (2*S*,5*R*)-5-hydroxypipecolic acid).

In order to determine the pattern of free amino acids in date fruits in relation to their darkening and maturation process, Rinderknecht (1959) investigated 70 % ethanolic extracts of dates of the Deglet Noor cultivar at various stages of ripening (green, yellow, red, brown, and dark brown) by two-dimensional paper chromatography. Besides various common proteinogenic amino acids, he detected 5-hydroxypipecolic acid and lower amounts of pipecolic acid in dates of all stages. Trace amounts of baikiain were found only in yellow-coloured dates. Furthermore, GABA and Cit were recognized as well as oxidized glutathione. The presence of homoserine and Hyl, as reported by Grobbelaar et al. (1955), could not be confirmed.

Remarkably, these early reports on the occurrence of non-proteinogenic AAs in date fruits were almost neglected and not confirmed in subsequent works focussing on nutritional aspects of proteinogenic AAs. This is attributed to the establishment of commercial amino acid analyzers according to Spackman et al. (1958), enabling the quantitation of proteinogenic AAs following post-column derivatization with ninhydrin. These instruments are commonly run in the short hydrolysate mode and not the high-resolution physiological mode, because such analyses are more costly in terms of time and expenditure. Consequently, date fruits were not analyzed for the presence of the aforementioned non-proteinogenic and minor AAs. This is surprising since possible health-related effects of 5-hydroxypipecolic acid and Cit had already been addressed by Rinderknecht (1959). With the exception of the detailed analysis of Pip(OH) by Witkop and Foltz (1956), no reports on the stereochemistry of AAs in date fruits came to our attention.

Therefore, our work was aimed in particular at the re-evaluation and analysis of minor non-proteinogenic AAs occurring in selected Saudi Arabian date fruit cultivars at the ripe (*Tamr*) stage. For optimal resolution, we used a modern instrument in the elaborative physiological mode being capable of separating at least 42 ninhydrin-positive compounds in a single run. However, assignment of amino compounds using IEC is based entirely on comparison of retention times with standards. In order to circumvent inherent problems of this method (for a discussion see Kaspar et al. 2009), IEC was complemented for two selected samples with HPLC following pre-column derivatization of amino compounds with the fluorescent reagent AQC. Moreover, GC–MS on chiral stationary phases was used for the assignment of the stereochemistry of amino acids resulting from total hydrolysates of date fruits.

Materials and methods

Origin, treatment and characterization of date fruit samples

Named cultivars of date fruits were purchased from retail markets in the central region of the Kingdom of Saudi Arabia. Fruits had been harvested at the *Tamr* stage, representing full ripeness. For names and specification see Table 1. The flesh (pericarp) of each five date fruit cultivars was separated manually from the pits, cut into small pieces with a sterilized knife and mixed in order to get an average sample. Samples were freeze-dried in a model Mobile 12 SL Freezer (The Virtis Company Inc., Gardiner, New York, USA).

Amino acid analysis using automated ion-exchange chromatography (IEC) and ninhydrin derivatization

A Model LC 3000 amino acid analyzer (Eppendorf-Biotronik, Hamburg, Germany) was used and run in the mode for physiological amino acids. A 125 × 4 mm i.d. column was used packed with cation exchanger resin of 4 μm particle size (Li^+-form) equipped with a pre-column 53 × 4 mm i.d., 11 μm particle size (Li^+-form). The separation column was heated in an oven the temperature of which was ramped from 33 to 80 °C. Samples were stored at 8–10 °C in a refrigerator and injected automatically using a 20 μL sample loop. Amino acids were eluted using five-step gradients of Li-acetate buffers. The flow rate of the

Table 1 Names and specifications of date fruits nos. 1–12

No.	Name	Specification of the mature fruit
1	Ajwa–Al Madina	Dried date, semi-soft at harvest season, black and shiny in colour, small fruit, round and sweet
2	Nabtat Ali	Dried date, high moisture at harvest
3	Sukkari	Fresh consumption, very sweet and soft, medium-sized
4	Segae	Dried date, low in moisture content
5	Shaihee	Dried date, sugar content medium, not too sweet, date golden in colour
6	Ruthana	Dried date, consumption at all three stages of ripening, famous flavour
7	Rushodia	Dried date, large fruit, semi hard and sweet, light brown in colour
8	Khalas	Dried date, medium sized, golden in colour, low fibre, very sweet
9	Barhi	Fresh consumption at Khala stage; sweet and juicy, yellow in colour
10	Barhi	Processed in KSU date factory
11	Segae	Processed in KSU date factory
12	Khalas	Processed in KSU date factory

Table 2 Elution time, buffer composition and temperature programme for amino acid analyzer (see also Fig. 2)

Step	Time (min)	Buffer	Temp. (°C)
1	12.0	A	33
2	17.0	A	33
3	27.0	B	33
4	7.5	C	39
5	9.0	C	42
6	9.0	C	50
7	10.0	D	54
8	11.0	D	60
9	16.0	E	66
10	10.0	E	70
11	8.0	F	80
12	5.0	A	80
13	8.0	A	55

Buffer A (0.15 M LiAc, pH 2.92), Buffer B (0.18 M LiAc, pH 3.30), Buffer C (0.20 M LiAc, pH 4.25), Buffer D (0.29 M LiAc, pH 7.85), Buffer E (0.37 M LiAc, pH 10.60), Regenerant F (0.4 M LiOH)

Flow rate 0.20 mL/min; sample injection at step 2, *LiAc* lithium acetate, *LiOH* lithium hydroxide

buffer was 0.2 mL/min. For buffer and temperature programme, see Table 2.

Reaction products formed by post-column derivatization with ninhydrin reagent at 125 °C in an electrically heated compartment were determined photometrically at 570 and 440 nm using a dual-wavelength filter photometer comprising a photometric cell of 11 µL volume and 16 mm path length. Data acquisition was performed using Biotronik WinLC™ controlled software and Chromstar 6.0 data handling software under Microsoft™ Windows 2000 environment.

The derivatizing reagent was prepared from 20.0 g ninhydrin, 0.60 g hydrindantin, and 150.0 g potassium acetate altogether dissolved in 450 mL ethylene glycol and filled up to 1000 mL with deionized water (all chemicals from Merck, Darmstadt).

For calibrations, external standard mixtures composed of physiological amino acids (Sigma, catalogue number A9906) were used. Calibration standards for special amino acids were prepared separately. The AAs Pip, Acc and L-*allo*-Ile were purchased from Sigma; (2*S*,3*R*)-5-hydroxy-pipecolic acid hydrochloride (purity >98 %) was from CHIRALIX B.V., Nijmegen, The Netherlands.

Extraction and release of amino components from date fruits for analysis by IEC

For total hydrolysis, about 63 mg of date samples was totally hydrolysed in closed vessels with 6 M HCl (3 mL) at 110 °C for 24 h, then evaporated to dryness in a vacuum evaporator, the remaining residues dissolved in 2 mL Li-acetate buffer and 20 µL aliquots used for analyses. For abundant amino components, dilutions 1:2 and 1:5 (v/v) were prepared and analyzed accordingly.

Analysis of date fruits by HPLC after pre-column derivatization with AQC

Amounts of 79.7 mg hydrolysates (sample 1) and 72.2 mg (sample 2) were dissolved in deionized water with addition of 1 mL 0.1 M HCl and filled up in a volumetric flask to 10 mL. Complete dissolution was achieved by ultrasonic treatment. Aliquots of the solutions were subjected to derivatization and subsequent HPLC analysis according to the improved method suitable for plasma AAs as described in detail by Jaworska et al. (2012). Briefly, analyte solutions were passed through 10 kD cut-off ultra-filtrate membranes and 50-µL aliquots derivatized with 6-amino-quinoyl-carbamyl-*N*-hydroxysuccinimidyl carbamate (AQC) using the AccQ-Tag™ Reagent Kit supplied by Waters (Waters, Milford, MA, USA). The chromatographic separation of the derivatives was accomplished by HPLC using a reversed phase AccQ-Tag™ column (150 × 3.9 mm i.d., 4 µm particle size) equipped with a Nova-Pak™ C18 guard column (20 × 3.9 mm; Waters). For elution a ternary gradient generated from (A) water, (B) acetonitrile, and (C) 50 mM triethylamine buffer (pH 5.1) containing 2 mM dimethyloctylamine (DMOA) as the counter ion was used (Jaworska et al. 2012). For quantification of derivatives, fluorescence detection at excitation at 250 nm and emission at 395 nm was used. For comparison a standard (c = 25 nmol/mL) was prepared, composed of 18 AAs commonly found in protein hydrolysates and enforced with the physiological AAs β-Ala, GABA, Tau, and Orn, the amino alcohol Eta, and Nva as internal standard. Peak assignment of the analytes was made by comparison of the retention times.

GC–MS of trimethylsilylated 5-hydroxypipecolic acid resulting from total hydrolysates

GC–MS for the derivatization and detection of Pip(OH) in date fruits No. 1 and 2 was performed as described by Dettmer et al. (2011). An Agilent 6890 GC (Agilent, Palo Alto, CA, USA) equipped with a mass selective detector (5975 Inert XL) was used. Analytes were separated on an RXI-5MS column (30 m × 0.25 mm i.d.; 0.25 μm film thickness; Restek GmbH, Bad Homburg, Germany). The analytical column was connected to a 2 m × 0.25 mm i.d. deactivated pre-column. The initial oven temperature was set to 50 °C for 1 min, ramped at 8 °C/min to 300 °C, and held for 15 min. Sample injection was performed in splitless mode at 280 °C using an injection volume of 1 μL. Helium was used as carrier gas at a flow rate of 0.7 mL/min. The transfer line to the mass spectrometer was kept at 310 °C. The mass spectrometer was run under standard 70 eV electron ionization conditions and operated in full scan mode from *m/z* 50 to 550 with a scan time of 0.5 s. The solvent delay was 8 min, and the source temperature was 240 °C. To the total hydrolysates of date fruits No. 1 and 2 was added 50 μL of 20 mg/mL methoxylamine hydrochloride in pyridine, followed by heating at 60 °C for 60 min. Then 50 μL of MSTFA was added, and the mixture was heated again at 60 °C for 60 min. Typically, 1 μL aliquots were injected into the instrument as described above, and the presence of 5-hydroxy-L-pipecolic acid was detected by the characteristic fragment ion at *m/z* 244 and comparison with reference spectra.

GC–MS of total hydrolysis of date fruit samples No. 1 and No. 2 and analyses of derivatives on Chirasil®-L-Val

Freeze-dried date fruits Nos. 1 and 2 (about 11 mg) were hydrolyzed in sealed flasks (1.5 mL) under vacuum in 500 μL of 6 M DCl in D_2O (>99.9 % D; Sigma) for 24 h at 110 °C. Samples were evaporated to dryness in a Savant SpeedVac® Concentrator (Thermo Fisher Scientific, Karlsruhe, Germany). Amounts of 500 μL dist. water and 10 μL 6N DCl/D_2O were added, the mixture was passed through a Bond-Elut-SCX cation-exchanger column (Agilent), washed with water and compounds displaced with 1M NH_4OH. To the dry residue, 250 μL mixture of 4 N DCl in EtOD was added and heated for 20 min at 110 °C in sealed vials. Solvents were removed in a cold stream of dry nitrogen. For acylation, 250 μL TFAA/ethyl trifluoroacetate (1:2, v/v) was added, and the mixture was heated in the closed vial at 130 °C for 10 min. Solvents were removed in a cold stream of nitrogen; about 150 μL of dichloromethane was added and aliquots of 1 μL injected automatically onto the capillary column in the split mode (ratio ca. 1:10) via the injector heated at 190 °C (detector 230 °C). For His, a separate analysis including an additional derivatization step of the *N*-imidazole group was used employing isopropyl chloroformate (Gerhardt and Nicholson 1994). Cysteine and Cystine were not determined by the method used.

The GC–MS used was a model 5973 MSD instrument with mass specific detector (Hewlett-Packard, Palo Alto, CA, USA) equipped with a 7683 autosampler and laboratory-made deactivated (diphenyltetramethyldisilazane) glass capillary column (20 m × 0.31 mm i.d.) coated with Chirasil®-L-Val of film thickness 0.25 μm (Frank et al. 1977). Hydrogen was used as a carrier gas, and the temperature was ramped from 70 °C (2 min isotherm) with a rate of 2.5 °C/min to 110 °C and with a rate of 7 °C/min to 190° (10 min isotherm). Assignment of enantiomers was performed in the total ion current (TIC) and in the selected ion monitoring (SIM) mode using the characteristic fragment ions.

For the resolution of stereoisomers of the (*N,O*)-TFA-*O*-ethyl esters of Pip and Hyp, a Lipodex® E γ-cyclodextrine capillary column (König et al. 1989) of 25 m × 0.25 mm i.d. (Macherey-Nagel, Düren, Germany) was used (Ali et al. 2006, 2010).

The relative amounts of D- and L-enantiomers were determined by monitoring the non-deuterated fragment ions of both enantiomers (Frank et al. 1979; Liardon et al. 1981; Gerhardt and Nicholson 1994, 2001).

Results

General

Twelve date fruit cultivars from the market were analyzed by IEC. Since date fruits 1 and 2 (see Table 1) were considered as representative for all fruits, they were analyzed by all methods described in the "Materials and methods" section in order to confirm the assignments of minor and special amino acids in all date fruits using IEC. Indeed, elution profiles of AAs from the twelve date samples were similar. Structures of the special amino compounds are discussed as follows and are displayed in Fig. 1.

Analysis by IEC

Quantities of AAs and Eta determined by IEC in total hydrolysates of the date fruits analysed (for characterization see Table 1) are compiled in Table 3, together with largest and lowest amounts detected. Amounts of Pip(OH), Acc, Glu and Asp were determined to be highest, followed by Pro, Ala, Gly, GABA, and Leu.

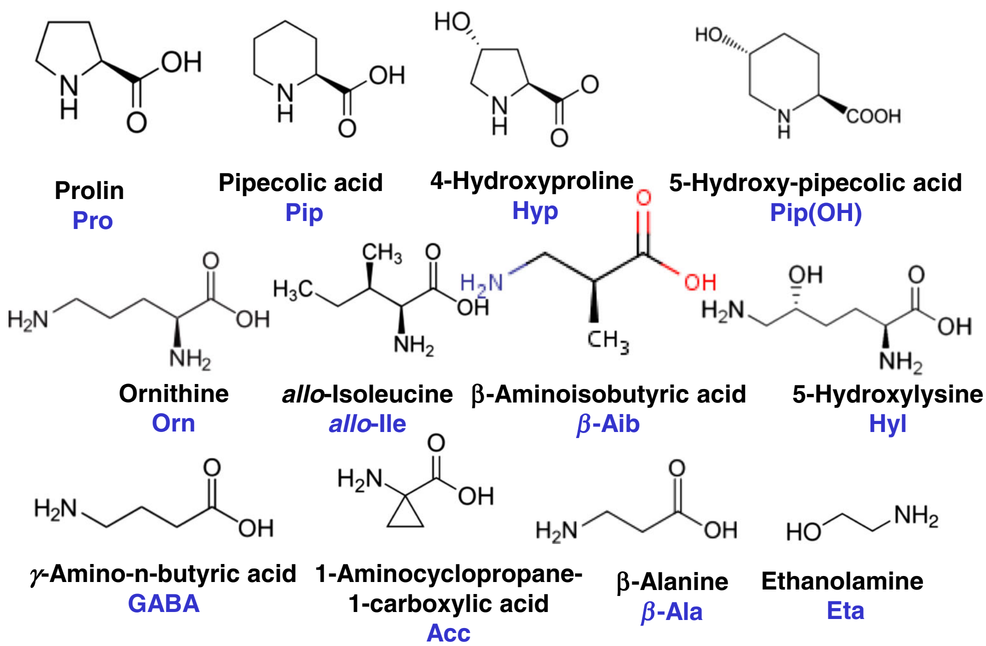

Fig. 1 Structures of special imino and amino acids detected in date fruits. For assignment of stereochemistry see text

Besides common proteinogenic AAs and relatively high amounts of the amino alcohol Eta, trace amounts of ß-Ala, β-Aib, Hyl, and Orn were detected. The representative chromatogram of date fruit sample No. 1 in comparison to a physiological standard, which has been used for calibration, is displayed in Fig. 2. Notably, peaks eluting very early in the chromatograms of the date hydrolysates and assigned as P-Ser, P-Eta and Tau in the physiological standard, could not be confirmed by the other analytical methods used (see below). Tau, in particular, is not present in date fruit hydrolysates despite elution of an intensive peak in analytes having the retention time of Tau in the IEC standard. A peak eluting at the retention time of Sar in the standard chromatogram was assigned to entirely represent Pip(OH) by comparison with a standard and confirmation of its presence and structure by GC–MS (see below). Thus, Tau and Sar could definitely be excluded as constituents of date fruits. Compounds P-Ser and P-Eta, if originally present in date fruits, are assumed to be decomposed by total hydrolysis, with release of Ser and Eta, both of which were detected in hydrolysates. Thus, minor peaks still eluting at the positions of P-Ser and P-Eta in total hydrolysates (see Fig. 2) represent rather unknown, ninhydrin-positive compounds.

Elution positions of Pip, Acc and L-*allo*-Ile resulting from IEC were assigned by comparison with authentic standards. The non-proteinogenic amino acid L-*allo*-Ile is difficult to detect and to quantify since in hydrolysates it is present in trace amounts only, and elutes as shoulder of abundant Met. The sterically constrained Acc provides a very low ninhydrin-colour yield of about 3 % at 570 nm in comparison to Leu and elutes as minor shoulder ahead of Ile. In routine IEC analyses, these compounds remain undetected but the elution positions are shown in an expanded plot of date fruit No. 1. The presence of Hyp and minor amounts of β-Ala, β-Aba, Hyl, and Orn was also deduced by comparison with elution positions of the standard. GABA, a common non-proteinogenic AA in organisms, is another major AA in date fruits. The presence of these amino acids was confirmed by applying those complimentary methods mentioned below.

A peak eluting at the position of α-Aaa was quantified only in sample No. 6 by IEC but traces were detected in date fruits No. 1 and 2 using the *i*TRAQ™ approach. Thus, traces of this AA appear to be present in all date fruits.

Among the proteinogenic AAs, the presence of noticeable quantities of cystine in hydrolysed date fruits are noteworthy (up to 446 mg/kg DM), which might be released, at least partly, from the tripeptide γ-L-Glu-L-Cys-Gly (glutathione) that has been detected in the oxidized (dimeric) form in ethanolic extracts of date fruits by Rinderknecht (1959).

Analysis of amino compounds in date fruit hydrolysates by HPLC and derivatization with AQC

Identical elution times of standard and samples cannot be considered a definitive prove for the identity in particular of minor amino compounds. Therefore, HPLC of amino acids in total hydrolysates of samples 1 and 2, after precolumn derivatization with AQC in comparison to a standard was performed (Fig. 3). Lack of Tau and Sar in the hydrolysates was definitely proven, but presence of non-proteinogenic Eta, Hyp, b-Ala (β-Ala), GABA, Hyl, and

Table 3 Quantities of amino acids (AA) and ethanolamine in nmol and mg per gram dry matter (DM) in date fruits 1–12 (see Table 1 for specification) determined by ion-exchange chromatography

AA	MM	Date number												Range	
		1	2	3	4	5	6	7	8	9	10	11	12	nmol/g DM	mg/g DM
Asp	133.11	14312	11155	20547	13353	13961	21033	16374	11957	19157	13578	9672	14468	9672–21033	1.29–2.80
Hyp	131.13	1391	1236	1164	1242	1417	979	1337	1782	1194	1377	1265	1106	979–1782	0.13–0.23
Thr	119.12	5669	4919	5345	5015	5735	6277	6771	6128	7079	5833	5714	6009	4919–6771	0.59–0.81
Ser	105.09	5731	4715	5767	4533	5628	5096	5582	6145	7023	5516	5349	5704	4533–7023	0.48–0.74
Glu	147.13	18006	14498	23026	15995	18291	25728	20785	14527	17375	16060	11976	16613	11976–25728	1.76–3.79
Pip(OH)	145.16	22532	13074	18600	23316	21256	26854	19287	27678	9661	23625	16729	9806	9661–27678	1.40–4.02
Pro	115.13	13663	14041	10599	10183	10931	12434	12924	11698	17156	11432	9043	10817	9043–17156	1.04–1.98
Gly	75.07	13043	12284	11886	11604	13351	15486	13824	12183	14719	13059	9958	13611	9958–15486	0.75–1.16
Ala	89.10	12271	13121	14112	12375	15430	16349	15494	11449	13150	12701	9745	13563	9745–15494	0.89–1.38
Val	117.15	7966	6989	6191	5641	7546	7414	8146	7678	7842	6837	6647	7268	5641–8146	0.66–0.95
$(Cys)_2$	240.30	704	654	958	811	1093	1856	1475	686	1290	841	469	981	469–1856	0.11–0.45
Pip	129.16	809	328	580	498	572	598	1202	420	616	546	508	1056	328–1056	0.04–0.14
Met	149.21	1549	1273	1153	197	1303	1092	949	1245	1145	1123	760	1485	197–1549	0.03–0.23
allo-Ile	131.18	ND	ND	ND	53	80	55	57	83	83	71	76	67	ND–83	ND–0.01
Acc	101.10	14375	13238	23638	21953	25804	25288	25712	24315	23788	16981	15104	20129	13238–25804	1.34–2.61
Ile	131.18	5403	4615	4382	4217	5164	4564	5181	5511	6076	5229	4736	5733	4217–6076	0.55–0.80
Leu	131.18	8604	7654	7066	6807	8462	8572	9381	9078	10084	8683	8218	9554	6807–10084	0.89–1.32
Tyr	181.19	2558	2213	1762	1207	1885	2239	2688	2341	2838	1768	1720	2244	1207–2838	0.22–0.51
Phe	165.19	4558	3838	3875	3726	4541	4431	4835	4837	5278	4573	4283	4799	3726–5278	0.62–0.87
β-Ala	89.10	248	332	571	390	240	561	518	179	495	357	115	389	115–571	0.01–0.05
β-Aib	103.12	93	ND	170	216	ND	ND	ND	221	207	155	91	127	ND–221	ND–0.02
GABA	103.12	10023	7375	11923	7240	6012	8120	7883	7295	9766	8854	5223	6844	5223–11923	0.54–1.23
His	155.16	2487	2765	2714	2604	2847	3438	3462	3144	3250	2540	2320	2646	2320–3462	0.36–0.54
Hyl	162.19	153	178	155	166	240	366	266	256	252	186	146	225	146–366	0.02–0.06
Orn	132.16	234	152	364	165	138	515	303	118	88	242	100	122	100–515	0.01–0.07
Lys	146.19	5862	6706	6267	5494	6918	7821	7628	7103	7184	5839	5150	6070	5150–7821	0.75–1.14
NH_3	17.03	26750	28148	17951	11171	12472	18325	17943	10307	18475	11276	8601	11306	8601–28148	0.15–0.48
Eta	61.08	2232	1777	2557	1481	1504	2667	2657	1443	1815	1440	629	1256	629–2667	0.04–0.16
Arg	174.20	4376	5991	2557	5000	5388	7088	6701	6123	4434	3808	3327	4070	2557–7088	0.45–1.23

ND, below detection limit; Pro, Hyp, Pip, and Pip(OH) calculated from absorption at 440 nm; others at absorption at 570 nm; peaks eluting before Asp were not assigned; Pip in sample No. 3 was estimated. Range refers to lowest and largest amounts

MM molecular mass

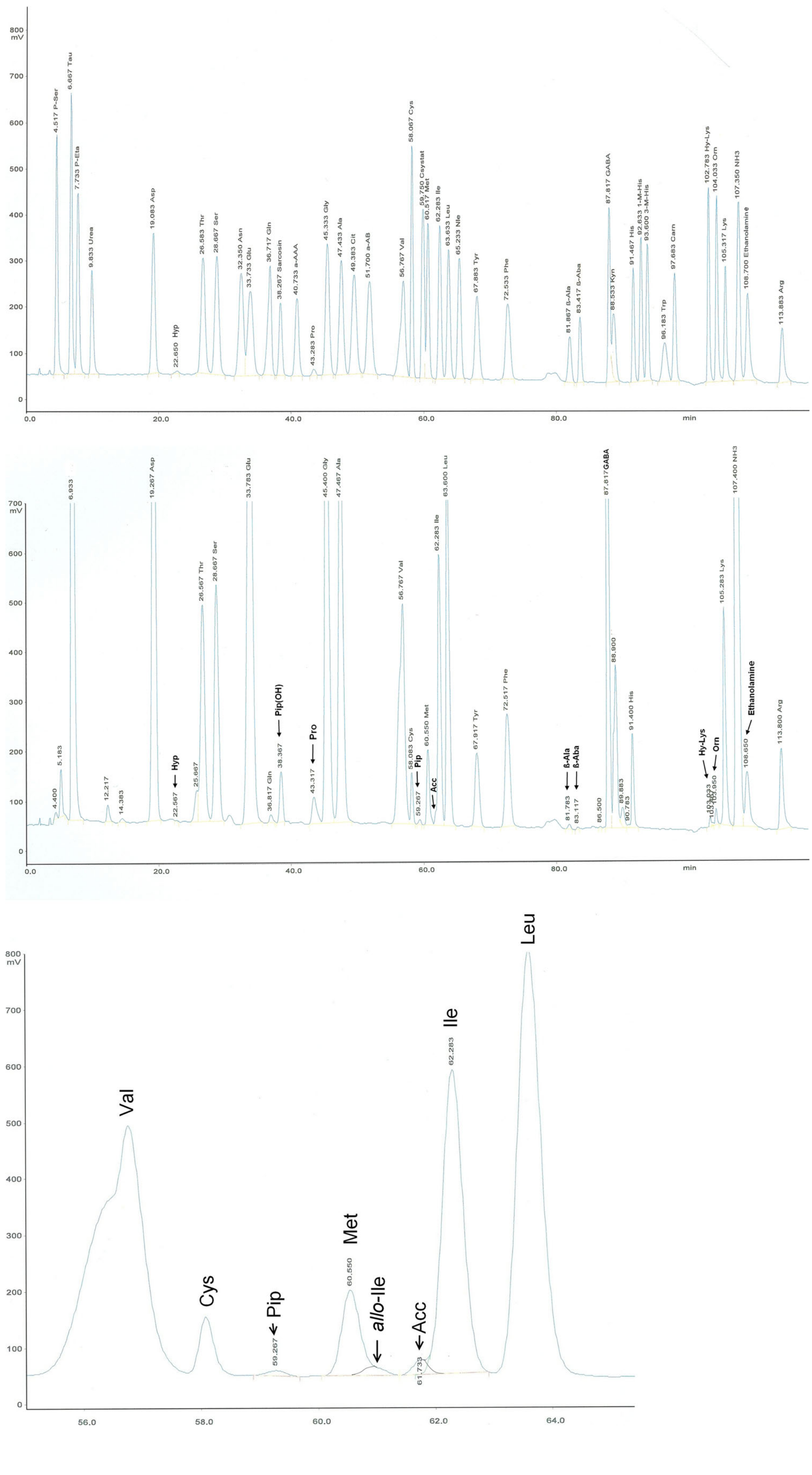

Fig. 2 Ion-exchange chromatogram (IEC) of a physiological standard of amino acids (*top*) and of a total hydrolysate of date fruit No. 1 (*middle*), and an expanded section (*bottom*) of the hydrolysate showing the elution positions of Pip, *allo*-Ile and Acc (shoulders of *allo*-Ile and Acc were completed for illustration by dashed lines). Post-column derivatization with ninhydrin and detection of derivatives at 570 nm. Special imino and amino acids are indicated by *arrows*. For abbreviations see text

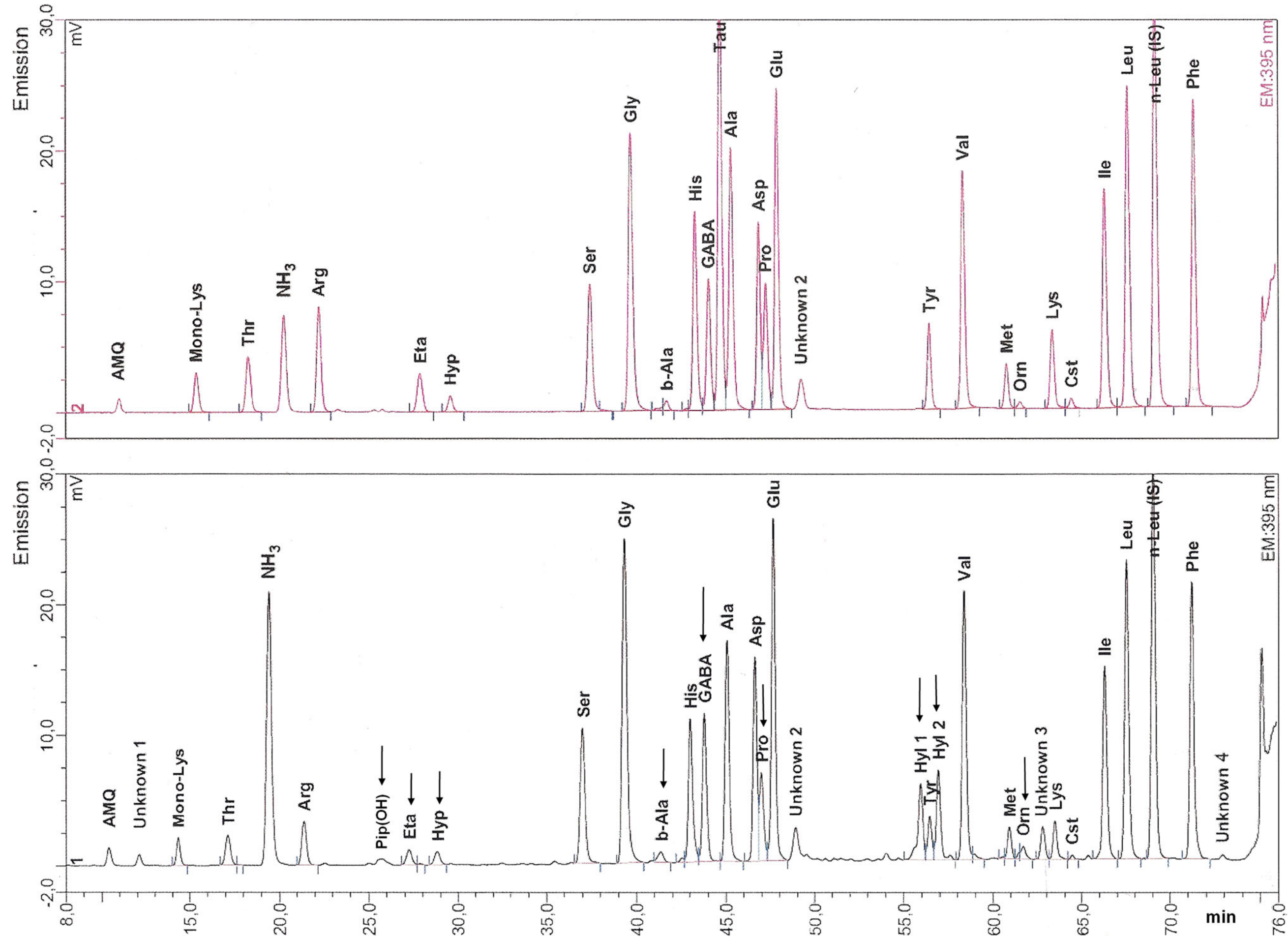

Fig. 3 HPLC of a standard of amino components (*above*), and of a total hydrolysate of date fruit No. 1 (*below*) after derivatization with AQC reagent and fluorescence detection (emission at 395 nm). Elution positions of special amino compounds are indicated by *arrows*. For abbreviations see text. Special abbreviations used are: *Mono-Lys* mono-derivatized Lys, *Hyl1*, *Hyl2* derivatives of hydroxylysine, *Cst* cystine, *n-Leu* norleucine (internal standard)

Orn (in the sequence of elution order) was established in accordance with IEC. The common proteinogenic amino acids Lys, Thr, Arg, Ser, Gly, His, Ala, Asp, Glu, Pro, Tyr, Val, Met, Lys, Cys, Ile, Leu, and Phe were also detected.

Under the derivatization conditions used, Lys was also mono-derivatized whereas Hyl gave rise to two derivatives. In addition, three peaks assigned as 'unknown 1, 2 and 3' were detected. Since 'unknown 2' also occurs in the standard, it is considered as 'system peak' or artefact. AMQ, resulting from hydrolysis of the excess of derivatizing reagent used, elutes at the very beginning of the chromatogram.

To sum up, the chromatographic data from IEC and HPLC established the presence of non-proteinogenic (non-coded) amino compounds, namely Pip(OH), Hyp, β-Ala, GABA, Hyl, Orn, and Eta and proved the absence of Tau and Sar in date fruits.

It should be noted in this context that analysis of hydrolysates of date fruits No. 1 and 2 employing the *i*TRAQ™ method (Kaspar et al. 2009; Dettmer et al. 2011) confirmed the presence of non-proteinogenic GABA, Hyp, Orn and Eta and of traces of Cit (20 nmol/g DM) and α-Aaa (<100 nmol/gDM) and possibly Hyl; the other non-coded AAs were not part of the standard used (data not shown). This is in accordance with the GC–MS data presented below.

Determination of Pip(OH) in date hydrolysates by GC–MS

Total hydrolysates of samples 1 and 2 were subjected to GC–MS after conversion of the amino acids into the corresponding trimethylsilyl esters by methoximation/silylation. Focus was put on the presence of Pip(OH). Based on the characteristic fragment ion at *m/z* 244, which results from loss of the trimethylsilyloxycarbonyl group from the pseudo molecular ion, the presence of Pip(OH) in both samples was definitely confirmed (Fig. 4) after comparison with NIST spectrum # 157429.

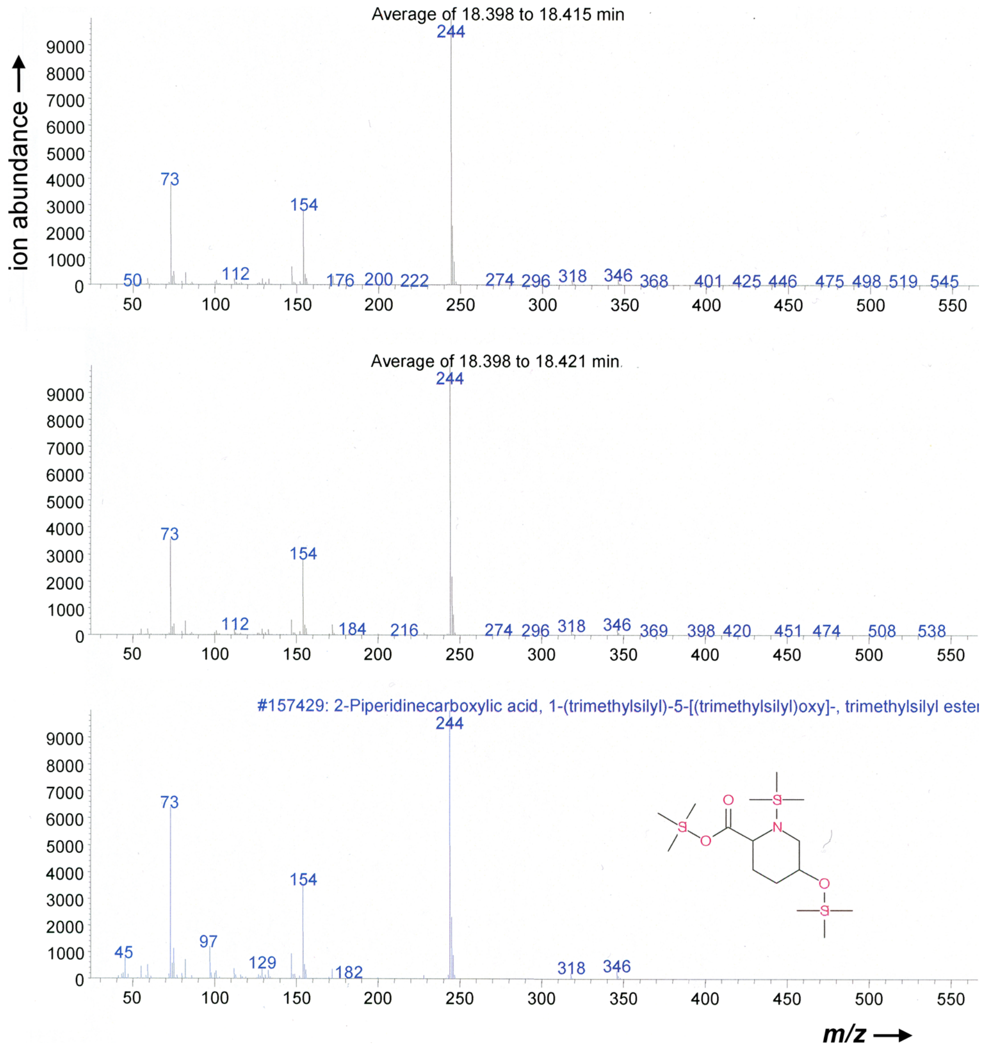

Fig. 4 GC–MS of trimethylsilylated 5-hydroxypipecolic acid (2-piperidinecarboxylic acid) from a total hydrolysate of date fruit sample No. 1 (*top*), date fruit No. 2 (*middle*) and the corresponding ion intensities of *m/z* 244 from authentic NIST standard (*bottom*); *m/z* 244 ([M–COOSiMe$_3$]$^+$)

Determination of amino acid stereoisomers and minor compounds in hydrolysates of date fruits by GC–MS

Amino acids occurring in the free or peptide-bound form were released from date fruit samples No. 1 and 2 by total hydrolysis using 6 M DCl in D_2O. Subsequently, they were converted into the corresponding *N*(*O*)-TFA-amino acid-(*O*)-ethyl esters, separated on the chiral stationary phase Chirasil®-L-Val (Frank et al. 1977) or, in the cases of Pip and Hyp, on Lipodex® E (König et al. 1989). Amino acids were identified by retention times and characteristic fragment ions. As expected, all common proteinogenic amino acids displayed the L-configuration.

Low amounts of D-amino acids, usually not exceeding 1 % relative to the corresponding proteinogenic L-amino acids, were detected and are included in Fig. 5.

Sar was not detected in both samples, thus corroborating its absence in date fruits. This method also confirmed presence and configuration of *trans*-4-L-Hyp, L-Pip, L-Orn and achiral β-Ala in both date samples. Traces of *β*-L-Aba (β-Aib) and Acc were found only in sample 1 but not in sample 2 (samples 3–12 were not analyzed by this method). Acc and β-Aib were detected in sample 1 but not in sample 2. Hyl was detected by IEC and HPLC (and traces by *i*TRAQ™) but not by chiral GC–MS. This is attributed to derivatization problems and low stability of the derivatives used for GC–MS. The detector response of Hyl is one to two orders of magnitude lower in comparison to Lys.

Small amounts of D-Ala, D-Asp, D-Glu, D-Ser and D-Phe were also detected, as well as trace amounts of some other amino acids. Representative, selected ion chromatograms of the enantiomers of Ala, Asp, Glu, Ser, Phe and Pip, and achiral derivatives of Acc, β-Ala, and GABA of date fruit No. 1 on Chirasil®-L-Val and Lipodex® E (for Pip) are displayed in Fig. 5.

Relevance of non-proteinogenic amino acids in date fruits

Basically, one has to distinguish between the relevance of non-proteinogenic amino acids and related

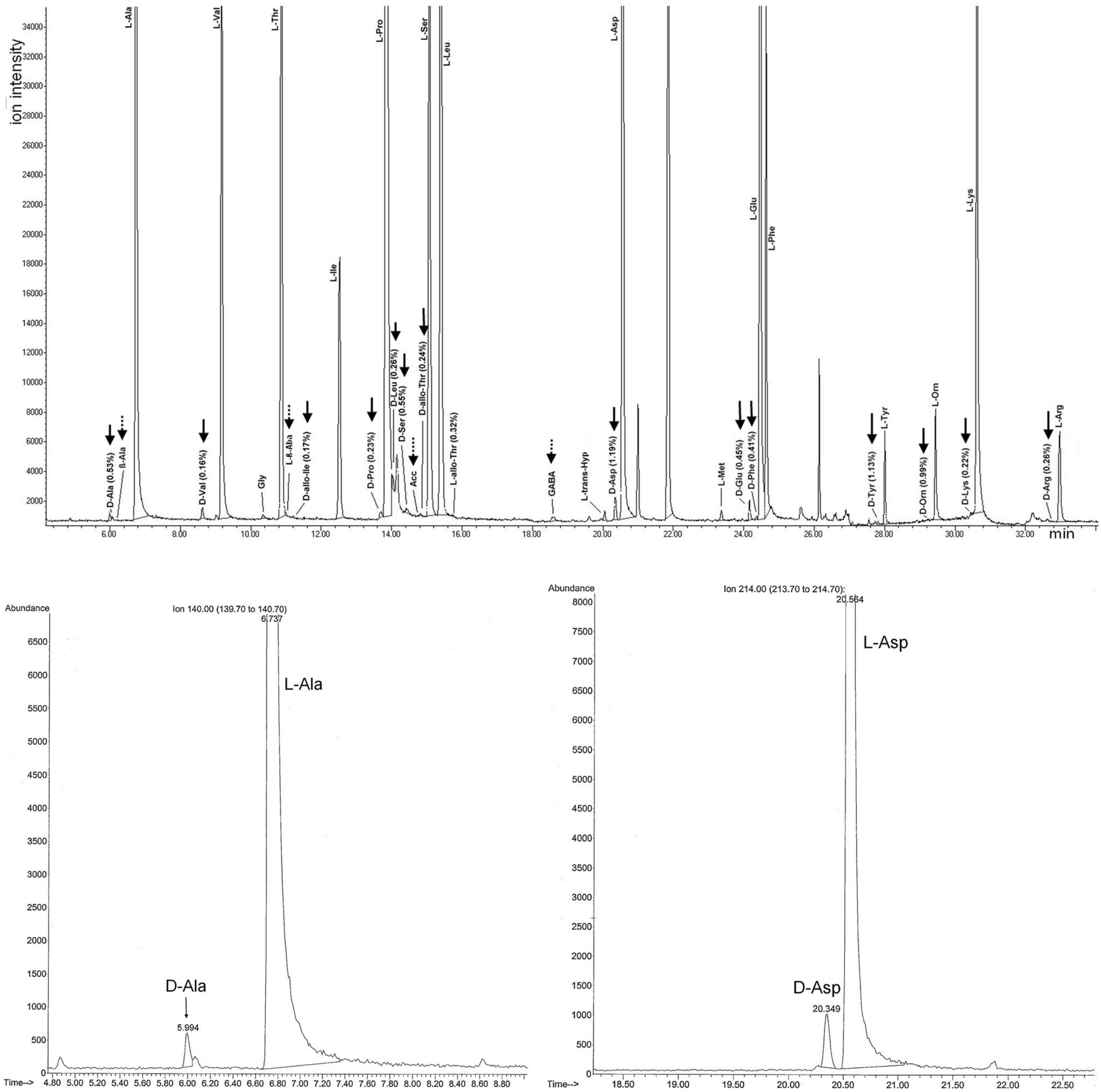

Fig. 5 *Above* GC-MS of $N(O)$-trifluoroacetyl-O-ethyl esters of amino acids resulting from a total hydrolysate (6 M DCl in D_2O) of date fruit No. 1 eluting from Chirasil®-L-Val; D-amino acids and relative % [%D = 100 D/(D + L) from peak areas] are indicated by *bold arrows* and special non-protein amino acids by *dashed arrows* [Pip and Pip(OH) not assigned; His not shown]. *Below* expanded sections of characteristic fragment ions showing, besides abundant L-amino acids, presence of selected D-amino acids: D-Ala (*m/z* 140), D-Asp (*m/z* 214), D-Glu (*m/z* 228), D-Ser (*m/z* 138), D-Phe (*m/z* 176), D- and L-Pip (*m/z* 180) (Pip enantiomers analysed on Lipodex® E column), and of achiral amino acids Acc (*m/z* 179), β-Ala (*m/z* 139), and GABA (*m/z* 182)

compounds for the biochemistry of the date palm, and possible nutritional and health effects on consumption of date fruits by human beings. In the following, both aspects are briefly outlined for those compounds found.

5-Hydroxypipecolic acid Pip(OH) and pipecolic acid (Pip)

Pip(OH) and its various stereoisomers and conjugates detected in plants (Kite and Ireland 2002) might be

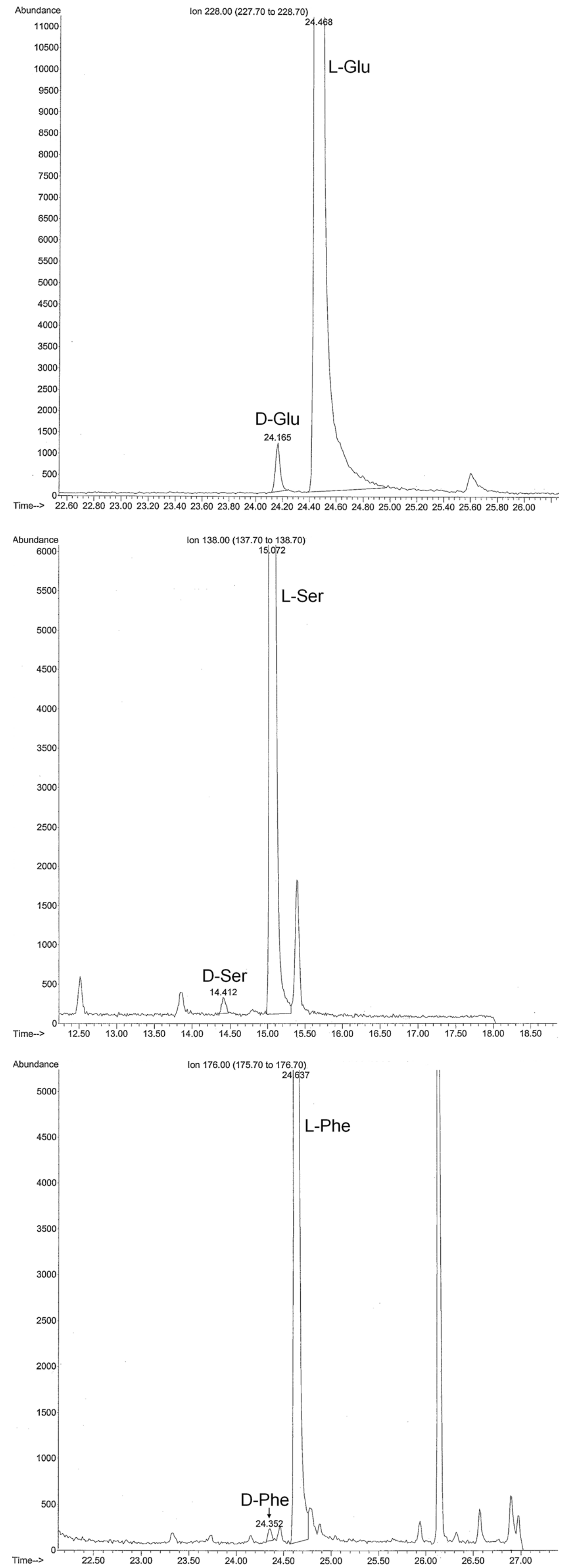

Fig. 5 continued

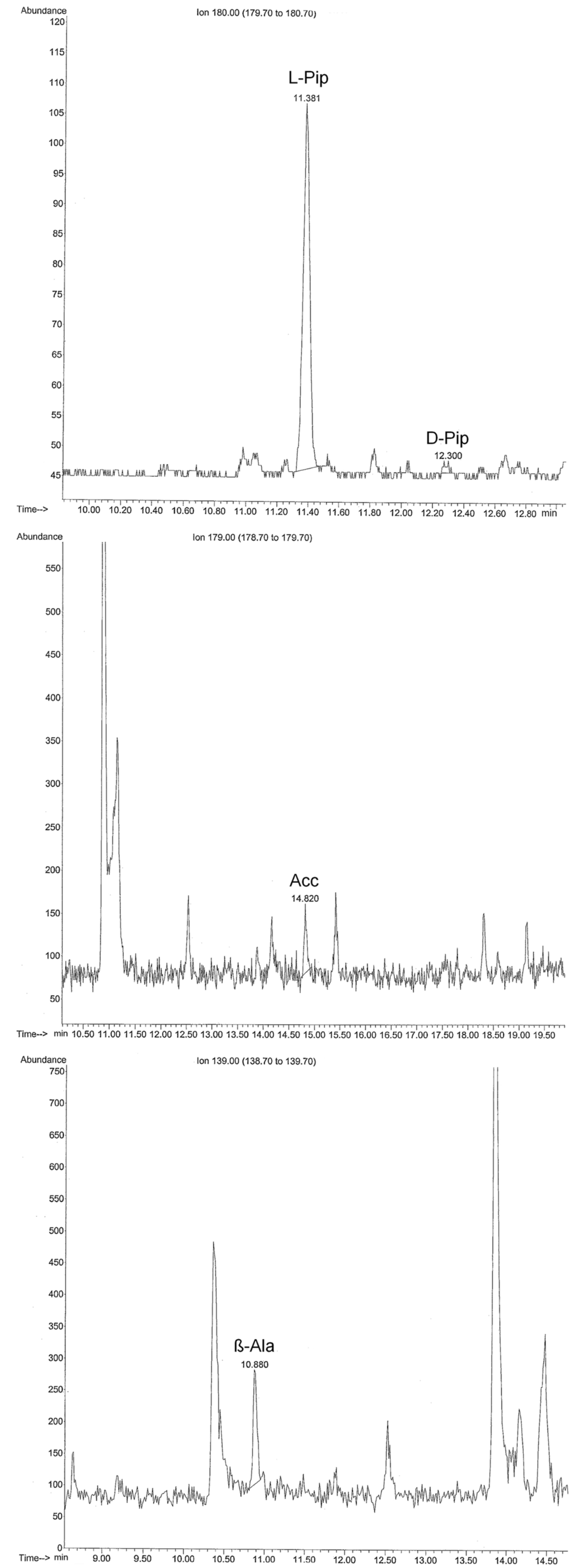

Fig. 5 continued

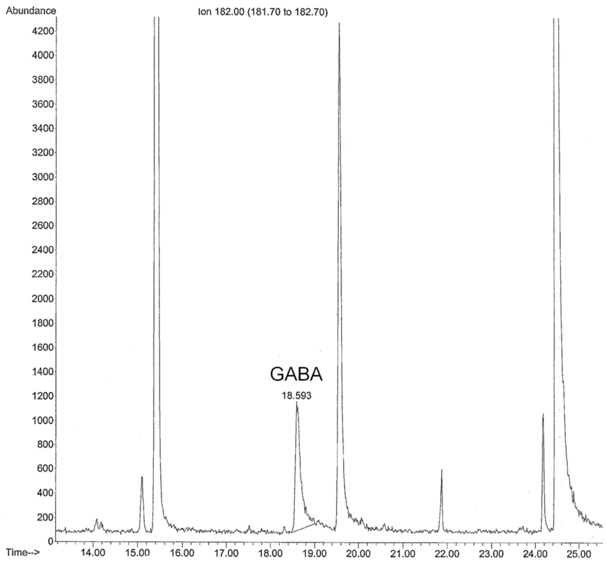

Fig. 5 continued

considered potential fungicides and insecticides (Brenner and Romeo 1986; Bell 2003). Pip(OH) has been described as powerful inhibitor of platelet aggregation induced by serotonin and hypothesized to be involved in the biological effects of the herbal drug *Xylia xylocarpa* (Mester et al. 1979).

L-as well as D-Pip have been recognized to be widely distributed in plants in the free and conjugated form (Dardenne and Sørensen 1974; Fujioka and Sakurai 1997) and both enantiomers occur in physiological fluids as outlined in a recent review by Vranova et al. (2013). L-Pip isolated from the edible mushroom *Sarcodon aspratus* showed moderate inhibitory effects on angiotensin I-convering enzyme (Kiyoto et al. 2008).

1-Aminocyclopropane-1-carboxylic acid (Acc)

Acc is of importance in plants as precursor of the plant hormone ethylene, which acts as fruit-ripening hormone and bloom stimulant. Ethylene plays a general role as a growth inhibitor in promoting leaf and flower senescence and abscission. Its occurrence in date fruits might be related to these effects. Free Acc has been isolated from perry pear juices (*Pyrus communis* L.) and cider apples (*Malus domesticus* Borkh.) (Burroughs 1957) as well as from cowberry (*Vaccinium vitis-idaea* L.) (Vähätalo and Virtanen 1957). However, Acc has also been detected as 1-(malonylamino)cyclopropane-1-carboxylic acid and as a constituent of the dipeptide γ-glutamyl-Acc in tomato plants (Peiser and Yang 1998). Conjugated Acc would have been released on total hydrolysis of date fruits.

With regards to physiological effects, Acc has been reported to exert antidepressant-like effects in animal models (Przegalinski et al. 1997) and to act as partial agonist of the *N*-methyl-D-aspartate (NMDA) receptor in electrophysiological studies (Cherkofsky 1995; Inanobe et al. 2005; Nahum-Levy et al. 1999).

γ-Aminobutyric acid (GABA)

In plants, animals, and microorganisms, GABA is formed from L-Glu by the action of glutamate decarboxlase (EC 4.1.1.15). Interest in the GABA shunt in plants emerged mainly from the observation that this amino acid is rapidly produced in response to biotic and abiotic stress such as microbial attack or drought (Bouché and Fromm 2004) and might serve as signalling molecule. Thus, GABA is of general interest for date palm cultivation.

Interest related to human beings results from occurrence in high levels in the brain indicating an important role in neurotransmission. It is also an integral part of the central nervous system. A number of commercial sources sell formulations of GABA as dietary supplements. Claims are made that these supplements have calming effects and promote sleep. Therefore, GABA is recommended as natural tranquilizer. Some studies showed that GABA acts as strong secretagogue of insulin from the pancreas, thus effectively preventing diabetes at least in rat (Adeghate and Ponery 2002; Hagiwara et al. 2004). One report claims that oral uptake of GABA increases production of growth hormones (Powers et al. 2008). Foodstuffs rich in GABA are considered as health foods, particularly in Asia. Uptake of large quantities of GABA with date fruits has to be considered in this respect.

Hydroxyproline (Hyp) and Hydroxylysine (Hyl)

Hyp and Hyl in proteins result from post-translational modification of Pro and Lys. Both amino acids are considered typical constituents of collagen. They are used as biomarkers for collagen turnover and related metabolic diseases. It is frequently overlooked that Hyp is also a major constituent of common plant cell wall glycoproteins, namely extensins, and Hyp-arabinogalactans (Kieliszewski and Lamport 1994). Therefore, the occurrence of those two amino acids in date fruits in moderate (Hyp) or trace amounts (Hyl) is of interest. Since Pip(OH) is considered to be synthesized from Hyl through cyclisation (Lindstedt and Lindstedt 1959), this might explain the detection of Hyl in date fruit hydrolysates.

β-Alanine (β-Ala), β-amino*iso*butyric acid (β-Aib), and ornithine (Orn)

In plants, β-Ala is synthesized *via* the uracil or propionate pathway (Rathinasabapathi 2002) and is used for the synthesis of pantothenic acid (vitamin B_5). In physiological fluids, β-Ala is found conjugated as a constituent of the dipeptides carnosine and anserine. As β-Ala is the rate-limiting precursor of carnosine, carnosine levels are limited by the amount of β-Ala. Thus, β-Ala is part of nutritional supplements, which are said to increase the carnitine concentration in muscles, thus increasing the exercise capacity of athletes (del Favero et al. 2011). β-Aib occurs in fluids but has also been detected in bulbs of *Iris tingitana*, together with β-Ala and GABA (Asen et al. 1959). Orn, detected by us in all hydrolysed date fruits, might arise from originally present Cit in fruits that is almost entirely converted into Orn under the conditions of acidic total hydrolysis as reported by Rinderknecht (1959). Indeed, trace amounts of remaining Cit were detected by *i*TRAQ™ in date fruits 1 and 2.

Ethanolamine (Eta)

Eta is widely distributed in plants in both free and bound form.

Remarkable quantities of the β-amino alcohol Eta were detected in all date fruits. This might be of interest as application of Eta to barley plants diminished drought stress (Bergmann et al. 1994). In plants, Eta is also formed by decarboxylation of Ser and P-Ser (Vance 2008).

In organisms, Eta occurs as ethanol phosphatidylserine and phosphatidylethanolamine (P-Eta). Both compounds are components of mammalian cell membranes and play important roles in biological processes such as apoptosis and cell signalling. They have also been shown to modulate the rate of rat hepatocyte proliferation in in vitro and in vivo (Sasaki et al. 1997). Thus, uptake of Eta via dates might have positive nutritional effects. On acidic total hydrolysis, Eta will be released from its respective conjugates.

D-Amino acids in date fruit hydrolysates

Low or trace amounts of free or conjugated D-amino acids (the stereoisomers or enantiomers of common L-amino acids) occur in all plants (see Brückner and Westhauser 2003, and references cited therein). D-AAs in unprocessed plants originate from a plant's endogenous racemase or are taken up from soil or rhizosphere bacteria. Another route for the formation of D-AAs, considered to play also a role in the ripening and processing of edible date fruits, is the *Maillard* or non-enzymatic browning reaction resulting from the interaction of amino acids and reducing sugars such as fructose and glucose (Ali et al. 2006, 2010; Pätzold and Brückner 2006; Kim and Lee 2009). The discussion related to possible toxic effects of D-AAs occurring in foods and beverages in recent years has changed entirely towards positive effects; and a fair number of D-AAs are used as medical drugs or food supplements (Friedman and Levin 2012; Brückner and Fujii 2011). This paradigm shift was also caused by findings that all physiological fluids and tissues of organisms contain D-AAs and that, in particular, microbially fermented foodstuffs of animal or plant origin contain relatively high concentrations of D-AAs as a result of the action of microbial racemase and epimerase. Consequently, there is a steady nutritional uptake of D-AAs. Certain amounts are converted by D-amino acid oxidases into alpha-keto acids that were reaminated to L-amino acids, whereas excesses of D-AAs are excreted with the urine.

Despite the low concentrations of D-AAs, some effects related to drugs or physiological effects are briefly discussed as follows.

D-Aspartic acid

D-Asp is an amino acid present in neuroendocrine tissues of invertebrates and vertebrates, including rodents and humans. In man and rat, D-Asp induces an enhancement of luteinizing hormone and testosterone (Nagata et al. 1999). The pituitary and testes possess a high capacity for trapping of circulating D-Asp, which has been formed endogenously in the body by an L-Asp racemase or by trapping circulating D-Asp taken up from endogenous sources like foodstuffs (Topo et al. 2009). D-Asp in human ovarian follicular fluid was aligned with oocyte quality (D'Aniello et al. 2007). The sodium salt of D-Asp is used as a drug to improve semen quality and testosterone level of man. D-Asp has been also associated with memory and learning (Topo et al. 2010). The Mg^{2+}-salts of L-Asp and DL-Asp are used as magnesium supplement (Iezhitsa et al. 2004). Even tumour growth inhibition in experimental rats by D-Asp has been described by Sasamura et al. (1998) but certainly needs confirmation.

D-Alanine

D-Ala is added to antipsychotics for the treatment of schizophrenia (Tsai et al. 2006).

D-Serine and D-Phe

Relative high amounts of 20 % D-Ser (related to L-Ser) were detected in human brain, and an L-Ser racemase has been localized in the human brain (Wolosker et al. 2000). D-Ser is used pharmacologically for treatment of schizophrenia. The Fe^{2+}-salt of DL-Ser is used pharmacologically as iron-supplement, and phosphono-DL-Ser is added to pharmaceutical preparations. DL-Phe is used medically for treatment of Parkinson's disease and has been reported to potentiate opiate analgesia—an example of nutrient/pharmaceutical up-regulation of the endogenous analgesia system (Russell and McCarty 2000).

Conclusions and outlook

(a) IEC running in the physiological mode is a suitable method for detection and quantification of non-proteinogenic amino acids in date fruits. However, chemical nature and elution positions of special, non-coded amino compounds have to be confirmed by other analytical methods.

(b) Besides common proteinogenic amino acids, the non-proteinogenic (non-coded) amino acids Pip(OH), Acc, and GABA have been detected in gram amounts per kilogram of dry date fruits. Moderate amounts of Pip and Hyp, were found and low amounts of β-Ala, β-Aib, and Orn. Low or trace amounts of D-amino acids were detected using chiral GC–MS.

(c) The relevance of the aforementioned compounds for date fruits and date palm biochemistry needs further exploration.

(d) Nutritional consequences and issues related to possible health benefits of non-proteinogenic amino acids occurring in date fruits, in particular with regard to the abundant Pip(OH), Acc, and GABA, require further investigations.

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Conflict of interest The authors declare that they have no conflict of interest.

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