Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 991 (2003) 143–150
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Short communication

Analysis of a ginger extract by high-performance liquid chromatography coupled to nuclear magnetic resonance spectroscopy using superheated deuterium oxide as the mobile phase

Shikha Saha[a], Roger M. Smith[a,*], Eva Lenz[b], Ian D. Wilson[b]
[a]*Department of Chemistry, Loughborough University, Loughborough, Leicestershire LE11 3TU, UK*
[b]*AstraZeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK*

Received 24 October 2002; received in revised form 22 January 2003; accepted 22 January 2003

Abstract

A methanolic extract of powdered ginger was separated on a Xterra RP 18 column using deuterium oxide as the eluent and a temperature gradient from 50 to 130 °C at 4 °C/min. On-line and off-line HPLC–NMR analysis yielded spectra for vanillin, dihydroferulic acid, zingerone and ferulic acid. The identification of dihydroferulic acid and zingerone were confirmed by mass spectroscopy.
© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Ginger; Superheated water chromatography; Natural products; Nuclear magnetic resonance spectroscopy

1. Introduction

Whilst the characterisation of the compounds present in plant extracts by HPLC can generally be achieved by the comparison of the retention times with authentic standards this does not provide an unequivocal method of identification. In addition, if they are not commercially available, the synthesis or isolation of these standards can be both time consuming and costly. Developments in the hyphenation of liquid chromatography and spectroscopy, particularly mass spectrometry (HPLC–MS) and nuclear magnetic resonance spectroscopy (HPLC–NMR) offer the potential for unknown peaks to be rapidly identified without the need for either extensive sample purification or synthetic standards. As such these techniques are playing an increasingly important role in the study of natural products [1].

The application of superheated water as the mobile phase in HPLC, whilst relatively new, is finding an increasing number of applications [2–5]. For HPLC–NMR the use of superheated deuterium oxide (D_2O) as the eluent is advantageous compared to conventional HPLC organic–aqueous eluents as this would avoid the signals from the modifiers, which could otherwise interfere with the spectra of the analytes [6–10]. Previously reported examples include the separation of barbiturates with both, on-flow and

*Corresponding author. Tel.: +44-1509-222-563; fax: +44-1509-223-935.
E-mail address: r.m.smith@lboro.ac.uk (R.M. Smith).

0021-9673/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0021-9673(03)00215-2

stop-flow modes of detection [6], and the HPLC–NMR–MS of model drugs [7] and sulphonamides [8]. Studies employing HPLC–IR–UV (DAD)–NMR–MS with superheated D_2O for a model drug mixture [9] and ecdysteroids [10] have also been described.

The present study examines the application of superheated water–NMR using deuterium oxide as the eluent for the separation and analysis of the constituents of ginger. Numerous methods (GC–MS, TLC, HPLC, and HPLC–MS) have previously been described for the examination of the components of ginger (for example, see Refs. [11–16]). Ambient temperature HPLC has been shown to be suitable for the analysis of ginger extracts [13,15,16]. However, it was found that the gingerols, which are the main pungent constituents, were thermally unstable and decomposed by a retro-aldol reaction under high temperatures during GC and GC–MS analyses [11,12,14].

2. Experimental

2.1. Materials

Vanillin, dihydroferulic acid and ferulic acid were obtained from Lancaster Chemicals (Lancaster Chemicals, Morecambe, UK). Zingerone was obtained from Pfaltz and Bauer (Waterbury, PA, USA). Deuterium oxide (99.8% isotopic purity) was obtained from Fluorochem (Glossop, UK) and HPLC grade methanol from Fisons (Loughborough, UK). Dry ginger powder was obtained from a supermarket (Sainsbury's, Loughborough, UK).

2.2. Extraction of ginger

First, 10.0 g of the dry ginger powder were extracted with methanol (20 ml) by stirring overnight at room temperature. The extraction solution was run onto a 10×0.5 cm column packed with silica gel (60–120 mesh) (BDH Chemicals, Poole, UK). The column was then eluted with methanol (20 ml). The combined eluent was evaporated to dryness under vacuum and taken up in a small volume of deuterated methanol (1 ml). Samples of the solution were separated by either conventional reversed-phase chromatography with UV detection or superheated (heavy) water chromatography with UV and NMR detection in on- and stop-flow modes.

2.3. Instrumentation

2.3.1. Superheated water chromatography–NMR spectroscopy

The superheated water chromatographic–NMR system (Fig. 1) comprised a LC-10AD Shimadzu pump (Shimadzu, Kyoto, Japan) which delivered the mobile phase at 1 ml/min. The mobile phase was

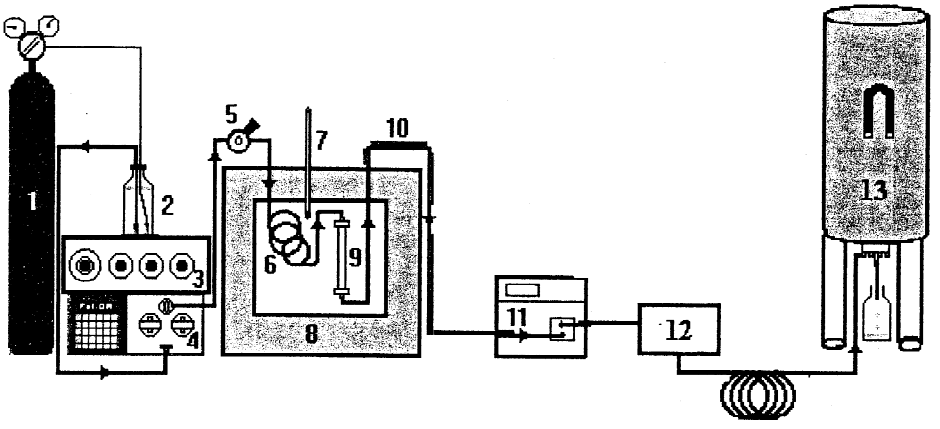

Fig. 1. The superheated water HPLC–NMR system. Components: 1=helium cylinder; 2=mobile phase reservoir; 3=oven temperature programmer; 4=LC pump; 5=injector loop; 6=preheating coil; 7=thermometer; 8=column oven; 9=column; 10=cooling fins; 11=UV–Visible detector; 12=12-loop trap; 13=NMR spectrometer.

continually degassed with nitrogen. The sample was injected using an injector (Model 7125, Rheodyne, Cotati, CA, USA) fitted with a 200-μl sample loop outside the oven and passed through a preheating coil made of stainless steel tubing (100 cm×0.5 mm I.D.) to the column. The column and preheating coil were placed in a Pye 104 oven (Pye Unicam, Cambridge, UK), whose temperature was controlled using a programmer (Series 104, Pye Unicam, Cambridge, UK). The separations was performed on an Xterra C_{18} bonded column (150×4.6 mm I.D., 5 μm particle size; Waters, Watford, UK) or a PDB–Zirconia column (150×4.6 mm I.D., 3 μm particle size; Zirchrom Separations, Anoka, MN, USA). The column outlet tubing was immersed in a beaker filled with ice-water (0–4 °C) and was connected by a 2 m×0.5 mm I.D. PEEK tubing to a Bischoff UV (DAD) detector (Bruker, Coventry, UK). This was connected to a BPSU-12 unit (Bruker Peak Sampling Unit with 12 collection loops) for off-line detection or directly by a 3 m×0.25 mm I.D PEEK tubing to the NMR flow-probe comprised a dual tunable $^{1}H/^{19}F$ probe with an active cell volume of 60 μl. The NMR spectra were measured using a DRX500 spectrometer (Bruker Biospin, Coventry, UK) operating at a ^{1}H resonance frequency of 500.13 MHz. In the on-flow mode, four free induction decays (FIDs) were collected over a spectral width of 8278.15 Hz into 8194 data points. A relaxation delay of 1 s was employed between successive scans, resulting in a pulse recycle time of 1.5 s. NMR analyses were also performed in the stop-flow mode on selected peaks after loop storage in the BPSU-12 (Bruker Peak Sampling Unit with 12 loops). Typically, depending on concentration, 128–4096 free induction decays (FIDs) were collected on each fraction over a spectral width of 8012.82 Hz into 16 384 data points. A relaxation delay of 2 s was employed between successive scans resulting in a pulse recycle time of 3.02 s.

2.3.2. SHW chromatography with UV detection

For superheated water chromatography with UV detection, a set of copper cooling fins (3×12 cm×0.05 mm) was attached to the oven outlet tubing to cool the mobile phase down to ambient temperature before it entered a Jasco Model 870 UV–Visible detector (Jasco, Kyoto, Japan). A back-pressure controller (Jasco 880/81) set at 35 kg/cm^2 (or a 0.13 mm I.D.×3 m length of PEEK tubing) was used to maintain the pressure of superheated water in the column. Eluent signals were recorded at 280 nm on an HP 3395 integrator.

2.3.3. Off-line identification of peaks by mass spectrometry

Following HPLC–NMR a number of peaks were collected as they eluted from the probe. These were freeze dried, redissolved in 2 μl chloroform and then subjected to mass spectroscopic analysis on a SX102 mass spectrometer (Jeol, Welwyn Garden City, UK) using direct injection and the electron impact (EI) mode

3. Results and discussion

3.1. Separations using superheated water

The chromatographic system used in this study was similar to that reported previously for superheated water separations [3]. Initial method development centred on the use of the PBD–zirconia phase, which had previously been successfully used superheated water chromatography for the separation of a wide range of aromatic compounds [4,6,17–19]. However, although a separation of the ginger extract was obtained with superheated water at 150 °C, the efficiencies of the separation was very poor. Separation of the samples on this column by conventional HPLC with a mobile phase of 35:65 (v/v) acetonitrile–water also yielded poor efficiency. Although good results for these compounds had been obtained previously on conventional ODS silica columns [13–15] these stationary phases are generally unstable at high temperatures. However, the recently introduced Xterra RP18 silica based phases are more robust and have been successfully used for superheated water chromatography [9,10]. Isothermal conditions gave a poor separation but a temperature gradient from 50 to 130 °C at 4 °C/min, yielded a good separation of the constituents of the extract (Fig. 2). This approach is equivalent to the use of gradient elution in RP-HPLC. When a sample of zingerone was examined under the same conditions it corresponded to the last peak and it was apparent that the components which

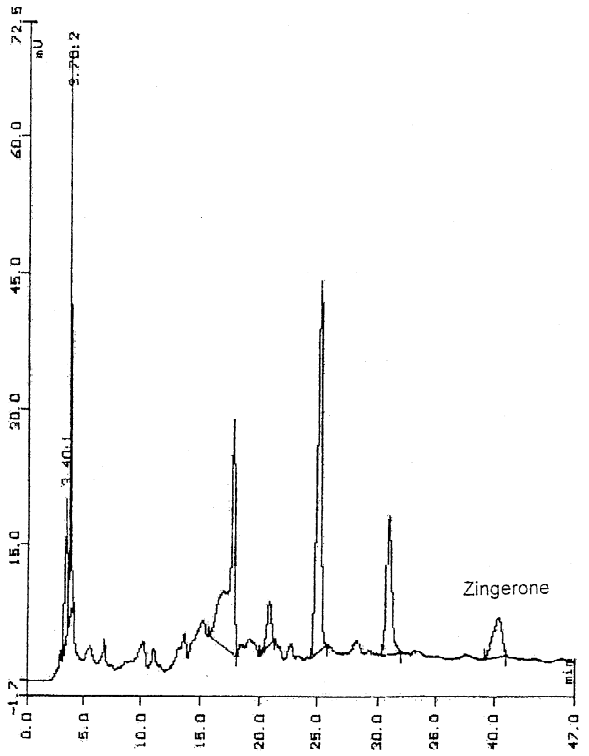

Fig. 2. Separation of the 30-μl of ginger extract on Xterra RP18 column using superheated deuterium oxide as the mobile phase. Conditions: temperature 50 to 130 °C at 4 °C/min; eluent, deuterium oxide at 0.5 ml/min; detection, 280 nm.

had been separated were probably not the relatively non-polar gingerol pungent principles but more polar components.

3.2. Coupling superheated water chromatography with nmr spectroscopy

In order to identify the components the separation was then examined by LC–^{1}H NMR using deuterium oxide as the eluent. Because of the need to position the LC system at least 2 m from the NMR magnet, a 2-m PEEK narrow bore tube was used to couple the HPLC system to the UV detector followed by additional 3 m length of PEEK tubing to the NMR flow cell. This PEEK tubing also created sufficient back-pressure to maintain a liquid state of the D_2O in the chromatographic column at the high temperatures employed, so that a separate back-pressure regulator was not required. Because of the relatively low sensitivity of the NMR detector the sample size (100 μl) and concentrations were increased resulting in a degree of column overloading and in distorted peak shapes (Fig. 3). However, in the on-flow mode, a reasonable spectrum was only obtained for peak 2 and this was identified as dihydroferulic acid (Fig. 4.). The spectrum displayed a doublet at 6.75 ppm ($^{3}J=8.13$ Hz), a doublet of doublets at 6.67 ppm ($^{3}J=8.13$ Hz and $^{4}J=1.96$ Hz) and a doublet at 6.84 ppm ($^{4}J=1.96$ Hz). The signals were assigned to an ABX system of three protons on an aromatic ring. Two triplets at 2.56 and 2.76 ppm corresponded to the $-CH_2$ groups of the propionic acid side chain. The methoxyl-group gave rise to a singlet at 3.75 ppm. The small singlet at 1.94 ppm is due to residual acetonitrile, which was present as a contaminant contained in the HPLC pump-head from previous analyses.

Although the spectrum of peak 2 closely resembled that of authentic dihydroferulic acid (in deuterium oxide), there were some differences in the chemical shifts especially of the aromatic protons. The signals for H5 and H6 in the spectrum of the standard resonated at 6.55 ppm and 6.53 ppm, and the doublet for H2 was at 6.58 ppm in contrast to the values in the extracted sample. However, when the pH of the solution was raised, the spectrum agreed closely with that from peak 2. It appeared that partial ionisation, which is not normally observed in non-aqueous NMR spectra, can be important in aqueous solutions and can even have a significant effect on the chemical shift of a proton remote from the carboxylic acid group.

The separation was then repeated and the peaks were collected using a 12-loop sample collector. Each fraction was then transferred in turn to the NMR spectrometer and examined off-line, which enables higher sensitivity to be obtained as more scans can be collected. In this way three discrete peaks were isolated. The spectra from peaks 1 (Fig. 5a) and 2 corresponded to vanillin and dihydroferulic acid, respectively. The spectrum of peak 1 displayed a doublet at 6.96 ($^{3}J=8.38$ Hz), a doublet of doublets at 7.44 ppm ($^{3}J=8.38$ Hz and $^{4}J=1.96$ Hz) and a singlet at 7.43 ppm ($^{4}J=$unresolved). These coupling patterns described an ABX-trisubstituted aromatic ring system. The singlet (3H) at 3.83 ppm corresponded to the methoxyl group ($-OCH_3$) and

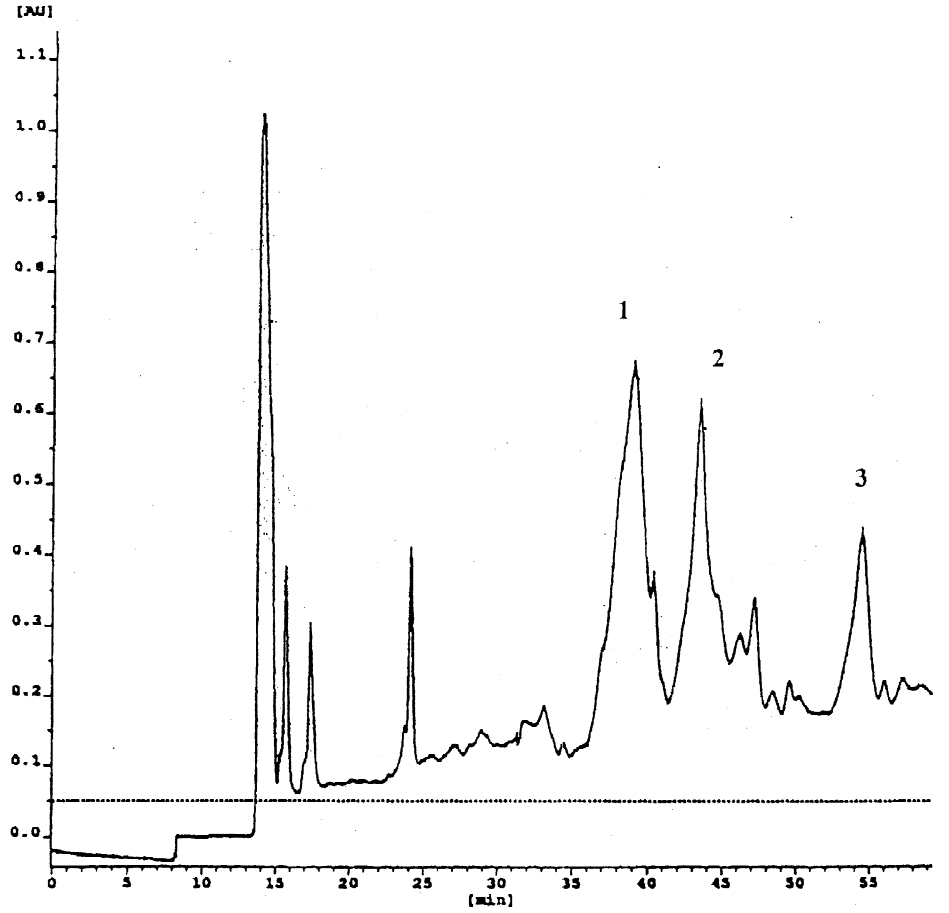

Fig. 3. Separation of 100 μl of high concentration of ginger extract on Xterra RP18 column. Conditions as Fig. 2.

the singlet at 9.6 ppm, was assigned as the aldehydic proton. The spectrum closely matched the spectrum of an authentic sample of vanillin run in D_2O.

Peak 2 gave the same spectrum as in on-flow mode, albeit with slight contamination with vanillin, probably due to tailing of peak 1, and matched that of the spectrum of dihydroferulic acid. The isomeric isodihydroferulic acid was also examined and was shown to have a different proton NMR spectrum.

Peak 3 was identified from the spectrum (Fig. 5b) as an unresolved mixture of zingerone and ferulic acid at a ratio of approx. 10:7 based on the signal intensities. The signals assigned to zingerone comprised a doublet at 6.72 ppm ($^3J=8.01$ Hz), a doublet of doublets at 6.63 ppm ($^3J=8.01$ Hz and $^4J=1.96$ Hz) and a doublet 6.81 ppm ($^4J=1.96$ Hz) for the three protons of the 1,2,4-substituted aromatic ring. The singlet (3H) at 3.73 ppm corresponded to the methoxyl protons, the two multiplets at 2.70 and 2.75 ppm to the methylenes in the $-CH_2CH_2COCH_3$ side chain and the singlet at 2.06 ppm to the methyl ketone group. The weaker signals corresponded to ferulic acid. The doublet at 6.85 ppm ($^3J=8.25$ Hz), the doublet of doublets at 7.09 ppm ($^3J=8.25$ Hz and $^4J=1.96$ Hz) and the doublet at 7.2 ppm ($^4J=$ 1.96 Hz) could be assigned to the three protons of the 1,2,4-substituted aromatic ring. The two doublets at 7.58 ppm and 6.3 ppm corresponded to the methene protons of the –CH=CHCOOH side chain. The coupling constant of $^3J=15.89$ Hz is diagnostic of the protons being in the *trans*-configuration. The singlet (3H) at 3.8 ppm could be assigned to the

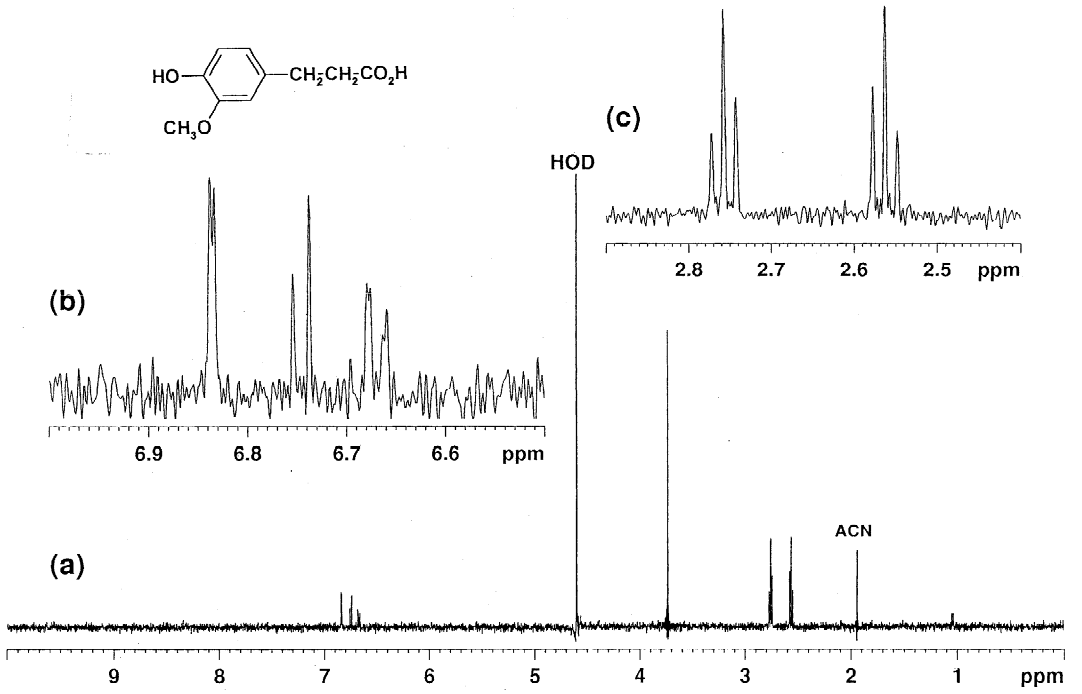

Fig. 4. ^{1}H NMR spectrum of peak 2 identified as dihydroferulic acid in the on-flow analysis (acquired with 4 scans/row). Key: a = the full spectral range between 10 and 0 ppm; b = expansion of spectral region between 7.0 and 6.5 ppm; c = expansion of spectral region between 2.9 and 2.4 ppm [HOD = residual water in D_2O; ACN = acetonitrile contamination from HPLC pump from previous analyses].

methoxyl protons on the aromatic ring. In both cases the spectra matched those of standard samples in deuterium oxide.

3.3. Identification of the chromatographic peaks by mass spectroscopy

To confirm the proton NMR identifications, the fractions from the loop collection were evaporated and analysed by EI–MS. No interpretable MS data were obtained for peak 1 (vanillin), however, for peak 2 the MS results supported the identification of dihydroferulic acid (M^+; m/z 196, relative abundance 31% and a tropylium ion with a hydroxyl and a methoxyl substituent on the phenyl ring, m/z 137, relative abundance 100%) and agreed with the spectrum of an authentic standard. The mass spectra from peak 3 showed molecular ions characteristic of zingerone (M^+; m/z 194, relative abundance 49% and the tropylium ion m/z 137, relative abundance 100%) and gave an exact match to an authentic sample. However, no peaks were observed for the less volatile ferulic acid.

Although zingerone has frequently been found as a constituent of ginger and vanillin has been reported by Connell and McLachlan [11], the other components found in the present study have not previously been detected from ginger. However, vanillin and ferulic acid were isolated by Masada et al. [20] from the degradation of the closely related compound curcumin, also found in the Zingiberaceae. In addition, ferulic and dihydroferulic acids have been demonstrated to be biosynthetic precursors of the gingerols by Whiting and co-workers [21,22].

4. Conclusion

The results of this study demonstrate the applicability and problems of superheated water HPLC–NMR (using superheated deuterium oxide as the mobile phase) for the identification of the components of a natural product. Direct on-flow ^{1}H

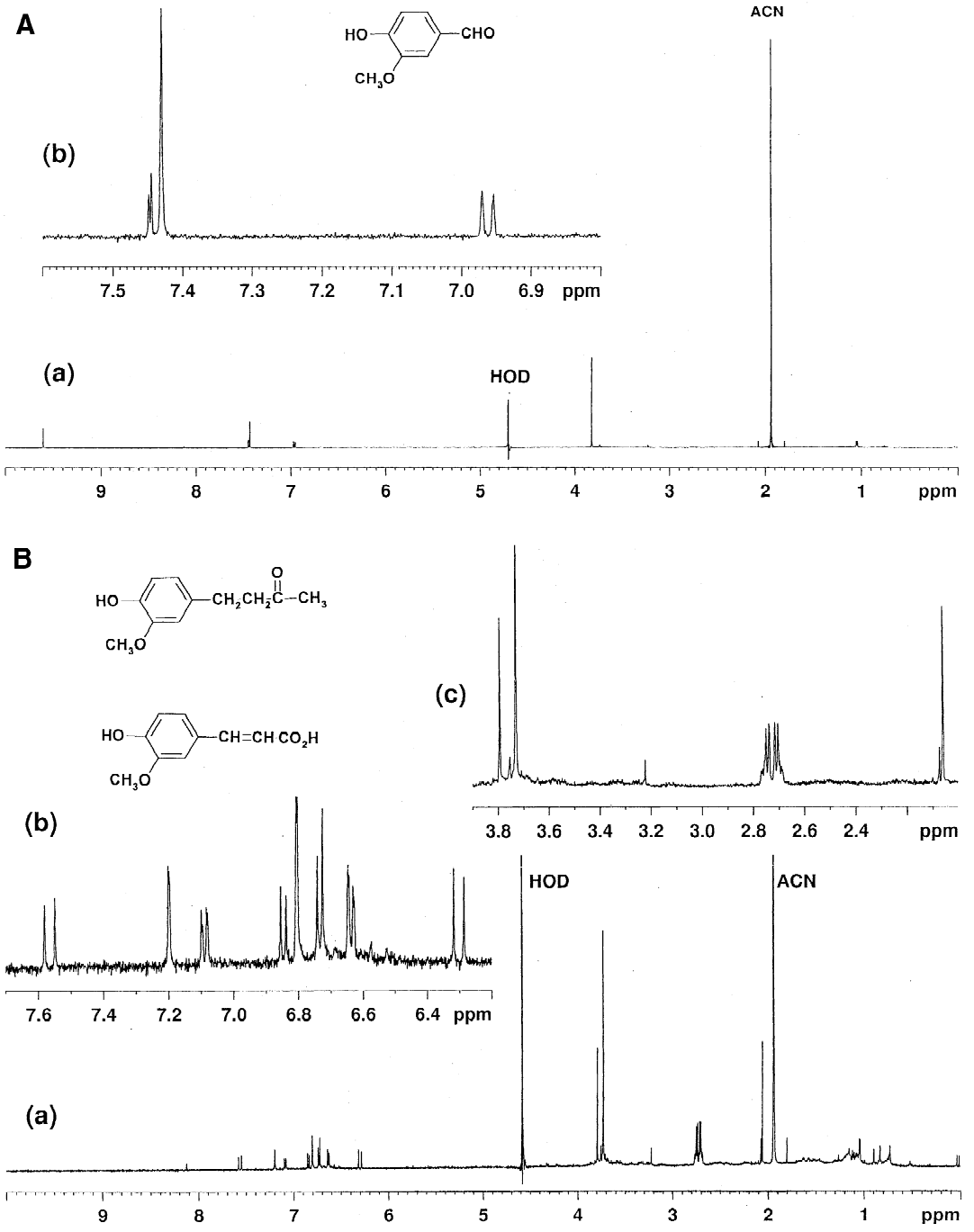

Fig. 5. Superheated water chromatography–500 MHz ^{1}H stop-flow NMR spectra obtained in off-line mode (following loop-collection); separation conditions as Fig. 4. (a) Peak 1 identified as vanillin. Key: a = the whole spectral range between 10 and 0 ppm; b = expansion of region between 7.6 and 6.8 ppm. (b) Peak 3 identified as a mixture of zingerone and ferulic acid. Key: a = the full spectral width of 10–0 ppm; b = expansion of the spectral range between 7.7 and 6.2 ppm; c = expansion of the spectral range between 3.9 and 2.0 ppm [HOD = residual water in D_2O; ACN = acetonitrile contamination from HPLC pump from previous analyses].

NMR analysis enabled the identification of dihydroferulic acid from ginger, however, loop collection was found to be the best method for obtaining NMR spectra of the less abundant components present in the extract. The stop-flow analyses provided spectra with good signal to noise and, hence, enabled structural identification of the additional residual components, vanillin, ferulic acid and zingerone.

Acknowledgements

The authors thanks the Government of Bangladesh for a studentship to SS and to the EPSRC for a research grant.

References

[1] J.L. Wolfender, K. Ndjoko, K. Hostettmann, Phytochem. Anal. 12 (2001) 2.
[2] R.M. Smith, R.J. Burgess, Anal. Commun. 33 (1996) 327.
[3] R.M. Smith, R.J. Burgess, J. Chromatogr. A 785 (1997) 49.
[4] R.M. Smith, R.J. Burgess, O. Chienthavorn, J.R. Stuttard, LC–GC Int. 12 (1999) 30.
[5] S.M. Fields, C.Q. Ye, D.D. Zhang, B.R. Branch, X.J. Zhang, N. Okafo, J. Chromatogr. A 913 (2001) 197.
[6] R.M. Smith, O. Chienthavorn, I.D. Wilson, B. Wright, Anal. Commun. 35 (1998) 261.
[7] R.M. Smith, O. Chienthavorn, I.D. Wilson, B. Wright, S.D. Taylor, Anal. Chem. 71 (1999) 4493.
[8] R.M. Smith, O. Chienthavorn, S. Saha, I.D. Wilson, B. Wright, S.D. Taylor, J. Chromatogr. A 886 (2000) 289.
[9] D. Louden, A. Handley, S. Taylor, I. Sinclair, E. Lenz, I.D. Wilson, Analyst 126 (2001) 1625.
[10] D. Louden, A. Handley, R. Lafont, S. Taylor, I. Sinclair, E. Lenz, T. Orton, I.D. Wilson, Anal. Chem. 74 (2002) 288.
[11] D.W. Connel, R. McLachlan, J. Chromatogr. 67 (1972) 29.
[12] D.J. Harvey, J. Chromatogr. 212 (1981) 75.
[13] R.M. Smith, Chromatographia 16 (1982) 155.
[14] C.-C. Chen, R. Rosen, C.-T. Ho, J. Chromatogr. 360 (1986) 163.
[15] C.-C. Chen, M.-C. Kuo, C.-T. Ho, J. Food Sci. 51 (1986) 1364.
[16] X.-G. He, M.W. Bernart, L.-Z. Lian, L.-Z. Lin, J. Chromatogr. A 796 (1998) 327.
[17] I.D. Wilson, Chromatographia 52 (2000) S28.
[18] C. Dunlap, C. McNeff, D. Stoll, P. Carr, Anal. Chem. 73 (2001) 598A.
[19] P.T. Jackson, P.W. Carr, J. Chromatogr. A 958 (2002) 121.
[20] T. Masada, K. Hidaka, A. Shinohara, T. Maekawa, Y. Takeda, H. Yamaguchi, J. Agric. Food Chem. 47 (1999) 71.
[21] P. Denniff, D.A. Whiting, J. Chem. Soc. Chem. Commum. (1976) 711.
[22] I. Macleod, D.A. Whiting, J. Chem. Soc. Chem. Commun. (1979) 1152.