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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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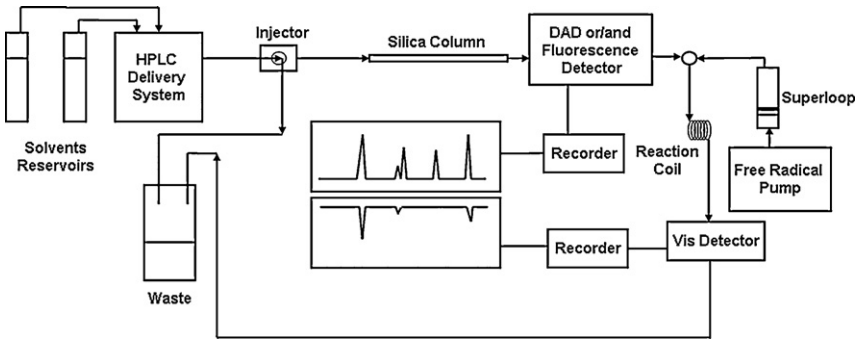

Fig. 1. Instrumental set-up for normal-phase HPLC with on-line detection of radical-scavenging compounds.

(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