Chromatographic Isolation of Antioxidants Guided by a Methyl Linoleate Assay

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A convenient and fast method for establishing the relative antioxidant activity of chromatographically purified fractions of plant extracts has been developed. The assay electrochemically determines the myoglobin-catalyzed oxygen consumption following addition of the fractions to methyl linoleate. Since the oxygen consumption rate decreases with increasing antioxidant activity, the method can be used for systematic screening of naturally occurring antioxidants. Applying this method to extracts of *Satureja hortensis* led to isolation of rosmarinic acid.

Keywords: *Antioxidant assay; assay-guided isolation; rosmarinic acid; Satureja hortensis*

The great majority of naturally occurring antioxidants isolated from higher plants are phenolic compounds related to cinnamic acid or flavonoids (Larson, 1988; Pratt, 1993). Although numerous procedures are available for the determination of the antioxidant activity for pure compounds [see e.g. Hudson (1990)], only a few methods exist for systematic assaying when purification of antioxidants from plant extracts is attempted. The usual method measures the autoxidation of linoleic acid or its esters by the decrease in oxygen tension (Le Tutour, 1990; Le Tutour and Guedon, 1992) or by the thiocyanate method (Masuda et al., 1993; Yagi et al., 1994; Jitoe et al., 1994). However, the former method depends upon a rather sophisticated apparatus and the latter may easily require 6 days. Recently, a method using methyl linoleate oxidation accelerated by elevated temperatures (110 °C) and abundant oxygen supply (7 mL/min flow rate) has been proposed, but even in this case each test required around 8 h (Cuvelier et al., 1990, 1994). More directly, the formation of hydroperoxides can be followed by monitoring the conjugated diene absorption at 235 μm (Torel et al., 1986), provided interfering absorption is absent. Thus, neither of these methods is optimal for use in systematic screenings which require a simple, rapid determination of the relative antioxidant activity of partially purified samples. Hence, we investigated the application of a method previously developed by Skibsted and his co-workers for predicting oxidative stability of processed meat and meat products (Mikkelsen et al., 1992; Jørgensen and Skibsted, 1993). This assay involves metmyoglobin (MMb)-catalyzed autoxidation of methyl linoleate stabilized with Tween 20 in air-saturated phosphate buffer and proceeds within 10 min at room temperature. The antioxidative efficiency is assessed by the oxygen consumption rate as monitored by a Clark electrode.

Summer savory, *Satureja hortensis* L., is a culinary herb widely used in the food industry. More than 100 constituents have been described in the essential oil (Chialva et al., 1980; Deans and Svoboda, 1989; Darbour et al., 1990; Svoboda et al., 1990; Zielinska-Stasiek and Cisowski, 1991; Zani et al., 1991; Regnault-Roger et al., 1993; Deighton et al., 1993). The potent antioxidant rosmarinic acid (Cuvelier et al., 1992) has been identified (0.23–2.5% wt/dry wt) by chromatographic methods (Herrmann, 1960, 1962; Lamaison et al., 1991). The ethanolic extract of summer savory therefore served as a convenient test for the antioxidative assay.

MATERIALS AND METHODS

Extraction with EtOH and Identification of Rosmarinic Acid as the Main Antioxidant. Summer savory (*S. hortensis* L.) (75 g, air-dried) was extracted with EtOH (96%), washed with heptane, and taken to dryness. The remanence was dissolved in EtOAc and washed with water, and the EtOAc extract was separated into five fractions by silica gel column chromatography (Büchi, Si60; acetone/EtOH). The fraction showing the best antioxidative activity was further purified by silica gel chromatography (Merck NP, B-column eluted successively with EtOAc, EtOH, MeOH, and H_2O) to give fractions 5a–5i. The strong antioxidant activity was concentrated in fractions 5e (122 mg) and some small fractions (5a, 5f, 5h, and 5i) as shown in Figure 2. Fraction 5e on RP-18 gave 55 mg of pure rosmarinic acid, showing ^{1}H and ^{13}C NMR data identical to published values (Kelley et al., 1975, 1976).

Apparatus for Antioxidative Assay. Measurement of the relative antioxidative activity of the chromatographic fractions was carried out according to the method of Mikkelsen et al. (1992) as further modified by Jørgensen and Skibsted (1993), and these papers should be consulted for details in the preparation of reagents, etc. The methyl linoleate emulsions were conveniently prepared as follows. In a nitrogen atmosphere 250 μL of a solution of methyl linoleate (80 mg) in MeOH (10 mL) was mixed with another 250 μL aliquot of a solution of Tween 20 (0.4 g) in MeOH (10 mL) and the mixture taken to dryness. Immediately before use, the remanence was stirred with an air-saturated phosphate buffer (5 mL, 5 mM, pH 5.8, 25.0 °C) to make a fine emulsion.

The apparatus consists of an oxygen electrode (Pt, Ag/AgCl) separated from the sample in the measuring cell by an oxygen permeable polyethylene membrane. For a description of equipment, maintenance, calibration, etc., see Siggaard-Andersen (1974). Prior to each test, the measuring cell is rinsed successively with water and buffer. When the sample is injected, care must be taken to ensure that all of the buffer

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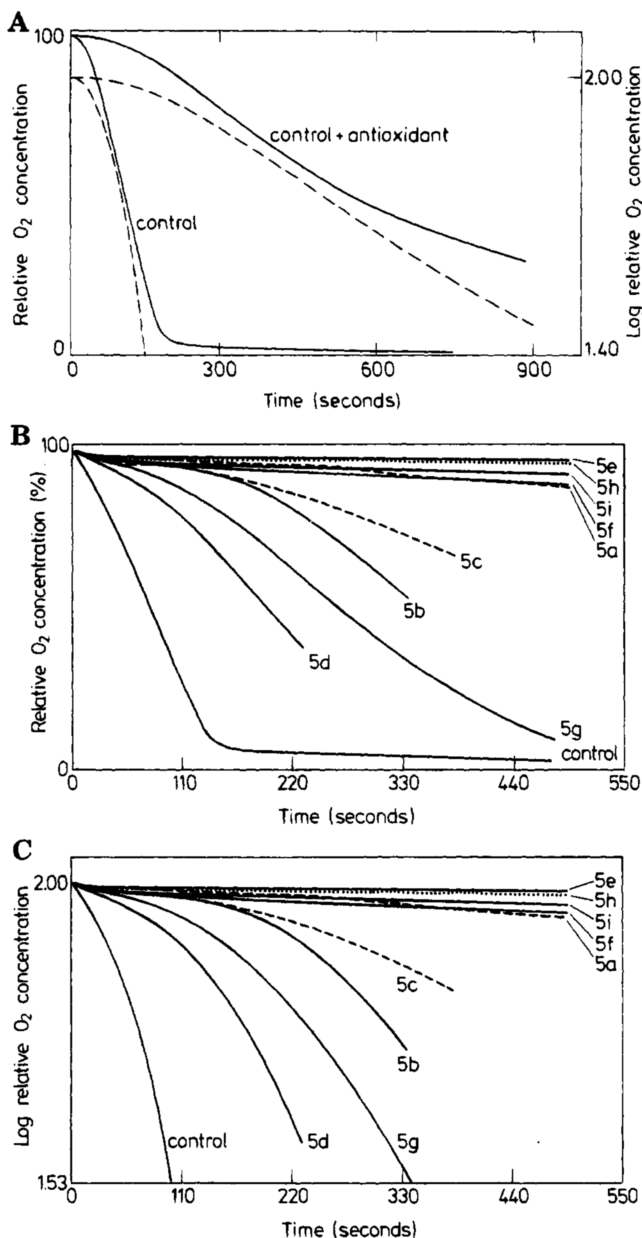

Figure 1. (A, Top) Oxygen concentration (—) and log oxygen concentration (– –) recorded as a function of time for the metmyoglobin catalyzed oxidation of methyl linoleate without (control) and with the addition of an antioxidant (control + antioxidant). (B, Middle) Oxygen concentration recorded as a function of time for the metmyoglobin-catalyzed oxidation of methyl linoleate without (control) and with the addition of successive fractions (5a–5i) from a chromatographic separation of an extract of *S. hortensis*. (C, Bottom) Logarithm of the data from (B).

solution is replaced by the test solution. In our experience a cell volume of 70 μL requires injection of 2 mL of test solution. The polyethylene membrane tolerates water and EtOH, and solutions in these can safely be tested.

Measurement of Relative Antioxidant Activity of Fractions. Each sample (0.1 mg) to be tested was dissolved in water or absolute EtOH (100 μL) and added to the methyl linoleate emulsion. The reaction was initiated by the addition of an aqueous solution of horse heart MMb (100 μL, 0.2 mM). The mixture was immediately injected into the thermostated measuring cell and the recording started. The electrode potential was recorded every 5 s and converted to a data file processed by a spreadsheet (SuperCalc 5.5). The values were displayed as a function of time by connecting the data points with straight lines, giving the curves shown in Figure 1A,B.

RESULTS AND DISCUSSION

Evaluation of Relative Antioxidant Activity. The experimental procedure described above under ideal conditions gives results shown as full curves in Figure 1A. The signal is a function of the rate of several simultaneous processes, such as reaction within the electrode, diffusion through the membrane, and the investigated chemical reaction (Ionescu et al., 1978). An initial phase is followed by the propagation phase with an almost steady state concentration of linoleate radicals ($R^\bullet$) and a rapid decrease in oxygen concentration (Mikkelsen et al., 1992). This terminates when the lower limit for oxygen detection by the electrode is approached, although this limit is seldom reached when extracts with antioxidative activity are tested. As a first approximation, the slope during propagation gives a qualitative measure of the antioxidative activity. Cuvelier et al. (1990) proposed the use of the half-reaction time (i.e. the time when the oxygen consumption rate had dropped to 50%) for this purpose.

Semiquantitative information of the relative rate constants for oxidation may also be obtained. The propagation part of the antioxidant curves reflects the reaction between linoleate radicals and oxygen with a very small activation barrier as rate-determining (Denisov and Khudyakov, 1987). Although the hydroperoxides formed may act as initiators, the MMb present is an efficient ROOH decomposer, accelerating the propagation step. Therefore, the ROOH concentration after an initial phase will become quasi-steady with the rate of formation being equal to the decomposition rate (Denisov and Khudyakov, 1987). The general rate expression

$$d[O_2]/dt = -k[R^\bullet][O_2] = -k'[O_2]$$

simplifies to the pseudo-first-order kinetics under such steady state conditions for production of linoleate radicals (Mikkelsen et al., 1992). Accordingly, a logarithmic transformation of the curves (dashed curves in Figure 1) gives approximately straight lines for the propagation phases with slopes reflecting the relative reaction rates.

When the extract from *S. hortensis* was assayed, these ideal conditions were not fullfilled as shown in Figure 1B,C. The rate constant may change with time since both the amount of MMb degradation (Mikkelsen and Skibsted, 1992; Mikkelsen et al., 1992) and secondary oxidation of linoleate (Halliwell and Gutteridge, 1989) increase and thus influence the concentration of radicals. This effect is observed as a deviation from linearity of the logarithmated curves. Nevertheless, the relative slopes during propagation still give a qualitative measure of the antioxidant activity of the fractions sufficient for systematic guidance of chromatographic separation of the active constituents. The pseudo-first-order rate constants k' were in this case taken as the slope of the curves in Figure 1C after 200 s, although the initial phase is not clearly separated from the propagation phase. Finally, when k' is plotted against fraction number (Figure 2) a conspicuous illustration of the antioxidant activity concentrated in fractions 5a, 5e, 5f, 5h, and 5i may be obtained.

Sources of Error. The reaction rate varies from day to day since the concentration of $R^\bullet$ varies with MMb activity and the concentration of lipid peroxide in the ester (Mikkelsen et al., 1992). All tests run on the same day are affected by the same factor, which means that the relative antioxidant activities are still correct.

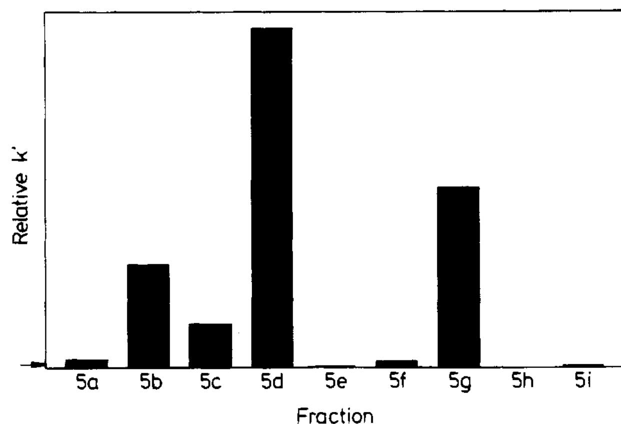

Figure 2. Antioxidative activity measured by the relative rate constants (k') for the same fractions (5a–5i) as shown in Figure 1B. The arrow indicates k' of the extract (5) before separation. The antioxidative activity is concentrated in fractions 5a, 5e, 5f, 5h, and 5i.

Comparison of tests run on different days requires a new measurement at any fraction previously measured for calibration. Some of the plant extracts may contain compounds interfering with oxygen consumption, but this error is negligible when small samples are used as in our routine (0.1 mg of sample compared to 2 mg of methyl linoleate). Like Uhegbu and Pardue (1990), we found that the electrode potential drifts by about 5% of the full scale per hour. However, if the curves are calibrated (taking the initial oxygen concentration as 100%), the relative k' values are not influenced. Ito and Yamamoto (1982) found that the apparent oxidation rate obtained for rapid reactions is underestimated due to the finite response time of the oxygen electrode, but since the delay is below 10 s, this error is negligible in the present case. The analytical coefficient of variation in repeated measurements with the oxygen electrode was found to be about 2% (Siggaard-Andersen, 1974), in agreement with our experience when the membrane is working ideally. Although the data points when connected with straight lines do not always give smooth curves, their numbers are sufficient for a reliable determination of k'. After some time, the membrane gives strongly oscillating data points and must be renewed.

Conclusions. The present assay has proved to be simple and reliable for systematic fractionation of ethanol-soluble antioxidant constituents of *S. hortensis*. In particular, it is superior to other related assays in that it records the antioxidative activity of a fraction within 10 min.

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Received for review September 26, 1994. Accepted February 27, 1995.[⊗]

JF940542Y

[⊗] Abstract published in *Advance ACS Abstracts*, April 15, 1995.