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Journal of Chromatography A, 912 (2001) 73–82

JOURNAL OF CHROMATOGRAPHY A

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Evaluation and comparison of two improved techniques for the on-line detection of antioxidants in liquid chromatography eluates

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Received 11 July 2000; received in revised form 16 November 2000; accepted 9 January 2001

Abstract

Two methods for the on-line detection in HPLC eluates of analytes possessing radical scavenging activity were improved and compared. The instrumental set-up of the method that is based on on-line inhibition of luminol chemiluminescence (CL) by antioxidants was improved using better quality syringe pumps, employing a diode array detector, and introducing a mixing/neutralisation coil and a pulse damper. Sensitivity of the HPLC–CL detection increased by a factor of 4. Post-column neutralisation of eluates improved compatibility of this detection method with acidified HPLC eluents. The second method, which is based on the post-column quenching of 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$), was improved by readjusting composition and flow-rate of the reagent, mounting an additional pulse damper and detecting unreacted DPPH$^{\cdot}$ with a detector equipped with a tungsten lamp. Purging of the DPPH$^{\cdot}$ solution with He gas prior to analysis was introduced. This led to 30-fold better detection limits. The improved methods were compared with respect to limits of detection, the radical scavenging mechanism involved, compatibility with common HPLC solvents and pH range, and some technical aspects. The techniques described have high potential for the rapid identification of radical scavengers in complex samples like plant extracts. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Radical scavengers; Luminol luminescence; Detection, LC; Antioxidants; 2,2′-Diphenyl-1-picrylhydrazyl

1. Introduction

Reactive intermediates in oxidation processes, particularly free radicals, are at present receiving increased attention in biology, medicine, food chemistry, and various industrial as well as environmental areas [1,2]. Radical species are involved in many oxidative chain reactions. A common example of such a process is lipid peroxidation in foods leading to rancidity. Food additives such as antioxidants can be applied to extend the shelf-life of foods and maintain their safety, nutritional quality, functionality and palatability. Antioxidants must be non-toxic, relatively inexpensive, effective, should possess carry-through effect during processing, and should not alter the quality of the end-product [3]. Currently, food manufacturers prefer additives labelled as “natural”. Therefore, there is a growing tendency to replace synthetic antioxidants by natural ones, rosemary extracts being a prime example. As a

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PII: S0021-9673(01)00548-9

result, new sources are screened for potential novel natural antioxidants.

Substances that retard oxidation processes by inactivating or scavenging free radicals are called primary antioxidants. Their radical scavenging activity may be evaluated directly in food products or in model systems. Sensory evaluation, measurement of hydroperoxides and numerous techniques that monitor secondary oxidation products under ambient or accelerated oxidation conditions are used in real-life foods [4–6]. However, results obtained from such tests are often difficult to interpret due to physical and chemical interactions of antioxidants with other food components. Furthermore, accelerated oxidation techniques do not always represent the real course of oxidation under normal storage conditions. Model oxidation systems alternatively used to assess activity of antioxidants include:

- β-carotene bleaching [7–9],
- methyl linoleate peroxidation [10,11],
- luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) chemiluminescence inhibition [1,12],
- tetraline chemiluminescence inhibition [13],
- 2,2′-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) bleaching [14–17],
- 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot +}$) bleaching [18,19], and
- inhibition of ferric thiocyanate formation [20].

Such model systems facilitate screening, identification and comparison of primary antioxidants. However due to their batch-like character they are unsuitable for identifying which individual compound(s) are responsible for the overall effect.

Some recent publications demonstrate the potential of merging the efficiency of HPLC separation with the convenience of on-line post-column detection of radical scavenging compounds based on a model oxidation system [21–23], i.e. luminol chemiluminescence inhibition and $DPPH^{\cdot}$ bleaching. Such a combination allows for rapid and convenient analysis of radical scavengers, even in complex mixtures (e.g. plant extracts, drugs, food systems, etc.). The on-line luminol chemiluminescence assay is based on scavenging of free radical species (including some reactive oxygen species) involved in the reaction sequence leading to luminol chemiluminescence [1,2,24,25]. The resulting inhibition of chemiluminescence is registered at 425 nm. The on-line $DPPH^{\cdot}$ bleaching assay is based on a decrease in $DPPH^{\cdot}$ absorbance at 517 nm, upon reduction of this relatively stable radical by an antioxidant [16].

Optimisation and evaluation of these two on-line methods, developed in our group, based on luminol chemiluminescence quenching and $DPPH^{\cdot}$ bleaching, respectively [21,23], are presented in this paper. Both methods are compared with respect to their sensitivity, selectivity, compatibility with common HPLC solvents, range of pH and some technical aspects.

2. Experimental

2.1. HPLC–CL instrumental set-up

The HPLC system with on-line chemiluminescence (CL) detection of radical scavengers is shown in Fig. 1. The binary gradient was formed in a Waters 600E Multisolvent Delivery System (Millipore, Waters Chromatography Division, Milford, MA). A pulse damper (toroid mixer, Scientific Systems, State College, PA) was introduced to improve pressure stability. Samples were injected using a Gilson 401 Dilutor (Gilson Medical Electronics, Middleton, WI) combined with a Gilson 231 Auto-Sampling Injector equipped with a 10-μl injection loop. Analytes were separated on an Alltima C_{18} 5U analytical column (250×4.6 mm, Alltech Associates, Deerfield, IL) and detected with a Waters 990 Series Photodiode Array Detector in the range λ=210–450 nm. Data were processed on a PC with original Waters software (version LCA–6.22a) and printed on a Waters 5200 Printer Plotter. On-line post-column addition of CL reagents was performed with two 45-ml laboratory-made syringe pumps (Free University, Amsterdam, The Netherlands). The CL was monitored with a fluorescence detector (Kratos Analytical, Ramsey, NJ) with the excitation lamp switched off. The CL signal obtained was recorded on a BD 40 recorder (Kipp and Zonen, Delft, The Netherlands). A 15-m long mixing/neutralisation coil of 0.25-mm I.D. PEEK tubing was introduced between two T junctions. A 60-cm reaction coil of 0.25-mm I.D. PEEK tubing was used to connect the syringe pump B with the fluorescence

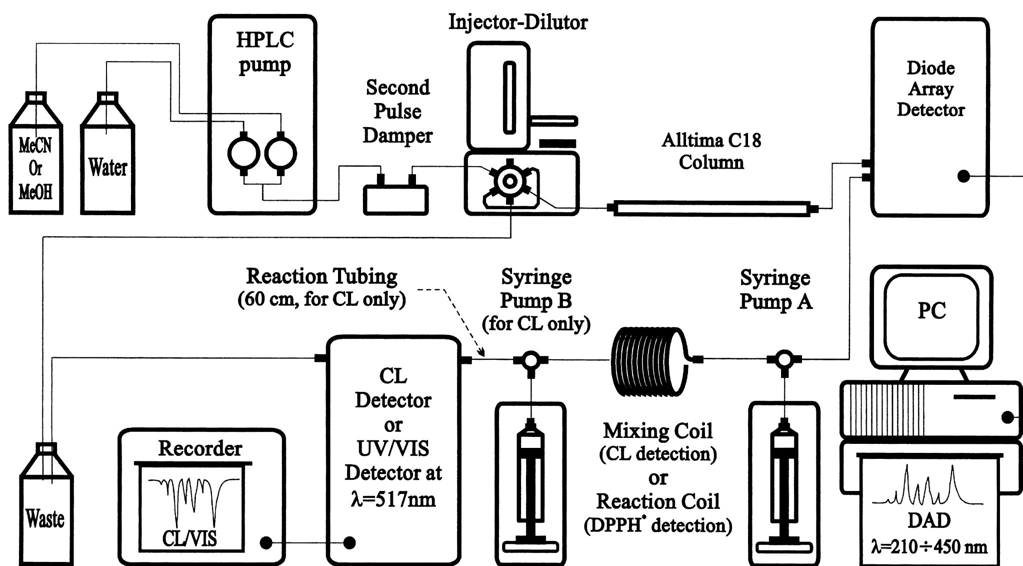

Fig. 1. Improved instrumental set-up for the on-line chemiluminescence/DPPH˙ detection of radical scavenging compounds.

detector. All other parts of the system were interconnected with stainless steel tubing of 0.2 mm I.D.

2.2. HPLC–CL reagents and gradient conditions

Ultra-pure water was mixed with HPLC grade acetonitrile (Lab-Scan Analytical Sciences, Dublin, Ireland) in a ratio of 3:1. The mixture was acidified with 0.25% (v/v) glacial acetic acid, membrane filtered and used as solvent A in the HPLC gradient program. HPLC grade filtered acetonitrile acidified with 0.25% (v/v) glacial acetic acid was introduced as solvent B. In linear binary gradient runs, solvent A decreased in 40 min from 95 to 10%, was held for the following 15 min, and then returned from 10 to 95% in 5 min. The combined flow of HPLC eluents was kept constant at 0.85 ml/min.

An aqueous 0.6-ml/l hydrogen peroxide was mixed with 1 *M* NaOH solution at a ratio of 4:10, and the solution was filtered and stored in a refrigerator prior to use with the first syringe pump (pump A). Horseradish microperoxidase sodium salt MP-11 (90%, Sigma–Aldrich) was dissolved in disodium tetraborate buffer at 5.7 mg/l. This buffer, which was brought to pH 10 by addition of 0.1 *M* of NaOH, contained 50 ml of 0.025 *M* disodium tetraborate decahydrate and 18.3 ml of 0.1 *M* NaOH solutions prepared in ultra-pure water. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, 97%, Sigma–Aldrich) was dissolved in HPLC grade methanol (Lab-Scan) at 1.6 m*M*. Stock solutions of MP-11 and luminol were stored at 4°C. MP-11 and luminol solutions were mixed at a ratio of 7:3 prior to use with the second syringe pump (pump B). Pumps A and B introduced streams of CL reagents at flow-rates of 0.02 and 0.22 ml/min, respectively.

2.3. HPLC–DPPH˙ instrumental set-up

The HPLC system adapted for DPPH˙ on-line detection of radical scavenging compounds differed from the HPLC–CL system in that pump B and the 60-cm reaction coil were removed and the fluorescence detector was replaced by a UV/VIS model 759A absorbance detector (Applied Biosystems, Foster City, CA) equipped with a tungsten lamp. Thus, the 15-m PEEK coil was connected directly to the absorbance detector working at λ=517 nm (0.05 range; 5.0 rise time) and functioned here as a reaction coil instead of a mixing coil.

2.4. HPLC–DPPH$^{\cdot}$ reagents and gradient conditions

Two binary HPLC gradients based on methanol (MeOH) or acetonitrile (MeCN) were tested with the HPLC–DPPH$^{\cdot}$ set-up.

For the MeOH/H_2O gradient, solvent A was prepared by mixing HPLC grade MeOH in ultra-pure water (MeOH/H_2O=35:65, v/v) and acidifying with glacial acetic acid (0.5 ml/l). Pure MeOH with 0.05% (v/v) glacial acetic acid was used as solvent B. The percentage of solvent A in the gradient was as follows: 0–4 min 85%, then from 85 to 50% in 10 min, hold for 3 min, then from 50 to 0% in 9 min, hold for 9 min, then return from 0 to 85% in 3 min. The combined total flow of HPLC solvents was 0.78 ml/min. The DPPH$^{\cdot}$ reagent (2,2′-diphenyl-1-picrylhydrazyl radical, 95%, Sigma–Aldrich) was dissolved in MeOH at a concentration of 5.8 mg/l. This solution was mixed with citric acid–disodium hydrogen phosphate buffer at a ratio of 3:1. The buffer, pH 7.6, contained 6.8 ml of 0.01 *M* citric acid and 93.2 ml of 0.02 *M* disodium hydrogen phosphate solutions prepared in ultra-pure water. The stream of buffered DPPH$^{\cdot}$ solution was introduced by the syringe pump at a flow of 0.5 ml/min.

For the MeCN/H_2O gradient, solvent A was 25% (v/v) HPLC grade MeCN in ultra-pure water, which was acidified with glacial acetic acid (2.5 ml/l). Pure MeCN mixed with 0.25% (v/v) acetic acid was used as solvent B. The percentage of solvent A in the gradient was as follows: 0–3 min 100%, then to 65% in 11 min, hold for 7 min, then from 65 to 5% in 7 min, hold for 12 min, then from 5 to 0% in 5 min, hold for 2 min, then back from 0 to 100% in 5 min. The buffered DPPH$^{\cdot}$ solution was prepared similarly to that used in MeOH/H_2O gradient runs with the only difference being that the reagent was dissolved in MeCN. The flow-rates of HPLC eluents and the DPPH$^{\cdot}$ mixture were identical to those of MeOH/H_2O gradient. Prior to use, all solvents as well as DPPH$^{\cdot}$ mixtures were filtered through a 0.45-μm membrane filter and during the runs, they were sparged with helium at 25 ml/min.

2.5. Sample preparation

Samples of thyme (*Thymus vulgaris* L.) were obtained from the Lithuanian Institute of Horticulture (Babtai, Lithuania) and dried in a drying cabinet with forced ventilation at ambient temperature for 2 days. A total of 50 g of herb material was ground and extracted with 500 ml of redistilled MeOH in an Erlenmeyer flask with a magnetic stirrer under N_2 during 72 h. After filtration, the extract obtained was vacuum-dried at 45°C. Obtained extracts were dissolved in MeOH/H_2O (1:1) at 2.5 mg/ml for the HPLC–DPPH$^{\cdot}$ analysis and in MeCN/H_2O (1:1) at 0.5 mg/ml for the HPLC–CL assessment. Extract solutions were membrane filtered before injection into the HPLC system.

Standards of some antioxidative compounds were purchased from the following suppliers: eugenol, isoeugenol, quercetin dihydrate, α-tocopherol, ascorbic acid, Trolox, 2,6-di-*tert*.-butyl-*p*-hydroxytoluene (BHT), *tert*.-butylhydroquinone (TBHQ), thymol, and carvacrol from Sigma–Aldrich; rosmarinic acid from Extrasynthèse (Genay, France); rutin trihydrate from Fluka (Buchs, Switzerland); chlorogenic acid from Koch-Light (Colnbrook, England); and (±)-taxifolin and eriodictyol from Rotichrom® (Carl Roth, Karlsruhe, Germany). Carnosic acid was isolated in our laboratory.

2.6. Detection limits, minimum detectable amounts (MDA) and minimum detectable concentrations (MDC)

The HPLC column was connected between the pulse damper and the injector-dilutor. Unbuffered MeOH/H_2O eluents were used with DPPH$^{\cdot}$ detection, while with chemiluminescence unbuffered MeCN/H_2O elution was used. The percentage of eluent A in the isocratical flow solvents for each compound is given in Table 1. From eight to 12 serial dilutions of antioxidant compounds were prepared in the corresponding mixture of eluents and tested in triplicates. Detection limits (L_d, in arbitrary units) were calculated for each compound and method as described by Koleva et al. [23]:

$$L_d = -2t[\textit{standard deviation of blank signal}]$$

in which $t=1.725$ for 20 measurements of blank signal with a confidence interval of 90%.

MDC (in μmol/l) values were calculated for each

Table 1
HPLC eluent composition and calculated MDA and MDC values for on-line DPPH˙/CL detection of some antioxidants

Compound	HPLC–DPPH˙ detection[a]			HPLC–CL detection[b]		
	Eluent A, %	MDC, μmol/l[c]	MDA, ng[c,d]	Eluent A, %	MDC, μmol/l[c]	MDA, ng[c,d]
α-Tocopherol	0	1.3	5.6	3	1.7	7.2
Ascorbic acid	90	2.0	3.6	90	0.2	0.4
BHT	0	128	283	5	8.7	19.2
Carnosic acid	5	0.1	0.4	5	1.7	5.8
Carvacrol	10	1600	2400	5	4.2	6.4
Chlorogenic acid	90	0.2	0.5	90	0.1	0.3
Eriodictyol	40	1.5	4.4	40	0.6	1.8
Eugenol	10	27	44	5	0.1	0.2
Isoeugenol	10	0.8	1.4	5	2.1	3.5
Quercetin	0	0.3	1.0	5	1.2	4.1
Rosmarinic acid	40	0.02	0.06	40	0.1	0.4
Rutin	10	0.7	4.6	40	2.5	16.4
Taxifolin	40	5.8	17.6	40	0.5	1.7
TBHQ	30	9.1	15.2	40	2.5	4.1
Thymol	10	1450	2180	5	4.7	7.0
Trolox	20	0.3	0.9	40	2.2	5.4

[a] Eluent A: 35% MeOH in water; eluent B: 100% MeOH.
[b] Eluent A: 25% MeCN in water; eluent B: 100% MeCN.
[c] Mean value of three measurements.
[d] Determined with a 10-μl injection loop.

antioxidant and each detection method by regression using SPSS 8.0 for Windows software (SPSS, Chicago, IL). Equations were fitted to the different antioxidant concentrations (x) and the corresponding detector responses (y) by the least-squares method. These equations were used to calculate the MDC using the previously calculated L_d value as y. Minimum detectable amounts (in ng) were calculated from the MDA values taking into account the loop size used.

2.7. Statistical analysis

The data obtained for both detection methods under study were submitted to ANOVA using SPSS 8.0 for Windows software.

3. Results and discussion

3.1. Improvements in DPPH˙ and luminol CL detection

The sensitivity of both on-line detection methods, which were previously developed by our group [21,23], was largely dependent on flow stability of both HPLC eluents and post-column reaction reagents. In this study, the instrumental set-up underwent several alterations in order to improve flow stability and to achieve better L_d values than those reported in our previous papers, as follows:

- introduction of an additional pulse damper between the eluent pump and the injector;
- use of more stable syringe pumps for post-column reagent addition in the luminol chemiluminescence method; and
- increasing detector lamp intensity at the detection wavelength (517 nm) for the DPPH˙ method.

Other changes that were applied in this study to both radical scavenger detection techniques are discussed below in more detail.

3.2. The luminol chemiluminescence quenching method [21]

Several alterations in the CL detection method presented earlier [21] were made in order to improve chromatographic separation of herb extract con-

stituents and increase detection sensitivity of their antioxidant activity.

Changing the acetonitrile/water gradient program and lowering pH of HPLC solvents resulted in a better separation efficiency especially for polar (acidic) analytes. However, partial pH neutralisation was required to allow for the post-column CL reaction, which favors alkaline conditions. This was accomplished by adding 1 *M* NaOH to the H_2O_2 solution at a ratio of 2:5. Attempts to combine a methanol/water gradient with post-column luminol CL detection were not successful, due to unacceptable chemiluminescence baseline drift. This was caused by rising backpressure during gradient runs, which in turn derived from viscosity differences of methanol–water mixtures.

Modification of the instrumental set-up and introduction of acidified eluents made it necessary to change the concentrations and flow-rates of CL reagents. The H_2O_2 concentration was increased three times, whereas the H_2O_2 solution flow-rate was increased 4.4 times. This was carried out to improve the signal-to-noise ratio and to produce a CL signal of similar intensity to that reported in our previous paper.

After introducing the above changes in set-up and flows, the sensitivity of the CL detection improved on average 4.2 times. The biggest changes were observed for thymol, carvacrol and TBHQ, whose MDA decreased approximately ten, six and nine times, respectively. An example of the improved separation efficiency for a methanolic thyme extract with combined UV/CL detection is presented in Fig. 2a. A variable sensitivity of CL detection to individual eluted compounds was observed, i.e. UV response did not correlate well with CL quenching. Due to its sensitivity, the CL method registered broadened peaks. This is possibly caused by minimal tailing from the HPLC column [21]. Active compounds detected by CL inhibition differed qualitatively and quantitatively from those obtained by DPPH˙ bleaching method. A greater number of non-polar compounds of thyme extract were detected by CL.

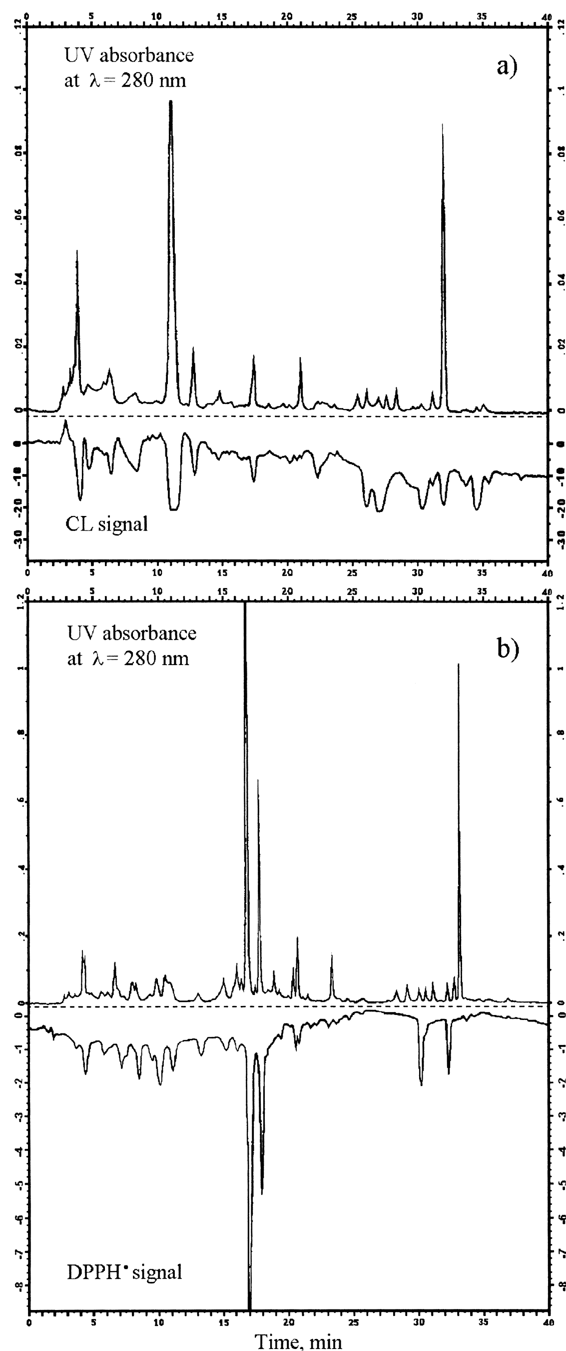

Fig. 2. On-line assessment of radical scavengers from thyme (*T. vulgaris*) methanol extract: (a) modified UV/CL detection with a post-column neutralisation of acidic HPLC eluents by mixing 1 *M* NaOH with hydrogen peroxide solution (pump A) at a ratio of 4:10; (b) modified UV/DPPH˙ detection using acidified HPLC eluents and buffered (pH 7.6, citric acid–disodium hydrogen phosphate buffer) DPPH˙ solution.

3.3. The DPPH˙ bleaching method [23]

Chromatographic separation efficiency for a herb

extract in the "mid-polarity range" was improved by applying both acetonitrile/water as well as methanol/water gradients and lowering eluent pH to 3.0–3.2. The corresponding drop in DPPH˙ bleaching detection sensitivity was compensated by buffering the DPPH˙ reagent flow with citric acid–disodium hydrogen phosphate buffer (pH 7.6). In order to obtain higher detection sensitivity, the previously reported [23] concentration of the DPPH˙ solution and its flow-rate were also subjected to minor changes. Purging the DPPH˙ solution with He gas before use reduced earlier observed baseline drift. This is probably due to elimination of interferences in the detection process, caused by dissolved oxygen. The result of these improvements is shown in Fig. 2b. It should be noted that an acetonitrile gradient was preferred over a methanol gradient, for reasons of diminished pressure variations during gradient runs, stability of the DPPH˙ solution and separation efficiency.

3.4. Consequences of eluent composition and eluent and reagent solution pH for MDA values with DPPH˙ detection

Minimum detectable amounts (MDAs) for two model compounds (carvacrol and rosmarinic acid) differing in their polarity were calculated by:

- applying two different eluents (methanol/water and acetonitrile/water) that were individually prepared for each model compound as indicated in Table 1;
- assessing neutral eluents and eluents with glacial acetic acid added (0.5 ml/l for methanol/water and 2.5 ml/l for acetonitrile/water); and
- using non-buffered DPPH˙ solution and DPPH˙ solution buffered with citric acid–disodium hydrogen phosphate buffer (pH 7.6).

Significantly different MDA values ($P<0.05$) were obtained (Fig. 3). These differences do not show a similar trend for both model compounds (Fig. 3a vs. 3b). However, a general tendency towards less favorable MDA values, if the eluent is acidified, can be observed. In combination with a methanol/water gradient, the MDA values for carvacrol and rosmarinic acid increased almost nine-fold and 500-fold, respectively, upon addition of acid to the eluent. These findings as well as herb extract analy-

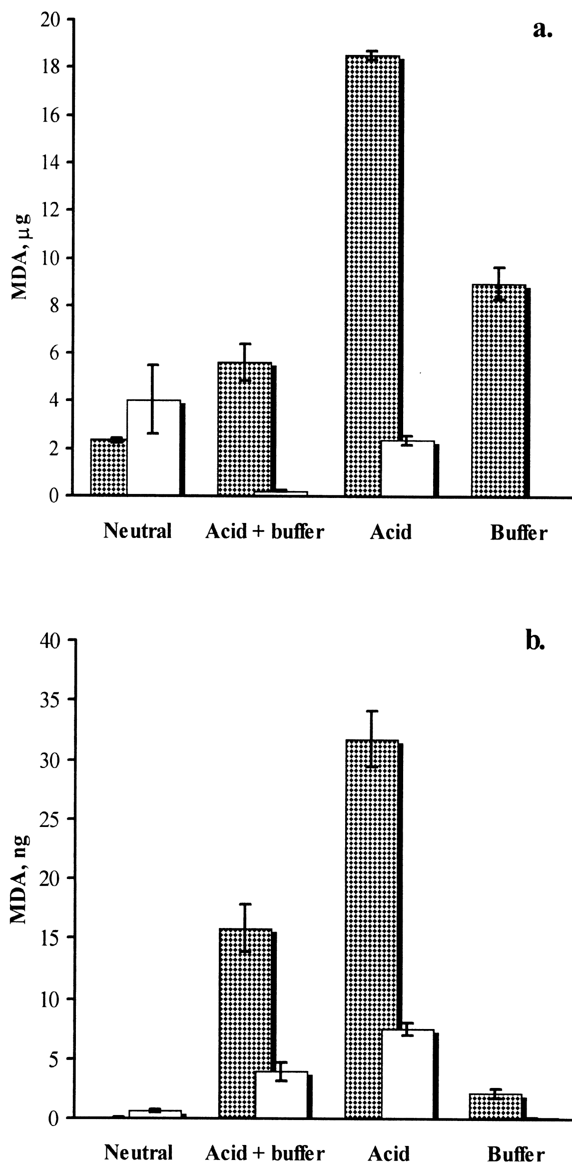

Fig. 3. Sensitivity of DPPH˙ detection assessed for (a) carvacrol and (b) rosmarinic acid with neutral eluents, acidified eluents+ buffered DPPH˙ solution, acidified eluents, and buffered DPPH˙ solution. Acidification of HPLC eluents with glacial acetic acid to pH 3.0–3.2; buffering DPPH˙ solution with pH 7.6 citric acid–disodium hydrogen phosphate buffer. Methanol/water elution is represented by columns in squares; acetonitrile/water by blank columns. Data on MDA of carvacrol eluted with acetonitrile/water and detected with buffered DPPH˙ solution are not available due to clogging of the reaction coil.

ses demonstrated that DPPH˙ bleaching should preferably be performed under ''neutral'' (i.e. unbuffered) conditions (either by operating the whole system under neutral conditions, or by post-column neutralisation of the eluent). However care should be exercised whenever post-column neutralisation is applied. Addition of a buffered DPPH˙ solution into a stream of an acetonitrile/water eluent with 96% (v/v) acetonitrile gave rise to clogging of the reaction coil. This in turn adversely affected the MDA values.

With carvacrol the most favorable MDA (0.28 μg) was obtained with an acetonitrile/water gradient, acidified with acetic acid (pH 3.2), and a DPPH˙ reagent flow buffered with citric acid–disodium hydrogen phosphate buffer (pH 7.6). In contrast, with rosmarinic acid the most favorable MDA (62 pg) was obtained with a methanol/water gradient (eluent- and reagent-flow pH unadjusted). However, it should be noted that the MDA with an acetonitrile/water gradient (eluent pH unadjusted and reagent flow buffered), was only slightly higher (101 pg).

3.5. Comparison of MDC and MDA values for both methods

Clearly, MDA and MDC values observed are strongly pH dependent, as was shown above and observed when screening thyme extracts with both methods. This is in accordance with earlier findings [21,23,26]. It was therefore concluded that a comparison of MDA and MDC values, for both methods, should be performed under neutral conditions (i.e. when eluents and reagent-flows were unbuffered and not pH adjusted). Under such conditions MDA and MDC values were calculated for 16 antioxidative compounds.

The organic solvent used (methanol and acetonitrile) was chosen to give the best sensitivity with DPPH˙ bleaching detection. The MDA value for carvacrol was 1.7 times lower with methanol/water than with acetonitrile/water, while for rosmarinic acid methanol/water elution allowed ten times lower MDA. Therefore, a methanol/water eluent was chosen with DPPH˙ bleaching detection. With luminol chemiluminescence quenching detection, acetonitrile/water elution was preferred, based on considerations of detector baseline stability during gradient runs. Table 1 summarises the MDA and MDC values calculated.

No correlation between MDA and MDC values measured with either detection method was observed. In general, lower MDA values were obtained with luminol than with DPPH˙ detection (nine vs. seven). However, the DPPH˙ bleaching system demonstrated significantly superior sensitivity to certain radical scavenging compounds (e.g. rosmarinic acid, carnosic acid, Trolox, isoeugenol, and rutin). Various explanations can account for the observed lack of correlation, as mentioned below.

A broader range of antioxidant mechanisms may be involved in quenching of luminol chemiluminescence [1,24,25].

Differences in reactivity between the DPPH˙ radical and radical species involved in luminol chemiluminescence may lead to differences in the rate of hydrogen abstraction, depending on the antioxidant involved. The activity of phenolic radical scavengers depends on O–H bond dissociation energy, resonance delocalisation of the phenolic radical and steric effects [17].

Low reactivity compounds (with more than one electron involved in the reaction with DPPH˙) may not have sufficient time to complete their reaction in an on-line system. In such a case, the incomplete detection of radical scavenging activity would yield non-representative MDA values.

Reproducibility of MDA and MDC values, obtained with both methods, was similar: RSD values larger than 20% were only obtained for five compounds with DPPH˙ bleaching and for seven compounds with luminol chemiluminescence quenching. These larger standard deviations were mainly caused by instrumental parameters, related to auto-sampler and HPLC pump performance. Furthermore, with DPPH˙ bleaching detection, gradual deterioration of the reagent solution during measurements might have played a negative role as well.

4. Conclusions

The two improved on-line HPLC radical scavenging detection methods (based on luminol chemiluminescence quenching and DPPH˙ bleaching) allow for direct qualitative determination of radical

Table 2
Advantages and disadvantages of CL and DPPH˙ on-line detection techniques for radical scavengers

HPLC–DPPH˙	HPLC–CL
+ Determination of radical scavengers in HPLC eluates + Compatible with common HPLC eluents at pH 3–7 + Simple instrumental set-up and easy to perform + Applicable in kinetic studies of radical scavengers + Stable baseline − Lower reproducibility due to high flow-rates of reagents, frequent re-filling	+ Determination of radical scavengers (including some reactive oxygen species) in HPLC eluates + Good reproducibility, low reagent flow-rates + Applicability in determination of total antioxidant capacity in physiological fluids + Applicability in studies of lipid oxidation/antioxidation mechanisms − Incompatible with water–methanol gradient − Two syringe pumps necessary

scavengers in complex mixtures using isocratic or gradient elution. Both methods are applicable for on-line screening of samples (e.g. extracts of spices and herbs, oils, juices, wine, hydrolysed protein products, etc.) for natural radical scavengers. Furthermore, both methods can be applied for the elucidation of lipid oxidation/antioxidation mechanisms and radical scavenging processes in general. Most of these analytical tasks can be carried out with minimal sample preparation, thus saving time and costs. It was found that the HPLC–CL system is more sensitive but also more prone to distortion. Baseline stability problems may be encountered [27]. Other disadvantages of the CL method are its incompatibility with water–methanol gradients and the more complex set-up. The advantages and disadvantages of the two methods have been summarised in Table 2. Both techniques can greatly speed up the identification of radical scavengers in complex samples with sufficient sensitivity. Of the two, the DPPH˙ method seems to be the more robust.

Acknowledgements

The authors acknowledge the help of E.C.J. van der Klift for his assistance in assembling the instrumental set-up and G.P. Lelyveld for the isolation of carnosic acid.

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