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Development of a triple hyphenated HPLC–radical scavenging detection–DAD–SPE–NMR system for the rapid identification of antioxidants in complex plant extracts

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Abstract

A rapid method for the simultaneous detection and identification of radical scavenging compounds in plant extracts was developed by combining an HPLC with on-line radical scavenging using DPPH• as a model radical and an HPLC–DAD–SPE–NMR system. Using this method a commercial rosemary extract was investigated. All major compounds present in the extract were collected on SPE cartridges after their separation. Advantages of on-line SPE peak trapping are the possibility to perform HPLC with non-deuterated solvents, a concentration effect and being able to record NMR spectra in pure 100% deuterated solvents. After comparing DAD and DPPH scavenging chromatograms, ^{1}H NMR spectra of compounds having radical scavenging activities were recorded. Afterwards all compounds were collected and infused into an ESI-MS. The five main active compounds – carnosol, carnosic acid carnosaldehyde, 12-methoxycarnosic acid and epiisorosmanol – could be identified from the combined UV, NMR and mass spectral data without actually isolating them. It was possible to record on-line an HMBC spectrum of carnosic acid. Also one compound was tentatively identified as epirosmanol methyl ether.

Keywords: Rosemary; Radical scavengers; On-line detection; Identification; LC–NMR

1. Introduction

There is more and more evidence that oxidised lipids could have negative health implications, for instance in the development of coronary heart disease, carcinogenesis and even ageing [1]. To retard the oxidation process and prolong the shelf life of food containing (multiple) unsaturated fats, antioxidants are frequently added. Purified antioxidative extracts from rosemary leaves have achieved widespread commercial application as a food additive [2–13]. In some products rosemary extracts gave results similar to those of synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole BHA [1]. Crude rosemary extracts possess a green colour and a rather strong odour. Therefore, usually processed extracts are used which are more neutral with regard to colour, taste and smell. In order to avoid losing antioxidants during the processing, knowledge on the activity and identity of individual antioxidants is essential. Thus much work has been carried out on the chemical composition of rosemary extracts [14–21]. Compounds responsible for the antioxidant properties are rosmarinic acid and phenolic diterpenes, such as carnosol, carnosic acid, rosmanol, epirosmanol and isorosmanol [7,20].

Obtaining information on the antioxidant activity and identity of individual constituents in complex plant extracts, such as rosemary, is normally a time-consuming task. Each compound has to be purified to homogeneity and then its activity and structure can be determined with off-line methods. Recently a technique has become available to measure the radical scavenging activity of individual compounds on-line when they elute from an HPLC column [22–24]. As all of

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the more powerful natural antioxidants are also radical scavengers, this technique makes it possible to directly identify active constituents. On-line spectroscopic methods like LC–UV and LC–MS are very sensitive and useful methods but for the exact identification of more complex natural products NMR remains frequently necessary. Therefore, LC–NMR is a logical development, which is gaining rapidly popularity even though sensitivity remains a problem [25–27]. To gain sensitivity, on-line solid phase (SPE) extraction is now available for focussing an analyte peak prior to introduction into the NMR measuring cell [28,29]. This can be done fully automatically without interruption of the column flow. An additional advantage of this system is that after drying the cartridge, analytes can be measured in fully deuterated solvents and HPLC solvents do not need to deuterated. To further increase sensitivity cryogenic flow probes are now being used. They can be combined with the SPE unit could [30]. In this paper we have coupled LC–DAD–radical scavenging detection (RSD) with LC–SPE–NMR. This hyphenated set-up provides in a single run retention times, radical scavenging activity, UV data and NMR data of individual peaks.

2. Experimental

2.1. Materials and chemicals

Rosemary extract RBT 255 (Robertet, Grasse, France) was used as sample. All solvents used for chromatography were of HPLC grade (Lab-Scan Analytical Sciences Ltd., Dublin, Ireland). Ultra pure water ($0.05\,\mu S\,cm^{-1}$) was obtained from a combined Seradest LFM 20 and Seralpur Pro 90 C apparatus (Seral, Ransbach-Baumbach, Germany). The following reagents and compounds were used: 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$, 95%), (trimethylsilyl)-diazomethane (2.0 M solution in hexane), carnosic acid, deuterated methanol and deuterated chloroform from Sigma–Aldrich Chemie (Steinheim, Germany) and ammonium acetate from Fluka Chemie (Buchs, Switzerland).

2.2. Sample preparation

The rosemary extract (0.5 g) was dissolved in 25 ml of hexane and successively extracted with five 10 mL portions of methanol–water (9:1). The five aqueous methanolic layers were combined and the solvent was removed in vacuum with a rotary evaporator. 0.058 g of dry material was obtained. This was dissolved in methanol at a concentration of 4% (w/v).

2.3. HPLC–RSD–DAD–SPE–NMR conditions and instrumental setup

Separation, radical scavenging detection (RSD), UV detection and recording of NMR spectra were carried out with the system schematically represented in Fig. 1. The linear binary gradient was formed with an LC 22 pump equipped with LC 225 gradient former (Bruker BioSpin GmbH, Rheinstetten, Germany), at a constant flow rate of $0.8\,mL\,min^{-1}$. Solvent A = 0.1% TFA, 1.0% acetonitrile, 97.9% water; solvent B = acetonitrile. At $t = 0$ min A = 70%, $t = 15$ min A = 50%, $t = 50$ min A = 45%, $t = 55$ min A = 35%, $t = 75$ min A = 10%, $t = 80$ min A = 0%, $t = 85$ min A = 0%, $t = 90$ min A = 70%. Analytes were injected with a Rheodyne model 7125 manual injector (Rheodyne, Rohnert Park, CA) equipped with a 100 μL injection loop (4 mg injected on column) and separated on an Alltima C18 5 μm analytical column (15 cm × 4.6 mm i.d. Alltech, Deerfield, IL). The compounds eluted from the column were split into two streams using an adjustable high-pressure stream splitter (Supelco Port, Bellefonte, PA). One part ($0.6\,mL\,min^{-1}$) went to a Bruker DAD detector (Bruker, Rheinstetten, Germany) operating at monitoring wavelengths of λ = 235 and 280 nm. After the detector, make-up water at a flow rate of $0.4\,mL\,min^{-1}$ was added to the eluent stream with a Knauer K-120 pump (Knauer, Berlin, Germany). This combined stream was entering an SPE unit Prospekt 2 (Spark Holland, Emmen, The Netherlands), where compounds, detected on the DAD, were collected on 10 mm × 2 mm cartridges with HySphere Resin SH 15–25 μm sorbent (Spark Holland, Emmen, The Netherlands). The other part of the column flow ($0.2\,mL\,min^{-1}$) was used for the radical scavenging detection. For this purpose 10^{-4} M DPPH solution in methanol, buffered with ammonium acetate (5×10^{-3} M) was added at a flow rate $0.4\,mL\,min^{-1}$ with a 50 mL syringe pump (laboratory made; Free University, Amsterdam, The Netherlands). After the addition of DPPH solution, the mixture passed a reaction coil made of peek tubing (4.4 m × 0.25 mm). The decrease of absorbance after the reaction was monitored with a 759A model visible light detector (Applied Biosystems, Foster City, CA) equipped with a tungsten lamp. Compounds having radical scavenging activities were detected as negative peaks in the RSD chromatogram. All SPE cartridges with trapped compounds were subsequently dried with nitrogen (30 min, at 0.5 MPa, at room temperature). Afterwards compounds having radical scavenging activity were transferred from the Prospekt 2 system to a Bruker DPX 400 spectrometer equipped with a 120 μl NMR flow probe (Bruker, Rheinstetten, Germany) with approximately 550 μl of deuterated chloroform or methanol.

NMR spectra were recorded at a probe temperature of 25 °C. Chemical shifts were expressed in ppm relative to internal methanol: 3.34 ppm for ^{1}H (or chloroform: 7.26 ppm for ^{1}H and 77.1 ppm for ^{13}C). The 1D ^{1}H proton spectra were recorded at 400.13 MHz. For the 2D HMBC spectrum a standard gradient enhanced 2D-HMQC pulse sequence delivered by Bruker was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 53 ms. For the HMBC experiment 1024 experiments of 2048 data points were performed with 128 scans per increment.

A 759A model UV detector (Applied Biosystems, Foster City, CA) was used to monitor the rosmarinic acid content

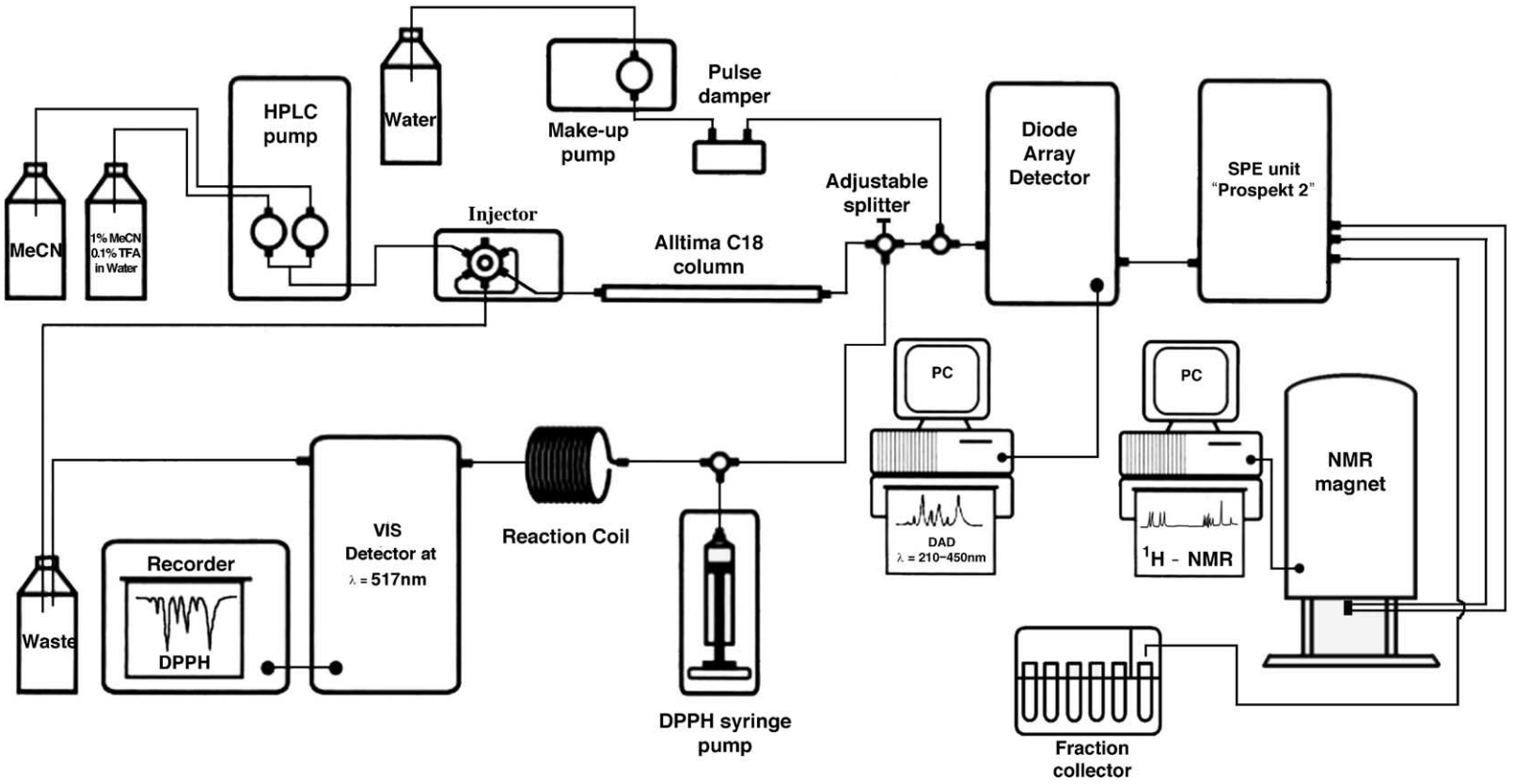

Fig. 1. Schematic HPLC–RSD–DAD–SPE–NMR instrumental set-up.

coming out of the SPE cartridges during method optimization. The in-line pulse damper (toroid mixer) connected to the make-up water pump was from Scientific Systems (State College, PA).

3. Results and discussion

The first parameters that were optimised were the different flows through the system. The total flow rate through the SPE unit should preferably not exceed 1.5 mL min^{-1}. Higher flow rates cause high back pressures in SPE cartridges and increase the possibility of damaging the UV cell. As the ratio between the make-up water flow rate necessary to reduce the eluent strength and the flow coming from the HPLC column is suggested as 4–1, frequently 2.1 mm i.d. columns and a flow rate of 0.2 mL min^{-1} of HPLC eluent are used. Then, the total flow going through the cartridge is 1 mL min^{-1}.

Since in our set-up (Fig. 1) part of the eluent was directed to the RSD reaction coil, it was necessary to increase the total amount of loaded sample and therefore a 4.6 mm i.d. column was used. The compound separation conditions were optimised for a flow rate of 0.8 mL min^{-1} [22]. The greater part of the flow was directed to the SPE unit, since RSD is much more sensitive than NMR detection. Under these conditions it was impossible to add make-up water at the suggested 4:1 ratio as the total flow rate had to be kept under 1.5 mL min^{-1}. So it was attempted to lower the flow rate of make-up water used for trapping the analytes.

The trapping abilities of the cartridges filled with highly non-polar polymeric stationary phase were tested at several different flow ratios using rosmarinic acid as a model antioxidant compound. This compound was chosen because of its relatively high polarity. The more polar a compound is, the more difficult it is to trap. As HPLC eluent 20% acetonitrile in water was used. The flow rate from the HPLC system was set to 0.6 mL min^{-1} and several make-up water flow rates (0.2, 0.4 and 0.8 mL min^{-1}) were chosen. A UV detector connected to the outlet of the cartridges was used to monitor any rosmarinic acid breakthrough. A make-up water flow rate of 0.4 mL min^{-1} was found to be sufficient for trapping rosmarinic acid into a cartridge for about 2 min and gave an acceptable backpressure. Since the make-up water

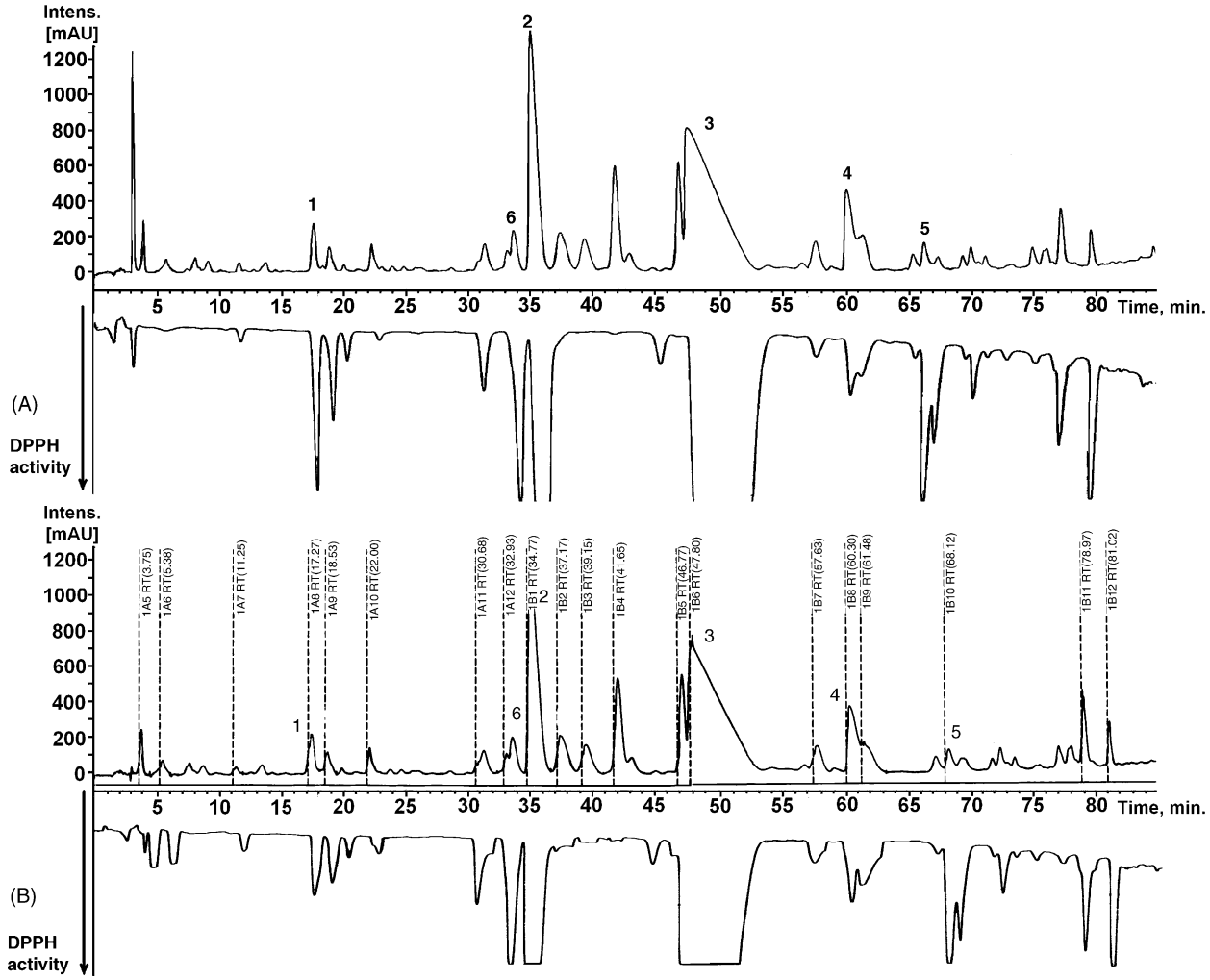

Fig. 2. HPLC UV and RSD profiles of rosemary extract: (A) no trapping performed and (B) compounds are trapped on cartridges.

pump gave relatively high pulsations disturbing the baseline in both the UV and RSD chromatograms, a pulse damper was connected to the make-up water stream.

Every SPE cartridge gives a slightly different backpressure, causing changes in flow rates and split ratios during the peak trapping process. When a frequently used cartridge was in line, the flow through the cartridge decreased and more eluent from the HPLC column was passing through the DPPH reaction coil. Because of this, DPPH solution at the moment of trapping was diluted and baseline stability was disturbed. Sometimes, because of the lower flow rates through the cartridge, not enough compound was trapped for recording an NMR spectrum. To ensure stable flow rates during the entire separation and compound collection process, an additional backpressure consisting of a piece of 13.5 m × 0.25 mm i.d. peek tubing was connected to the exit of the SPE unit. This significantly reduced base line disturbances and trapping problems during the time the cartridge was connected to the system.

UV and RSD chromatograms are shown in Fig. 2. Fig. 2A and B are identical except for the trapping effect of the trapping procedure on the baseline. In Fig. 2A no peaks were trapped. It shows that the trapping can be performed without markedly affecting peak resolution or peak intensity of both the UV and RSD signal. Only in the very beginning of the trapping chromatogram (Fig. 2B) two artificial peaks at 4 and 6 min, respectively, can be observed. All major (marked in Fig. 2B by the retention time and cartridge number) peaks appearing in the UV chromatogram were collected on SPE cartridges. After drying with nitrogen, compounds with radical scavenging activity were delivered to a 120 μL LC–NMR flow cell with fully deuterated solvents and ^{1}H NMR spectra were recorded. Because of the large sample size (4 mg) needed for LC–NMR, the major compound in the extract is clearly overloaded without however adversely affecting the separation of the other constituents.

Sufficient amounts of compounds were trapped into cartridges 1A8 (**1**), 1B1 (**2**), 1B6 (**3**), 1B8 (**4**), 1B10 (**5**), and 1A12 (**6**), to allow the recording of enough NMR data for their structure elucidation. In some cases two-dimensional proton NMR data (TOCSY and COSY) could be collected and one injection was sufficient to record an HMBC spectrum of the major compound.

The NMR spectrum of the most polar radical scavenging compound identified (trap 1A8) showed two methyl singlets at 0.90 and 1.02 ppm, a doublet integrating for six protons at 1.21 ppm, a 1H septet at 3.28 ppm and a 1H singlet at 6.77 ppm. This combination of signals is characteristic of a carnosic acid type diterpene. After pumping the pure compound out of the LC–NMR probe, evaporating the deuterated solvent and redissolving in methanol, infusion ESI-MS measurements in negative mode showed a pseudomolecular ion $[M-H]^-$ at *m/z* 345. This corresponded to a MW of 346 amu, i.e. a rosmanol isomer. The UV absorption maximum at 288 nm and a peak shoulder at about 225 nm were in accordance with the data presented by Cuvelier et al. [5]. A doublet of doublets at 4.30 ppm (J_1 = 4.3 Hz, J_2 = 4.2 Hz) coupled with a doublet at 5.13 ppm (J = 4.3 Hz), and a doublet at 1.38 ppm (J = 4.2 Hz) indicated that the compound was epiisorosmanol **1** [20]. In the case of rosmanol and epirosmanol no couplings between H-5 and H-6 should be observed because of the near 90° angle between them [20]. The completely assigned ^{1}H NMR spectrum of epiisorosmanol **1** is presented in Table 1.

Table 1
^{1}H NMR spectral data of identified compounds

H #	Epiisorosmanol **1** mult. (*J*, Hz)	Carnosol **2** mult. (*J*, Hz)	Carnosic acid **3** mult. (*J*, Hz)	12-Methoxy-carnosic acid **4** mult. (*J*, Hz)	Carnosaldehyde **5** mult. (*J*, Hz)
1α	2.78 d (14.4)	2.80 d (14.3)	1.12 m	1.10 ddd (12.9, 12.2, 4.4)	1.11 m[a]
1β	2.57 ddd (4.5, 13.3)	2.57 ddd (4.4, 14.1)	3.53 ddd (13.8, 3.4, 3.4)	3.64 m	3.57 d (13.4)
2α	1.87 m	1.89 m	2.08 d (13.0)	2.27 m	∼1.95–2.05 m[a]
2β	1.59 m	1.62 dt (13.7, 4.9)	1.5–1.6 m	1.53 m	1.45–1.65 m
3α	1.50 d (12.3)	1.51 d (13.1)	1.5–1.6 m	1.53 m	1.45–1.65 m
3β	1.31 m	1.32 ddd (13.5, 13.3, 3.1)	1.33 ddd (13.1, 13.4, 4.3)	1.32 m	∼1.22–1.45 m[a]
5	1.38 d (4.2)	1.69 dd (10.6, 5.7)	1.5–1.6 m	1.53 m	1.80 d (13.4)
6α	–	1.84 m	1.82 bd (13.3)	1.82 bd (12.0)	2.29 m
6β	4.30 dd (4.3, 4.2)	2.20 m	2.37 m	2.27 m	2.85 m
7α	5.13 d (4.3)	5.43 d (2.8)	2.78 m	2.80 m	1.45–1.65 m
7β	–	–	2.78 m	2.80 m	2.85 m
14	6.77 s	6.69 s	6.45 s	6.47 s	6.52 s
15	3.28 m	3.25 m	3.18 m	3.18 m	3.19 m
16	1.21 d (6.7)	1.20 d (6.7)	1.16 d (7.0)	1.17[a] d, (7.1)	1.17 d (7.0)
17	1.20 d (6.8)	1.19 d (6.6)	1.18 d (7.3)	1.19[a] d, (7.3)	1.18 d (6.8)
18	1.02 s	0.87 s	0.99 s	0.99 s	1.02 s
19	0.90 s	0.87 s	0.92 s	0.91 s	0.85 s
20	–	–	–	3.66 s	9.97 s
Solvent	CD_3OD				
NS	982	104	40	144	704

NS: Number of scans used for recording spectrum.
[a] Exact peak positions not clear due to peak overlap.

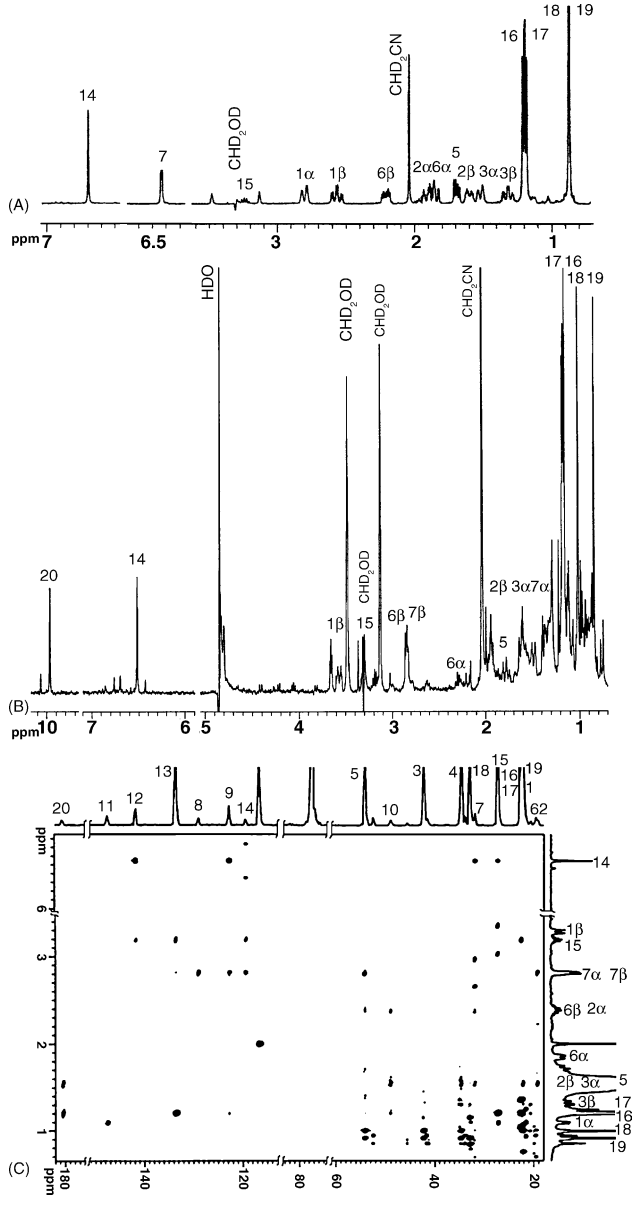

Fig. 3. (A) ^{1}H NMR spectrum of carnosol **2**; (B) ^{1}H NMR spectrum of carnosaldehyde **5** and (C) HMBC spectrum of carnosic acid **3**.

Similar to **1**, compound **2** (trap 1B1) showed signals characteristic of a carnosic acid type skeleton (Fig. 3A). However, in contrast to rosmanol type of compounds and carnosic acid, only one signal between 4 and 5.5 ppm was present. A comparison with literature NMR data [18,31] allowed the identification of **2** as carnosol, after carnosic acid the second most important antioxidant in rosemary extracts. Its ^{1}H NMR spectral data can be found in Table 1. All couplings in the COSY spectrum were in accordance with a carnosol structure. Mass spectral (pseudomolecular ions at *m*/*z* 331 $[M+H]^+$ and *m*/*z* 329 $[M-H]^-$ in positive ion (PI) and negative ion (NI) mode infusion ESI-MS, respectively) and UV data (λ_{max} 283 nm) further confirmed the assignment.

The overloaded compound in cartridge 1B6 was suspected to be carnosic acid, the main antioxidative compound in rosemary extracts. The ^{1}H NMR spectral data of **3** corresponded well with literature NMR data of carnosic acid [16,31]. Enough of **3** was collected to record an HMBC spectrum. Although not all H–C interactions were present in the spectrum, the two-dimensional spectrum clearly substantiated the structure of carnosic acid. The ^{13}C NMR shifts, obtained from the HMBC correlated well with the ones found in literature [6]. For instance the characteristic multiplet at 3.18 ppm assigned to H-15 had a correlation with the carbon signal at 27.2 ppm, which in turn showed cross peaks with the H-16 and H-17 methyl groups. This indicates an isopropyl side chain. Its position was proven by couplings between H-15 and C-14, and H-14 and C-15. Couplings of H-18 and H-19 with C-3, -4 and -5 confirmed that these methyl groups are both attached to C-4. Cross peaks of H-1 and H-2 with the carbonyl carbon also corresponded with the structure of carnosic acid. However, a mismatch of our shift for C-1 with the literature data was observed. A single bond coupling of H-1 with C-1 clearly indicated that C-1 was at 22.1 ppm (instead of 34–36 ppm given in literature [6,16]). The ^{13}C NMR shifts obtained from the HMBC spectrum of **3** were as follows: 19.2 (C-2); 19.5 (C-6); 22.1 (C-1, 19); 22.5 (C-16, 17); 27.2 (C-15); 31.9 (C-7); 33.0 (C-18); 34.5 (C-4); 42.4 (C-3); 48.6 (C-10); 54.1 (C-5); 119.5 (C-14); 122.8 (C-9); 129.1 (C-8); 133.4 (C-13); 142.2 (C-12); 147.5 (C-11); 180.5 (C-20). The HMBC spectrum of carnosic acid is given in Fig. 3C. Couplings obtained from COSY also corresponded with all proton assignments. Finally the ESI-MS measurements in negative mode (MW 332 amu) and the UV spectrum (λ_{max} 284 nm [31]) fully confirmed the prior identification on the basis of NMR.

The ^{1}H NMR shifts of compound **4** (Table 1) were very similar to those of carnosic acid **3**, i.e. no signals between 4 and 5.5 ppm. However, an additional singlet at 3.66 ppm, integrating for three protons, suggested the presence of a methoxy group. ESI-MS confirmed this as measurements in both PI and NI mode gave pseudomolecular ions $[M-H]^-$ at *m*/*z* 345 and $[M+H]^+$ at *m*/*z* 347 corresponding with the MW of 346 amu of methylated carnosic acid. The UV spectrum (λ_{max} at 225 and 282 nm) was identical with the literature values of carnosic acid methyl ester [31]. However, it was not possible to determine if the compound was really carnosic acid methyl ester, based only on molecular mass and UV data. To confirm that **4** was carnosic acid methyl ester methylation of carnosic acid was performed, as described by Hashimoto et al. [32]. Methyl carnosate, obtained after methylation was investigated with LC–MS under the same gradient as described for LC–NMR. Although the retention time of carnosic acid methyl ester was very close to that of **4** present in the extract, the MS spectrum was different. The major fragment in positive mode for carnosic acid methyl ester was the same as for carnosic acid (*m*/*z* = 287 amu), but the major fragment in the mass spectrum of **4** was 301 amu. This means that the methyl group is not split off with the loss of formic acid, so

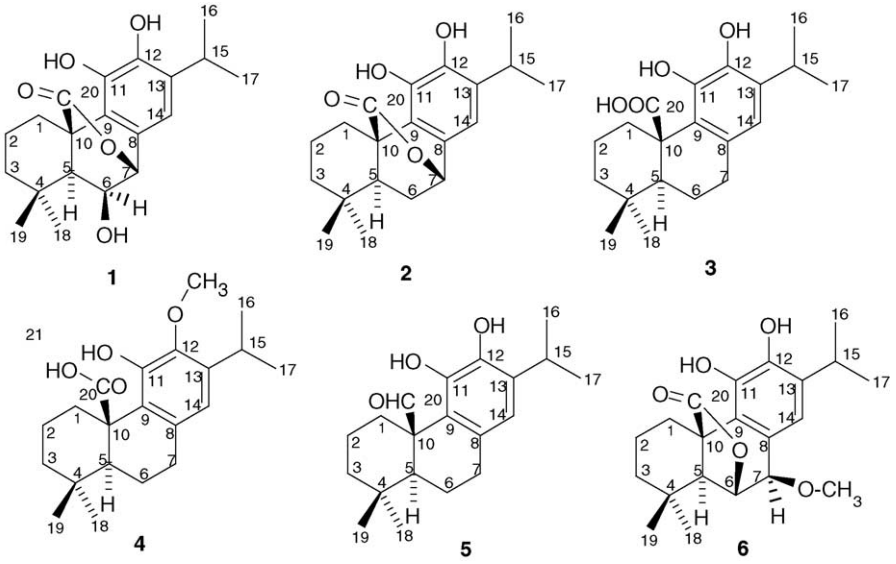

Fig. 4. Structural formulas of compounds **1**–**6**.

it should be attached to one of the hydroxyls. The chemical shift of the methoxy group in the NMR spectrum, recorded for **4** was identical to that of 12-methoxycarnosic acid [6,33]. Also chemical shift changes of 1β, 2α and 6α of **4** comparing with carnosic acid were identical to literature data [6]. Taking this into account compound **4** was identified as 12-methoxycarnosic acid. It should be noted, that assignments of 6α and 6β protons found in literature [6] are not correct. Since 6β has three large diaxial couplings, it is impossible that it appears as a broad doublet. Also the NMR spectrum of compound **5** (Fig. 3C) was almost identical to the one of carnosic acid **3**, except for a 1H singlet at 9.96 ppm, which could be explained by the presence of an aldehyde group. ESI-MS in NI mode suggested a MW of 316 amu corresponding to the replacement of the carboxylic acid group of carnosic acid by an aldehyde group. This compound has not yet been described in literature. Closely related aldehydic compounds are euphracal (11,12,15-trihydroxyabieta-8,11,13-trien-20-al) the aldehydic proton of which has a shift of 9.79 ppm in $CDCl_3$ [34] and 11,12,16-trihydroxyabieta-8,11,13-trien-20-al which has its aldehydic proton at 9.92 ppm in $CDCl_3$ [35]. The UV data was in correspondence with the one described for euphracal (λ_{max} 230 and 271 nm). Thus compound **5** was identified as carnosaldehyde (Fig. 4).

The proton NMR spectrum of compound **6** (trapped on a cartridge 1A12) showed two methyl singlets at 0.88 and 0.93 ppm, and a 3H double doublet at 1.18 ppm. Together with an aromatic proton signal at 6.77 ppm, these are characteristic for an aromatic diterpene skeleton. A signal at 3.66 ppm showed the presence of methoxy group. Unfortunately due to the small amount present the S/N ratio of the spectrum was rather low and not all signals could be clearly observed. However, a doublet (J = 3.2 Hz) at 4.28 ppm was clearly visible. ESI-MS gave a pseudomolecular ion peak at m/z 359 in negative mode (i.e. MW = 360) and a major fragment at m/z 283. This suggested the structure of epirosmanol methyl ether [5]. For this compound an additional doublet at 4.81 ppm [16] coupled with the signal at 4.28 ppm should be observed. Unfortunately the residual water signal overlapped the characteristic signal at 4.8 ppm. Taking into account all collected data this compound was tentatively identified as epirosmanol methyl ether **6**. It cannot be excluded that this compound is an artefact formed from epirosmanol and methanol which was used for dissolving the extract.

The injection of rosemary extract was repeated four times and each time the same compounds were trapped. In each case the NMR measurements gave the same spectrum, so it can be stated that the method is reproducible enough to perform simultaneous detection and identification of radical scavenging compounds in rather complex extracts.

4. Conclusions

A triple hyphenated HPLC–radical scavenging detection–DAD–SPE–NMR system was developed for the rapid identification of antioxidants in complex plant extracts. The SPE unit allowed temporary peak parking without peak broadening. Thus it was possible to first assess which peaks possessed radical scavenging and then at a later stage to measure only those compounds with NMR that showed activity. Additional advantages of on-line SPE–NMR relative to stop-flow or loop-storage LC–NMR is that normal non-deuterated solvents can be used for the HPLC separation and that spectra of trapped compounds can be recorded in fully deuterated solvents. Thus less solvent

suppression techniques are necessary and spectra are easier to compare with literature NMR data.

Analysis of a commercial rosemary extract with this set-up showed that it was possible to identify a significant number of constituents without having access to reference compounds and without prior isolation. The NMR spectra were decisive for the correct identification of some closely related compounds with the same mass, e.g. in the case of epi-isorosmanol. Molecular weight information could be simply obtained by infusion ESI-MS of the pure LC–NMR samples. Thus this method greatly improves and speeds up the identification of antioxidants, since it eliminates compound purification and activity assays of individual compounds, normally a very laborious task. Also, using this method, problems of compound degradation, occurring with intrinsically labile compounds such as antioxidants and the introduction of impurities prior to the NMR measurements are avoided. This method could be useful for monitoring the quality of antioxidative extracts, since it shows not only changes in antioxidant activity, but also in chemical composition of the extract. This enables to determine what could influence the quality of an extract (e.g. oxidation, hydrolysis, etc.).

In spite of the fact that it was feasible to record an HMBC spectrum of the main constituent, a disadvantage of the method remains the relatively poor sensitivity. For instance we were not able to record an intelligible NMR spectrum of the peak at 81 min that shows potent radical scavenging activity. To further increase the sensitivity of the NMR part of this technique several options are available: (1) changing the NMR flow cell from 120 to 30 μL, because in the SPE unit all trapped compounds are dried and then eluted with the first 40 μL of eluent; (2) using stronger magnets or a cryoprobe system; (3) using multiple trapping of peaks on the same cartridge after repeated injections. As our method is well reproducible this can certainly be done albeit at the expense of time; (4) using the LC–NMR option without simultaneous radical scavenging detection which effectively means that 30% more compound ends up in the LC–NMR probe; (5) prior enrichment of minor compounds of interest, e.g. by removing carnosic acid by partitioning or preparative HPLC. We are currently pursuing several of these possibilities.

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