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An on-line normal-phase high performance liquid chromatography method for the rapid detection of radical scavengers in non-polar food matrixes

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ABSTRACT

An on-line method for the rapid pinpointing of radical scavengers in non-polar mixtures like vegetable oils was developed. To avoid problems with dissolving the sample, normal-phase chromatography on bare silica gel was used with mixtures of hexane and methyl *tert*-butyl ether as the eluent. The high performance liquid chromatography-separated analytes are mixed post-column with a solution of stable free radicals in hexane. Reduced levels of the radical as a result of a reaction with a radical scavenger are detected as negative peaks by an absorbance detector. After investigating a number of different reagents, solvents, concentrations and solution flow rates an optimized instrumental set-up incorporating a superloop for pulse-free delivery of the reagent solution is presented. Both 2,2'-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) and 2,6-di-*tert*-butyl-α-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyloxy (galvinoxyl$^{\bullet}$) were used as stable free radicals. The method is suitable for both isocratic and gradient HPLC operation. The method has a simple experimental protocol, uses standard instruments and inexpensive and stable reagents, and accepts any hexane-soluble sample. It can also be used for semi-quantitative analysis. The method was applied to several pure, non-polar natural antioxidants, vegetable oils and lipid-soluble rosemary extract. The limits of detection varied from 0.2 to 176 μg/ml, depending on the compound tested.

1. Introduction

One of the causes of food spoilage is oxidation, a radical initiated reaction of oxygen with one or more of the ingredients of the food product. These radical chain reactions lead to bad taste, malodour, unpleasant appearance, colour changes, and decrease of nutritional value [1,2]. This is especially true for foods containing fats, like unsaturated vegetable oils. The shelf life of foods can be prolonged by the presence or addition of antioxidants [3–6]. Radical scavengers are an important class of antioxidants and can be present in foods as intrinsic constituents (vitamin C, vitamin E, flavonoids, tyrosol, rosmarinic acid) or can be added artificially (BHT, rosemary extract) [7]. Many assays for the assessment of radical-scavenging activity have been developed and compared [8–12]. Unfortunately such off-line assays only give the total activity and provide no information about the individual activity of the constituents in complex mixtures. In these assays both the short-lived radicals actually causing food deterioration (e.g. reactive oxygen species, superoxide anion radical, hydroxyl radical, peroxyl radicals) and more stable model radicals (mainly DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) are used [13,14]. Stable radicals have the advantage of commercial availability and easiness of handling. Both react within seconds with common antioxidants like ascorbic acid, tocopherol, quercetin, Trolox and carnosic acid yielding the reduced form of the radical and an oxidised phenol. As the reduced forms of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ absorb light at much lower wavelengths than the original radical, this allows a facile spectroscopic detection of any reaction. DPPH$^{\bullet}$ has been used as a spraying agent for the detection of antioxidants on TLC plates and this provided for the first time information on individual constituents. Koleva et al. [15,16] and Dapkevicius et al. [17] "translated" the TLC procedure to an on-line HPLC application. This approach has met considerable success and led to about 40 papers in this field by various groups [18]. For a review, see Niederländer et al. [19]. All used reversed-phase RP-HPLC, which unfortunately makes the analysis of highly non-polar samples such as fatty oils difficult because these samples generally have a very limited solubility in reversed-phase eluents. To analyse antioxidants like tocopherols in oil, both normal phase (NP) [20,21] and reversed-phase HPLC [22,23] have been used. Normal-phase HPLC provides better separation results [20]. Thus for such samples a normal-phase system appears more attractive as the sample can be dissolved in hexane and directly injected without any further sample pretreatment. In this contribution we report on the development and use of an on-line normal-phase

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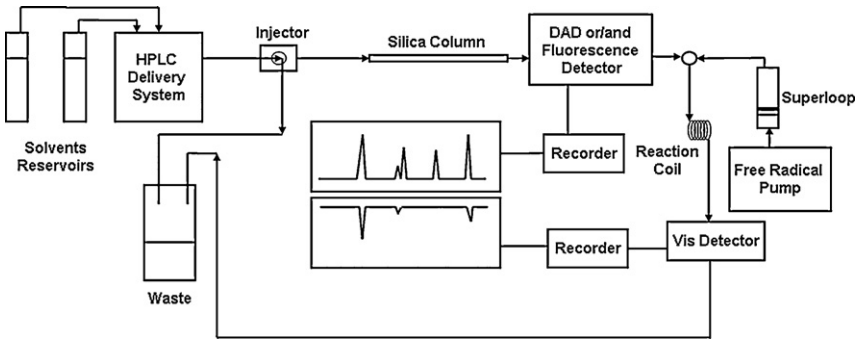

Fig. 1. Instrumental set-up for normal-phase HPLC with on-line detection of radical-scavenging compounds.

(NP)-HPLC method making use of relatively stable model radicals. The method is applied for the detection and, if possible, identification of antioxidants in various non-polar food matrixes.

2. Experimental

2.1. Solvents and chemicals

All solvents used were of HPLC grade (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Before use in the HPLC system, all sample solutions and solvents were membrane-filtered (0.45 μm, type RC 55; Schleicher & Schuell; Dassel, Germany). All solvents used were purged with nitrogen for at least 20 min before use.

The following reagents and compounds were used: 2,6-di-*tert*-butyl-α-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyloxy (galvinoxyl•), 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH•), 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), tris(4-bromophenyl)ammoniumyl hexachloroantimonate, eugenol (99%, GC), isoeugenol (98%), α-tocopherol (95%), all from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); 2,6-di-*tert*-butyl-4-methylphenol (BHT, p.a.) from Fluka (Buchs, Switzerland); carnosic acid from Extrasynthese (Genay, France). Wheat germ oil was purchased in the Netherlands; olive oil was produced in France; oil-soluble rosemary extract was from Robertet (Grasse, France).

DPPH• and galvinoxyl• solutions were freshly prepared every 2 days and kept at 4 °C, protected from light.

For the determination of the minimum detectable concentration (MDC), eight to ten different dilutions of each antioxidant in n-hexane or a mixture of n-hexane and *tert*-butyl methyl ether (MTBE) were prepared and sequentially injected under the following isocratic conditions: 100% n-hexane (BHT); n-hexane - MTBE (90:10, v:v) (α-tocopherol); n-hexane - MTBE (60:40, v:v) (isoeugenol, eugenol, carnosol); 100% MTBE (carnosic acid). Vegetable oils and rosemary extract solutions were prepared in hexane (10 mg/ml).

2.2. Instrumental set-up and operating conditions

The instrumental set-up is presented in Fig. 1. The HPLC-radical-scavenging system consisted of the following: an HP 1050 HPLC system (including an HPLC pump; an autosampler; a programmable diode array detector (data were processed using HP Chemstation software)), an HPLC pump (Gynkotek, 300) equipped with a 150 ml superloop (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for delivery of the free radical solution and a UV–vis absorbance detector (Applied Biosystems, model 785A, Forster City, USA) connected to a recorder (Kipp & Zonen BD40; Delft, The Netherlands) for recording the radical-scavenging signal. Separations were carried out on an Alltima silica HPLC column (3 μm, 150 mm × 4.6 mm i.d.). The reaction coils used were made of PEEK tubing of the following sizes: 3.5 m × 0.25 mm i.d. or 15.0 m × 0.25 mm i.d. Detection of DPPH• and galvinoxyl• reduction was carried out at 515 nm and 425 nm respectively with a UV–vis absorbance detector. The UV detection wavelengths for the test compounds were chosen according to their characteristic absorbance maxima.

Eluents for wheat germ oil and olive oil were n-hexane (A) and MTBE (B) with the following gradient: 0–15 min, 8–12% MTBE; 15–20 min, 12–20% MTBE; 20–30 min, 20–50% MTBE at a flow rate of 1.0 ml/min; 10 μl of sample was injected; the radical solution was 2 μM galvinoxyl• in hexane and was added post-column at a flow rate of 1.0 ml/min. Compounds were detected with UV at 292 nm and by fluorescence (excitation and emission at 290 nm and 326 nm respectively). Galvinoxyl• was detected at 425 nm. 0.1% Formic acid was added to n-hexane or MTBE as eluents for rosemary oil-soluble extract with the following gradient: 0–10 min, 3% MTBE; 10–20 min, 3–8% MTBE; 20–35 min, 8–35% MTBE; 35–50 min, 35–100% MTBE; 50–60 min, 100% MTBE. All other parameters were the same as for the vegetable oils except that 20 μl sample was injected and compounds were detected at 280 nm.

To investigate the possibility of identifying antioxidants in mixtures, the same column, eluents and gradients were used on an LC–DAD–MS instrument (Thermo Fisher Scientific Corp., Waltham, MA, US) using atmospheric pressure chemical ionization (APCI). The effluent from the HPLC column was divided into two parts by a splitter after the UV detector with 0.2 ml/min going to the MS via a 0.2 m × 48 μm i.d. fused silica capillary from Polymicro Technologies (Phoenix, AZ, USA) and 0.8 ml/min going to the reaction coil via a 0.8 m × 0.25 mm i.d PEEK tubing. The off-line UV–vis measurements were carried out on a Lambda 18 UV–vis spectrometer (Perkin-Elmer Corp., Norwalk, CT) at 515 nm for DPPH• and 425 nm for galvinoxyl•. All spectrometric measurements were carried out at least in triplicate.

MS settings. For vegetable oils positive mode was employed with the temperatures of the capillary and the APCI vaporizer set at 275 °C and 400 °C, respectively. Sheath gas flow, aux gas flow and sweep gas flow were 50, 5 and 0 (relative units), respectively. Source voltage was 6 kV and source current was 1 μA. The capillary voltage was zero. The instrument was scanned from *m/z* 350 to 450. For the rosemary oil-soluble extract, the MS settings were identical to those for the vegetable oils except that spectra were now recorded in negative mode, the capillary voltage was 12 V and spectra were scanned from *m/z* 110–1000.

3. Results and discussion

3.1. Choice of free radicals

Free radicals used in an on-line system need to be well soluble at the concentrations used, have a strong absorbance at a high

wavelength, no absorbance at this wavelength after reduction, a good stability, and react sensitively and rapidly with the antioxidants. DPPH• and ABTS•+ are two relatively stable free radicals widely used in on-line antioxidant measurements. ABTS•+ is more sensitive than DPPH• for most compounds [16], but it is insoluble in non-polar organic solvents like hexane and thus cannot be used in a normal-phase system. In order to find a good radical for use in an on-line NP-HPLC system, three other commercially available free radicals were investigated. Off-line measurements showed that the ε-value of TEMPO was too low, even at its maximum absorption wavelength (λ = 485 nm, in hexane). A very high concentration would be needed (above 1.5 mg/ml) which in turn would result in a poor sensitivity of the method. Tris(4-bromophenyl) ammoniumyl hexachloroantimonate showed a deep blue colour (λ_{max} = 705 nm) but was very sensitive to air. Its absorbance decreased so fast that it could not be used. Galvinoxyl• has a very strong absorbance at 425 nm in hexane and remained stable during two days in the dark. Galvinoxyl• is a classical reagent used in electron spin resonance spectroscopic measurements [24,25]. Smith and Hargis were the first to use it for measuring the radical-scavenging activity of antioxidants [26] based on the decrease in absorbance of galvinoxyl• in ethanol at 550 nm. In hexane, however, we failed to find an absorption maximum at 550 nm. DPPH• has been used in similar systems with RP-HPLC many times, and it also showed a sufficiently high solubility in non-polar solvents like hexane. Because of the poor sensitivity and stability, TEMPO and tris(4-bromophenyl) ammoniumyl hexachloroantimonate were discarded. DPPH• and galvinoxyl• were chosen as the free radicals to be investigated further.

3.2. Solvent choice

Hexane, MTBE, and acetone were employed as solvents. As Figs. 2 and 3 show, DPPH• and galvinoxyl• are very stable in the three solvents in the dark. A remarkable effect of the solvent was observed in off-line measurements of the reaction kinetics of the reaction of the radicals with α-tocopherol. DPPH• or galvinoxyl• showed the highest absorbance in MTBE but when reacting with α-tocopherol, the largest and fastest decrease of the absorbance was observed in hexane. When we applied DPPH• or galvinoxyl• solutions in hexane as post-column reagents in the on-line system, they reacted much faster with α-tocopherol resulting in a higher signal to noise (S/N) ratio for the α-tocopherol peak than in the other solvents (Fig. 4). Caldin et al. attribute this to less interaction of the phenol with non-polar solvents resulting in easier formation of a radical–phenol complex, within which the rate-limiting H-atom transfer occurs [27]. So in the following optimisation of the on-line experimental set-up, hexane was used as the solvent for DPPH• and galvinoxyl•.

3.3. Optimisation of on-line experimental set-up

From previous work it is known that both reagent concentration and reaction time influence the sensitivity and baseline noise of the method. These parameters were optimized with respect to the S/N ratio for α-tocopherol. Optimum values for the DPPH• and galvinoxyl• concentration and flow rate were determined under on-line conditions by monitoring the S/N ratio for 10 μl injections of a 500 μM α-tocopherol solution (triplicate measurements). The free radical concentrations in the reactor were changed by changing the concentration of the working solution (5 μM, 10 μM, 20 μM, 50 μM and 100 μM) and the flow rate (0.5, 0.8, 1.0 and 1.2 mL/min) of the pump delivering the free radical solution. In these experiments, free radical solutions were prepared in hexane. The HPLC mobile phase (hexane:MTBE = 90:10, v:v) was run at 1.0 mL/min. As can be seen from Fig. 5, the S/N ratio is significantly affected by

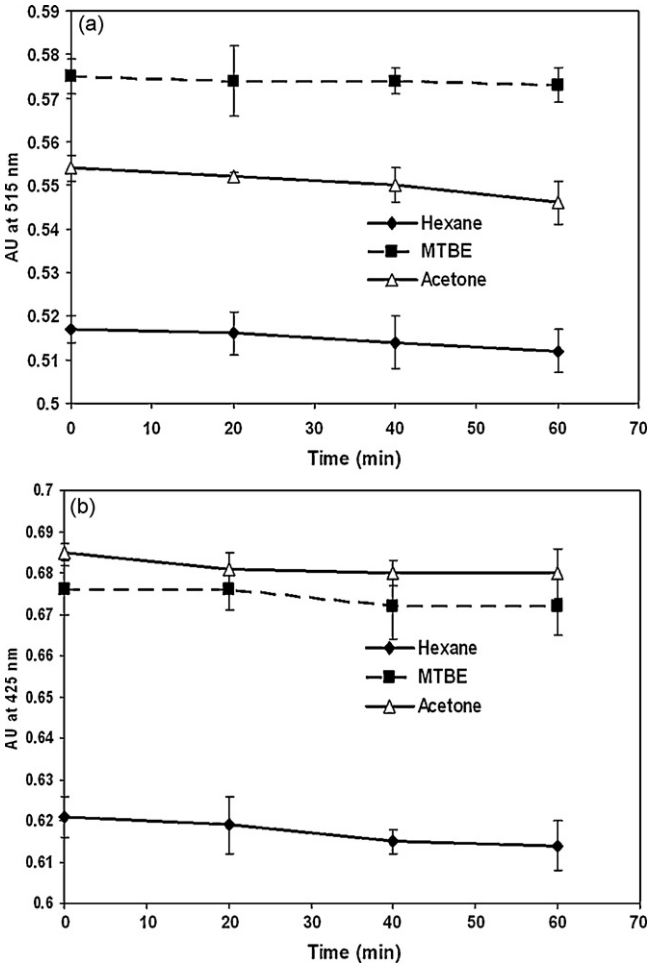

Fig. 2. (a) Decrease of absorbance of DPPH• in different solvents over time. (b) Decrease of absorbance of galvinoxyl• in different solvents over time. The initial concentrations of DPPH• and galvinoxyl• were 50 μM and 5.0 μM, respectively. Solutions were protected from light.

the concentration of the free radical solution. The noise decreased as the concentration decreased but at the same time the signal decreased as well. Optimum S/N ratios were obtained at 20 μM DPPH• and 2 μM galvinoxyl• at reagent and column flow rates of both 1.0 ml/min.

In off-line measurements, α-tocopherol reacted very rapidly with DPPH• and galvinoxyl• in hexane. In the on-line measurements, S/N values for α-tocopherol were determined with different reaction times (24 and 6 s, corresponding to reaction coils of 15 m and 3.5 m × 0.25 mm i.d. respectively). The results are given in Table 1. Shorter coils decreased the S/N ratio significantly. Coils longer than 15 m will increase the S/N value, but at the same time also increase the system back pressure and cause band broadening, which was not preferred. Based on these data, the reaction coil of 15 m × 0.25 mm was chosen for further experiments.

Table 1
S/N ratios determined for α-tocopherol peak at two reaction times with DPPH• and galvinoxyl•[a].

Reaction time (s)	DPPH•	Galvinoxyl•
6	11	59
24	69	206

[a] Concentration of α-tocopherol is 5×10^{-4} mol/l, concentrations of DPPH• and galvinoxyl• are 20 μM and 2.0 μM respectively. Reaction times of 24 and 6 s correspond to reaction coils of 15 m and 3.5 m × 0.25 mm i.d., respectively.

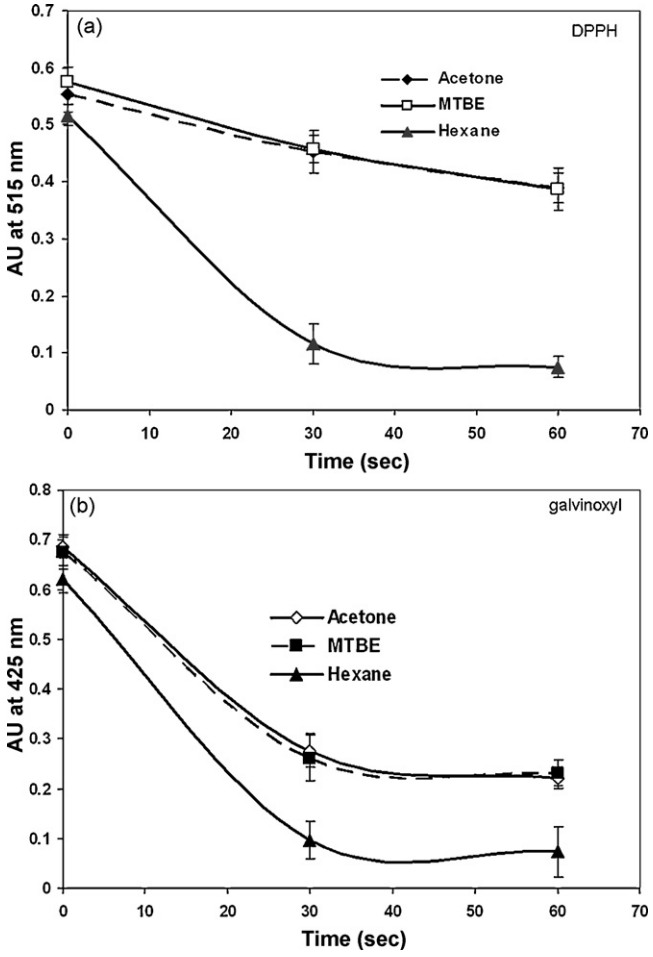

Fig. 3. Speed of reaction (measured as decrease in absorbance/time) of DPPH• and galvinoxyl• with α-tocopherol in different solvents. The concentration of α-tocopherol was 5×10^{-4} mol/l, and concentrations of DPPH• and galvinoxyl• were 50 μM and 5.0 μM, respectively. Solutions were protected from light.

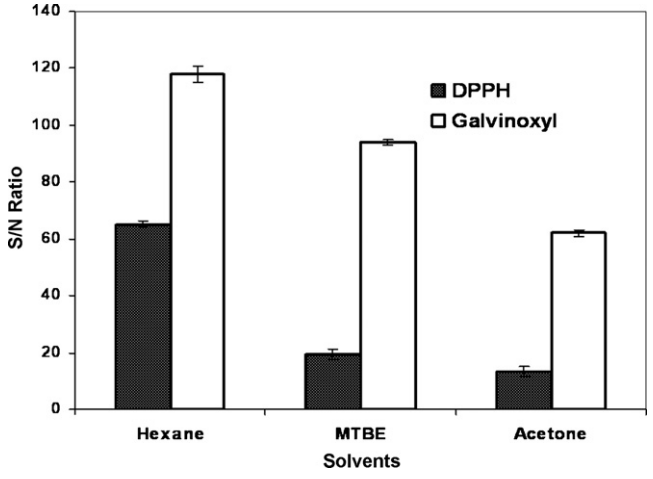

Fig. 4. Influence of the solvent on the S/N ratio of the α-tocopherol peak after reacting with DPPH• or galvinoxyl• in the on-line system. The concentration of α-tocopherol was 5×10^{-4} mol/l, and the concentrations of DPPH• and galvinoxyl• were 20 μM and 10 μM, respectively. The HPLC run was performed under isocratic conditions. More details are given in Section 2.

Table 2
S/N ratios determined for α-tocopherol peak with DPPH• and galvinoxyl• without and with superloop[a].

Free radical	Without superloop	With superloop
DPPH•	69	187
Galvinoxyl•	206	1250

[a] Concentration of α-tocopherol is 5×10^{-4} mol/l, concentrations of DPPH• and galvinoxyl• are 20 μM and 2.0 μM respectively.

To further lower the noise, a superloop reagent-delivery device was investigated. Installation of this piston-driven device can decrease the minor fluctuations of the reagent pump flow, thereby lowering the noise caused by irregular delivery of the radical solution. S/N ratios for α-tocopherol (0.1 mg/ml solution) in combination with DPPH• or galvinoxyl• are shown in Table 2 . Since the noise was much lower, the S/N ratio was much higher than before. In the optimised system the influence of the radical concentration was investigated once more. The optimum concentration of the radicals was the same as in the system without the superloop. In the optimised system the S/N value in combination with galvinoxyl• was much larger than with DPPH•. For this reason all further experiments were performed with galvinoxyl•. The optimized system used a 2 μM galvinoxyl• in hexane solution delivered by a pump equipped with a superloop at 1.0 ml/min and a reaction coil of 15 m × 0.25 mm.

3.4. Determination of minimum detectable amounts

For determination of the minimum detectable concentration (MDC, mol/l) and the minimum detectable amount (MDA, ng), the coefficient $t = 1.725$ for $n = 21$ measurements of the blank signal with a confidence interval of 90% was used, and the MDC was calculated as

$$\mathrm{MDC} = -2t\sigma_{\mathrm{blank}}$$

where σ_{blank} is the standard deviation of the blank signal and t is the Student's t statistic [16]. The sample for determining the blank signal consisted of the HPLC mobile phase used for elution of the test compound with the DPPH• or galvinoxyl• working solution at its optimum concentration and flow rate. The negative peak resulting from the reduction of radicals by a radical-scavenging compound was considered detectable if its height (depth) exceeded the calculated MDC value.

The limits of detection and minimum detectable amounts of several antioxidants were determined. The data obtained are presented in Table 3. A combined plot of the UV-based chromatogram and galvinoxyl• reduction profiles of 12 dilutions of α-tocopherol is presented in Fig. 6.

α-Tocopherol (vit. E) is a well-known natural antioxidant. It is the main antioxidant in vegetable oils. In previous work with the RP-HPLC-DPPH• system α-tocopherol was found to have an MDA of

Table 3
Detectable minimum concentration (MDC), detectable minimum amount (MDA) and calibration curve ($h = a \times c + b$; R^2) data of some antioxidants in the HPLC-galvinoxyl• on-line system under isocratic conditions[a].

Antioxidant	MDC (μM)	MDA (ng)	a	b	R^2
α-tocopherol	4.6×10^{-1}	2.0	1.1	0.13	0.9998
BHT	5.6×10^{2}	1.2×10^{3}	5.6×10^{-3}	−0.34	0.9924
Isoeugenol	1.0×10^{2}	1.6×10^{2}	3.1×10^{-2}	0.15	0.9983
Eugenol	1.1×10^{3}	1.8×10^{3}	5.6×10^{-3}	−0.64	0.9975
Carnosic acid	3.2	1.1×10	1.5	−0.97	0.9974
Carnosol	1.0	3.5	7.2×10^{-1}	0.10	0.9824

[a] Determined under optimal conditions. HPLC used isocratic conditions described in "Experimental". Free radical was dissolved in methanol in the case of carnosic acid.

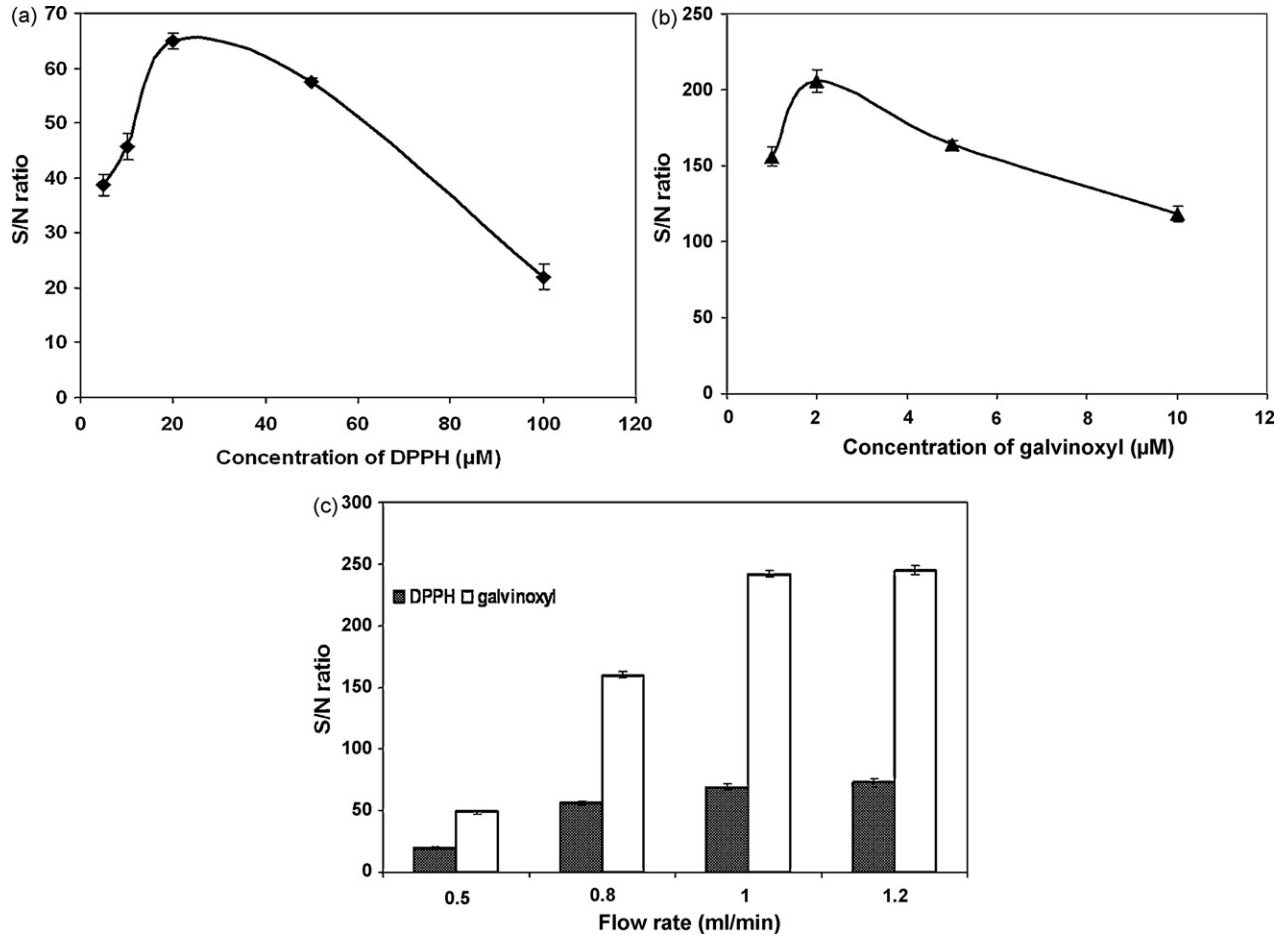

Fig. 5. S/N ratio of the α-tocopherol peak as a function of the DPPH• and galvinoxyl• concentration (a and b respectively) and flow rate (c). The concentration of α-tocopherol was 5×10^{-4} mol/l and the solvent for the DPPH• and galvinoxyl• solutions was hexane. In (a) and (b) the flow rate of the radical solutions was 1 ml/min. In (c) the concentrations of DPPH• and galvinoxyl• were 20 μM and 2.0 μM, respectively.

5.6 ng [17]. This value is similar to the value obtained in the current NP-HPLC-galvinoxyl• system. Although in off-line experiments in hexane, α-tocopherol reduces DPPH• faster than galvinoxyl• at the same molar ratio, galvinoxyl• has a much stronger absorbance at 425 nm than DPPH• at 515 nm. As a consequence of that, a ten times lower optimal concentration is found for galvinoxyl• in on-line measurements, which in turn causes the S/N ratio for α-tocopherol in combination with this radical to be larger than with DPPH•.

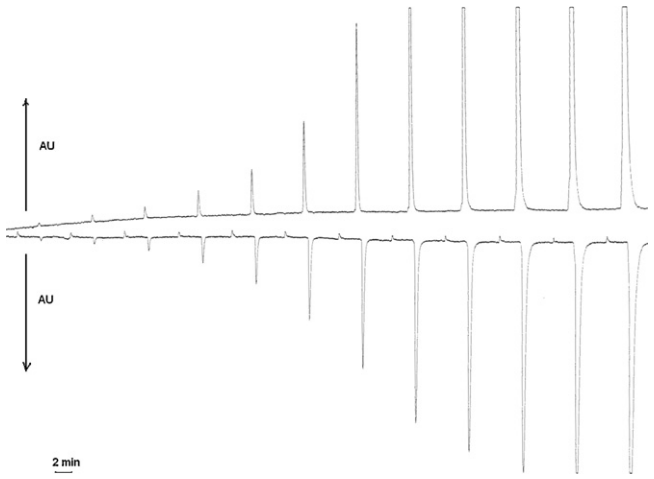

Fig. 6. Combined plot of the UV chromatogram and galvinoxyl• reduction profiles of different concentrations of α-tocopherol sequentially injected (left to right: 0.28, 0.56, 1.1, 2.25, 4.5, 9.0, 18, 36, 72, 144, 288, 576 μg/ml) under isocratic HPLC conditions. Further details are given in Sections 2 and 3.3.

Carnosic acid can be very sensitively detected in RP-HPLC with DPPH•, but in normal-phase solvents, it reacted much slower with DPPH• or galvinoxyl•, leading to a strongly reduced sensitivity. In a polar solvent like methanol, on the other hand, it reacts rapidly with galvinoxyl• leading to an increase in sensitivity. Litwinienko and Ingold [28] found that in alcohols some phenols can partially ionize and the resulting phenoxide anion rapidly transfers an electron to the DPPH• radical. This could explain the low reactivity of carnosic acid and galvinoxyl• in hexane, however it does not explain why the related carnosol shows the same sensitivity for NP-HPLC and RP-HPLC eluents. Isoeugenol, eugenol and BHT showed very poor sensitivity in any solvent and effectively cannot be determined at realistic levels. An explanation for this phenomenon could be that the galvinoxyl• radical is actually more stable than the radicals formed from isoeugenol, eugenol and BHT. Even if an equilibrium between these radical species is kinetically possible, hardly any H-transfer will take place. Thus the equilibrium will be in favour of galvinoxyl• and this leads to a very poor sensitivity.

To investigate the influence of temperature on the sensitivity, the reaction coil was placed in a water bath of 40 °C, 50 °C or 60 °C. For α-tocopherol temperature did not affect the sensitivity. Larger negative peaks were observed for injections of 0.1 mg/ml BHT or 0.1 mg/ml isoeugenol. The magnitude of the negative peak increased twice at 60 °C compared to that at room temperature (data not shown), with noise being unaffected by temperature.

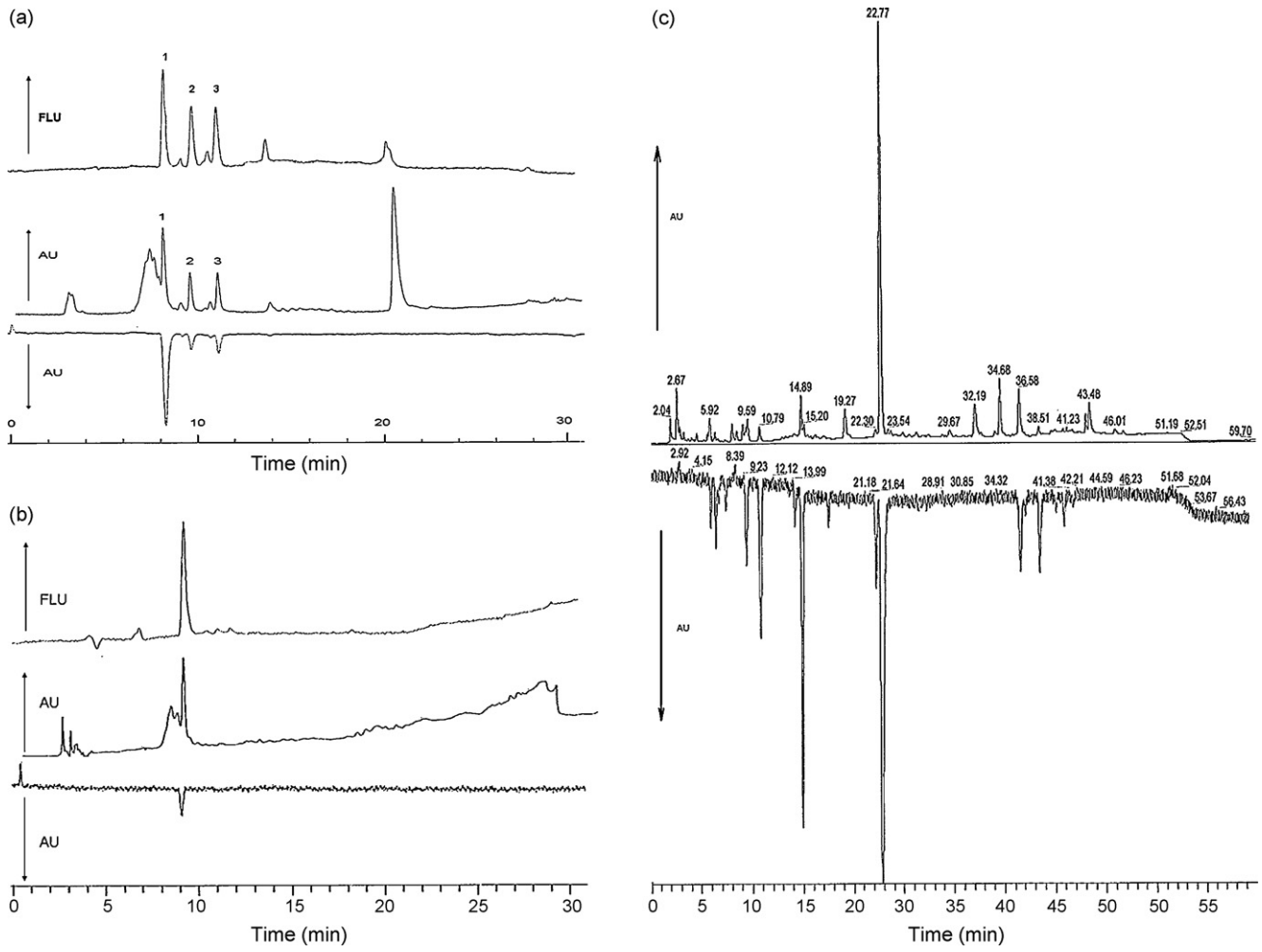

Fig. 7. (A) Combined plot of the UV and fluorescence profiles and galvinoxyl• reduction profiles of wheat germ oil (10 mg/ml hexane). Compounds were detected with UV detection at 292 nm and by fluorescence (λ_{ex} = 290 nm, λ_{em} = 326 nm). Galvinoxyl• reduction was detected at 425 nm. (B) Olive oil, experimental conditions were the same as in (A). (C) Combined plot of the UV profile and galvinoxyl• reduction profiles of rosemary extract (10 mg/ml hexane). UV detection at 280 nm and galvinoxyl• reduction was detected at 425 nm. For chromatographic details see Section 2.

Heating the reaction coil can hence increase the sensitivity for at least some compounds in this system.

3.5. Interfacing with MS

The standard NP-HPLC set-up uses two detectors, either fluorescence and radical scavenging or diode array and radical scavenging. For tocopherols fluorescence detection is much more sensitive than UV. It was investigated if a mass spectrometer with APCI interface could be added for obtaining additional spectral information. To this purpose, a 4:1 splitter was inserted after the DAD with 1 part going to the MS and 4 parts to the reaction coil. The settings for the APCI experiments were optimized with α-tocopherol and carnosic acid. After interfacing with the MS, UV, MS and radical-scavenging activity data of every peak could be obtained with one injection. Good spectra could be obtained with the used NP eluents.

3.6. Application of the on-line method to real samples

The method developed here can be used for a rapid detection of antioxidants (radical scavengers) in vegetable oils and non-polar plant extracts. Combined UV chromatograms and galvinoxyl• reduction profiles of wheat germ oil, olive oil and oil-soluble rosemary extract recorded under gradient conditions are presented in Fig. 7a–c, respectively. No sample preparation was necessary, confirming that the method can save time, labour and chemicals. In Fig. 7a three negative peaks are visible. Several other components, although showing strong UV absorbance and/or fluorescence, did not reduce galvinoxyl•. Based on a comparison with retention times of standard compounds, UV and MS data, the antioxidants in wheat germ oil could be identified as α-tocopherol (1st peak), β-tocopherol (2nd peak) and γ-tocopherol (3rd peak). Olive oil could also be directly analysed (Fig. 7b). Here only one negative peak from α-tocopherol was obtained. In Fig. 7c more than ten negative peaks can be observed confirming that oil-soluble rosemary extract contains many antioxidants. The MS data and a retention time comparison with pure carnosol and carnosic acid showed that the main antioxidant, the peak at a retention time of 22.77 min, is carnosic acid. The negative peak at a retention time of 36.58 min was found to be caused by carnosol. Interesting was the strong negative peak at 14.89 min. As it elutes prior to both carnosic acid and carnosol, this implies it is probably even more non-polar and might have been missed in RP-HPLC studies due to its late elution. This was confirmed by collecting the peak and re-injecting it in an RP-HPLC system where it eluted later than carnosic acid. The λ_{max} of this peak is 268 and 234 nm, which indicates a chromophore different from carnosol and carnosic acid. With APCI-MS it showed pseudomolecular ions at *m/z* 285 and 287 in (−) and (+)-mode respectively suggesting a molecular of *m/z* 286. A literature search yielded no result and this gathered information was on its

own not sufficient to elucidate the structure. A preparative isolation followed by NMR will be pursued. The peak at 10.79 min was identified as α-tocopherol by its UV and mass spectra and R_t. Other radical scavengers could not be identified solely on the basis of their mass spectra and UV data. For this, preparative HPLC on silica followed by NMR is the logical next step during which the currently developed method can be used to monitor different fractions.

4. Conclusion

A rapid normal-phase HPLC-DAD method for on-line detection of antioxidants in complex non-polar mixtures was developed. The stable radical galvinoxyl• was applied in this system because leads to higher S/N ratios than DPPH•. The use of a superloop in the system for post-column radical addition decreased the noise remarkably. In the optimized instrumental set-up a radical-scavenging solution of 2 μM galvinoxyl• in hexane is delivered by a pump equipped with a superloop at 1.0 ml/min into a reaction coil of 15 m × 0.25 mm. Heating the reaction coil increases the sensitivity for some compounds. The system can be equipped with a splitter to direct part of the column effluent to an MS. Hence, structural data and antioxidant activity from mixtures can be obtained in one analysis. The method is simple and rugged. It can be applied for the direct screening of non-polar samples like vegetable oils and lipid-soluble plant extracts for relatively non-polar antioxidants like tocopherols without any prior sample preparation. The sensitivity for α-tocopherol is in the low nanogram range.

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References

[1] H.-D. Belitz, W. Grosch, P. Schibele, Food Chemistry, 3rd ed, Springer-Verlag, Berlin, 2004, p. 190.
[2] P.B. Addis, Food. Chem. Toxicol. 24 (1986) 1021.
[3] D. Rekika, S. Khanizadeh, M. Deschênes, A. Levasseur, M.T. Charles, R. Tsao, R. Yang, HortScience 40 (2005) 1777.
[4] A.B. Martín-Diana, D. Rico, C. Barry-Ryan, Innov. Food Sci. Emerg. Technol. 9 (2008) 593.
[5] B. Nanditha, P. Prabhasankar, Crit. Rev. Food Sci. Nutr. 49 (2009) 1.
[6] J. Pokorny, Electr. J. Environ. Agric. Food Chem. 7 (2008) 3320.
[7] E.N. Frankel, Food Chem. 57 (1996) 51.
[8] S. Singh, R.P. Singh, Food Rev. Int. 24 (2008) 392.
[9] L.M. Magalhães, M.A. Segundo, S. Reis, J.L.F.C. Lima, Anal. Chim. Acta 613 (2008) 1.
[10] I.I. Koleva, T.A. van Beek, J.P.H. Linssen, Æ. de Groot, L.N. Evstatieva, Phytochem. Anal. 13 (2002) 8.
[11] E.N. Frankel, J.W. Finley, J. Agric. Food Chem. 56 (2008) 4901.
[12] J.-K. Moon, T. Shibamoto, J. Agric. Food Chem. 57 (2009) 1655.
[13] R.L. Prior, X. Wu, K. Schaich, J. Agric. Food Chem. 53 (2005) 4290.
[14] V. Poginsky, E.A. Lissi, Food Chem. 92 (2005) 235.
[15] I.I. Koleva, H.A.G. Niederländer, T.A. van Beek, Anal. Chem. 72 (2000) 2323.
[16] I.I. Koleva, H.A.G. Niederländer, T.A. van Beek, Anal. Chem. 73 (2001) 3373.
[17] A. Dapkevicius, T.A. van Beek, H.A.G. Niederländer, J. Chromatogr. A 912 (2001) 73.
[18] V. Exarchou, Y.C. Fiamegos, T.A. van Beek, C. Nanos, J. Vervoort, J. Chromatogr. A 1112 (2006) 293.
[19] H.A.G. Niederländer, T.A. van Beek, A. Bartasiute, I.I. Koleva, J. Chromatogr. A 1210 (2008) 121.
[20] A. Kamal-Eldin, S. Görgen, J. Pettersson, A.-M. Lampi, J. Chromatogr. A 881 (2000) 217.
[21] S.L. Abidi, T.L. Mounts, J. Chromatogr. A 670 (1994) 67.
[22] E. Gimeno, A.I. Castellote, R.M. Lamuela-Raventós, M.C. de la Torre, M.C. López-Sabater, J. Chromatogr. A 881 (2000) 251.
[23] A. Gliszczyńska-Świgło, E. Sikorska, J. Chromatogr. A 1048 (2004) 195.
[24] P.T. Gardner, D.B. McPhail, G.G. Duthie, J. Sci. Food Agric. 76 (1998) 257.
[25] M.F. Ramadan, L.W. Kroh, J.-T. Mörsel, J. Agric. Food Chem. 51 (2003) 6961.
[26] R.C. Smith, J.H. Hargis, Microchem. J. 31 (1985) 18.
[27] E.F. Caldin, S.P. Dagnall, M.K.S. Mak, D.N. Brooke, Faraday Discuss. Chem. Soc. 74 (1982) 215.
[28] G. Litwinienko, K.U. Ingold, J. Org. Chem. 68 (2003) 3433.