Determination of Amines in Fresh and Modified Atmosphere Packaged Fruits Using Electrochemical Biosensors

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"Natural" amines in plants, endogenous constituents of tissues, are involved in different physiological processes such as fruit development and senescence. Their levels can vary depending on variety, ripening, and storage conditions. Moreover, microbial contamination results in the increase of the "biogenic" amine content of fruits and vegetables. Electrochemical biosensors for the determination of the amine content in fruits have been assembled using diamine oxidase and polyamine oxidase covalently immobilized onto polymeric membranes. Both enzymes in the presence of their substrates produced H_2O_2 that was detected at a platinum electrode polarized at +650 mV versus Ag/AgCl. The best analytical conditions for the two enzyme electrodes have been optimized. The main positive effect of packaging fresh fruits with polymeric films is the provision of a barrier to the gas diffusion; the reduced O_2 and increased CO_2 concentrations slow the respiration rate. The films also allow the maintenance of high relative humidity with reduction of water loss. To investigate the postharvest life of apricots and sweet cherries in modified atmosphere storage at 0 °C, the concentration of amines in fruits packaged using different polymeric films was monitored.

Keywords: *Modified atmosphere packaging (MAP); polymeric film; electrochemical biosensor; amine; diamine oxidase; polyamine oxidase; sweet cherry; apricot fruit; postharvest*

INTRODUCTION

The diamine putrescine (Put) and the polyamines spermidine (Spd) and spermine (Spm), "natural" amines in plants, are endogenous constituents of tissues and are involved in different physiological processes such as fruit development and senescence (Smith, 1985). Their levels can vary depending on variety, maturity, and storage conditions (Winer and Apelbaum, 1986; Biasi et al., 1988; Kushad et al., 1988; Botondi et al., 1996; Escribano et al., 1996).

Polyamines and ethylene (a ripening hormone) biosynthesis have a common intermediate, *S*-adenosylmethionine (SAM) (Lombardini and Talalay, 1971; Cantoni, 1975; Smith, 1975; Adams and Yang, 1977; Bakanashvili et al., 1987), thus the total amine concentration is expected to decrease as soon as fruits begin to synthesize ethylene (Toumadje and Richardson, 1988; Casas et al., 1990; Saftner and Baldi, 1990). Because ethylene and polyamine are known to have opposite effects in relation to fruit ripening and senescence (Dibble et al., 1988; Rastogi and Davies, 1989; Wang and Faust, 1992), exogenous application of polyamines to delay fruit ripening (Apelbaum et al., 1981; Law et al., 1991) has been used.

Modified atmosphere packaging (MAP) in conjunction with low-temperature storage has been recognized as a promising and inexpensive way to improve the shelf life of fresh fruits and vegetables while minimizing product quality impairment (Kader, 1986; Kader et al., 1989; Zagory and Kader, 1989). The main positive effects of packaging fresh product with polymeric films are the maintenance of high relative humidity with the reduction of water loss and the provision of a barrier to the gas diffusion. The consequent reduction of O_2 and increase of CO_2 slow the respiration rate (Ke et al., 1991; Ke and Kader, 1992). The application of MAP, in conjunction with temperature control, for the preservation of the commercial quality of some stone fruits, such as apricots and sweet cherries, for at least 2−3 weeks after harvesting has been considered. The preservation allows distribution over long distances and delayed postharvest commercialization if necessary (i.e., in case of saturation of the market).

Scarce information is available on the levels of polyamines in vegetable food and in MAP-stored fruits (Kramer et al., 1989; Kramer and Wang, 1989). We were interested in monitoring the variation of amine content during sweet cherry and apricot fruit ripening in modified atmosphere storage using different polymeric films in conjunction with storage at 0 °C. For this reason we have assembled electrochemical biosensors using diamine oxidase (DAO) to measure the total amine content and polyamine oxidase (PAO) for specific determination of spermidine and spermine. These innovative analytical systems, based on fast and selective devices that require very little or no sample pretreatment, could result in useful tools to monitor the variation of the amine content in MAP-stored fruits.

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10.1021/jf980159j CCC: $15.00
Published on Web 09/24/1998

EXPERIMENTAL PROCEDURES

Materials. *Apricots.* Fruits of Boccuccia liscia and Pellecchiella varieties were picked early in the morning from a commercial orchard in Caserta (Italy) and transported to the Centro Ricerche Bonomo (CRB) postharvest laboratory within 4 h. The same day, fruits were sorted to remove those with defects or decay and divided into two maturation stages according to peel color (breaker as stage 1 and fully ripe as stage 2). Samples of ~1 kg were prepared and placed in plastic crates. Each one of the crates was then wrapped in plastic bags using low-density polyethylene (LDPE) and Super L films, both from Grace Italiana (Milano, Italy). Control samples were left unpackaged. All fruits were then placed in a cold room at 0 °C. Three replicate samples for each different treatment were taken initially and after 20 days of storage for amine determination.

Sweet Cherries. Fully ripe Ferrovia sweet cherries were picked early in the morning from a commercial orchard in Bari (Italy) and transported to the CRB laboratory within 2 h. The same day fruits were sorted to eliminate those with defects or decay and divided into 2 kg samples in plastic crates. Samples were wrapped and sealed in plastic bags prepared using either MR and Super L films or left unpackaged as control samples and then stored at 0 °C. Three replicate samples for each different treatment were taken initially and after 20 days of storage for amine determination. Additional samples were taken after fruit exposure in air at 20 °C for 24 h.

Chemicals. DAO (cicer seedling; specific activity = 25 U/mg of protein) was obtained following the procedure of Padiglia et al. (1991), and PAO (maize; specific activity = 32 U/mg of protein) was purified according to Suzuki's method (Suzuki and Hirasawa, 1973). Putrescine, spermidine, and spermine were purchased from Sigma Chemical Co. (St. Louis, MO). Cellulose acetate membranes were prepared in our laboratory according to a procedure reported in the literature (Mascini and Mazzei, 1987). Microporous polycarbonate membranes (0.03 and 0.1 μm pores size) were obtained from Nuclepore (Pleasanton, CA); the Immobilon-AV affinity membrane (0.65 μm pore size, 125 μm thick) was from Millipore (Bedford, MA). A commercially available nylon net membrane was also used as a support of the enzymatic mixture.

Methods. *Apparatus.* Electrochemical measurements were carried out with an amperometric biosensor detector (ABD) from Universal Sensors (Metaire, LA). A platinum hydrogen peroxide probe maintained at +650 mV applied potential versus a silver/silver chloride cathode from Universal Sensors was used. Currents were recorded with a model 868 AMEL recorder (Milan, Italy).

Enzyme Immobilization Procedures. Immobilization of DAO was carried out as follows: 16 μL of enzymatic solution was mixed with 4 μL of 0.25% glutaraldehyde, and the mixture was immediately put onto the nylon membrane (used only as physical support) and allowed to dry. The membrane was finally extensively washed (30 min) with glycine (0.1 mol/L) to eliminate the excess of glutaraldehyde.

PAO was immobilized by pipetting 10 μL of enzymatic solution onto one side of the Immobilon membrane [hydrophilic poly(vinylidene difluoride), chemically derivatized to allow covalent protein immobilization through the ϵ-amino groups of lysines] and allowed to react for 30 min. The membrane was then washed with citrate buffer (0.1 mol/L, pH 5.5).

Biosensor Assembling. The probes were assembled by placing the membranes onto a jacket provided with the probe in the given order: a cellulose acetate membrane, the enzymatic membrane, and a polycarbonate membrane (0.03 and 0.1 μm pore size for DAO and PAO biosensors, respectively). The membranes were secured with an O-ring. The jacket was filled with 0.1 mol/L potassium chloride (supporting electrolyte), and the electrode was then inserted into the jacket and screwed down until the tip of the platinum was firmly in contact with the cellulose acetate membrane.

Assay Reaction Scheme. The general reaction catalyzed by DAO is the following:

$$\mathrm{R\text{-}CH_2\text{-}NH_2 + O_2 \xrightarrow{DAO} R\text{-}CHO + H_2O_2 + NH_3}$$

The reactions catalyzed by PAO in the presence of spermidine (Spd) and spermine (Spm) are the following:

$$\mathrm{NH_2(CH_2)_3NH\text{—}(CH_2)_4NH_2 + O_2 + H_2O \xrightarrow{(spd)\ PAO} NH_2(CH_2)_3CHO + H_2O_2 + Dap}$$

$$\mathrm{NH_2(CH_2)_3NH\text{—}(CH_2)_4NH(CH_2)_3NH_2 + O_2 + H_2O \xrightarrow{(spm)\ PAO} NH_2(CH_2)_3NH(CH_2)_3CHO + H_2O_2 + Dap}$$

where Dap is diaminopropane. The enzyme reactions produce H_2O_2, which can be oxidized at the electrode, and the current output is related to the amine content of a standard or sample solution.

Sample Treatment and Procedure of Analysis. Ten apricots and 30 cherries per replicate were stoned and ground to a puree in a Waring blender (Waring, New Hartford, CT). The homogenate was filtered, and the resulting juice was used for the analysis. The content of amines was measured as follows: the biosensor was allowed to equilibrate in 3 mL of working buffer at room temperature and under magnetic stirring until a steady current baseline was reached. This took ~10 min. Three hundred microliters of the juice obtained after filtration was then injected and a current change due to the H_2O_2 production recorded. When the current reached 95% of the new steady-state current, a Put standard solution (at a concentration comparable to that of the sample) was added and the current change recorded again. The signals due to the sample and the standard were used to calculate the concentration of the amine in the original sample.

Measurements with the DAO biosensor were carried out in a 0.1 mol/L phosphate buffer, pH 8 (Draisci et al., 1998). Phosphate buffer was selected because it has been reported as the best of this enzyme (Padiglia et al., 1991). As shown in Figure 1a, Put gave a high and stable current signal in all pH ranges tested, whereas for Spm and Spd the response increased slightly and strongly, respectively, with pH. A more homogeneous amine response at pH 8 was observed.

Measurements with the PAO biosensor were performed in a 0.1 mol/L citrate buffer, pH 5.5. The effect of pH and buffer on the response of the PAO biosensor was studied in the 4.2−9.3 range using phthalate buffer (4.2−5.2), citrate buffer (5.2−5.9), phosphate buffer (5.6−7.4), and borax buffer (8.0−9.3). The concentration used for all of the buffers used was 0.1 mol/L.

As shown in Figure 1b, the probe was able to perform the analysis of both the polyamines over a wide pH range. No significant change in sensitivity was observed with any of the buffers tested in the 5.2−8.0 pH range. Citrate buffer, which gave the most similar response for Spd and Spm (at pH 5.5) was selected for further experiments.

RESULTS AND DISCUSSION

Assay Optimization. The chosen biosensor configuration (cellulose acetate/enzyme/polycarbonate membrane) has been already used for the measurement of several analytes in food matrices (Palleschi et al., 1992; Volpe and Mascini, 1996; Esti et al., 1997). Selectivity for H_2O_2 is achieved by the use of the inner cellulose acetate membrane having a nominal molecular weight cutoff of 100 Da and a thickness of ≈200 μm (as wet cellulose acetate film). However, preliminary tests, run

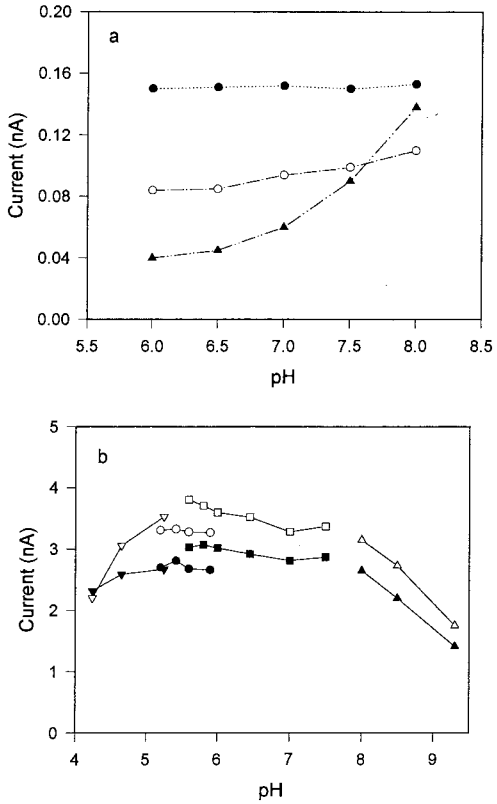

Figure 1. (a) pH profile of DAO biosensor in the 6−8 range for (●) Put, (▲) Spd, and (○) Spm. (b) pH profile of PAO biosensor in the 4.2−9.3 range for Spm (solid symbols) and Spd (open symbols): pH 4.2−5.2 phthalate buffer (▼, ▽); pH 5.2−5.9 citrate buffer (●, ○); pH 5.6−7.4 phosphate buffer (■, □); pH 8.0−9.3 borax buffer (▲, △).

with a probe with no enzymatic membrane and with a 1:10 dilution of the sample in the working buffer, gave rise to current signals due to electrochemical interferents (mainly ascorbic acid and phenolic compounds) both for cherries and for apricots. A higher reduction of interfering signal was obtained by increasing the thickness of the cellulose acetate membrane. Membranes of different thicknesses have been tested using a standard solution of H_2O_2 (2×10^{-6} mol/L) and diluted samples (1:10); the results are reported in Figure 1. Using an 800 μm thick cellulose acetate membrane, the current signal generated by the samples was greatly reduced (97%) with respect to that of a 200 μm membrane. As expected, a decreased sensitivity to H_2O_2 was also observed with this membrane, which resulted in respect to the 200 μm membrane; however, the probe was still able to detect 2×10^{-7} mol/L H_2O_2. Eight hundred micrometer cellulose acetate membranes were used for the amine biosensors, which were assembled with DAO and PAO. These enzymes catalyze the oxidative deamination of the substrates, giving an aldehyde and H_2O_2 as products (see Experimental Procedures) with different substrate specificities.

A previous work carried out by our group demonstrated that immobilized DAO can react with a large number of amines such as Put, Spd, cadaverine, istamine, and tyramine (Draisci et al., 1998). On the other hand, PAO, immobilized onto an Immobilon-AV membrane, was specific for Spd and Spm. Among all of the amines, only Put, Spd, and Spm, which are involved in the physiological processes of plant tissues, were selected and tested with the DAO biosensor. Detection limit, linear range, repeatability ($n = 5$, at 10^{-5} mol/L),

Table 1. Linear Range, Detection Limit, Repeatability, and Response Time for Put, Spd, and Spm, Using DAO and PAO Biosensors

amine	linear range (mol/L)	detection limit (mol/L)	RSD%[a]	response time (min)
	(A) DAO Biosensor			
Put	$2 \times 10^{-6} - 2 \times 10^{-3}$	10^{-6}	2.0	3
Spm	$2 \times 10^{-6} - 5 \times 10^{-5}$	10^{-6}	3.2	3
Spd	$2 \times 10^{-6} - 5 \times 10^{-5}$	10^{-6}	2.5	3
	(B) PAO Biosensor			
Spd	$2 \times 10^{-6} - 1 \times 10^{-3}$	10^{-6}	3.4	2
Spm	$2 \times 10^{-6} - 7 \times 10^{-4}$	10^{-6}	2.8	2

[a] RSD% was calculated at 10^{-5} mol/L of each amine ($n = 5$).

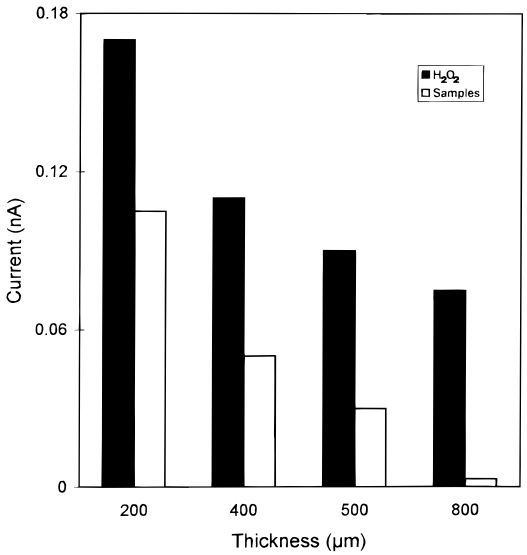

Figure 2. Comparison of the current signals generated by samples diluted 1:10 and H_2O_2 at a concentration of 2×10^{-6} mol/L, using different cellulose acetate thicknesses.

and response time for each amine (90% of the steady state) using both biosensors are reported in Table 1. Parts a and b of Figure 3 report the typical calibration curves obtained using DAO and PAO biosensors, respectively. The linear range common to Put, Spd, and Spm was 2×10^{-6}−5×10^{-5} mol/L (Figure 3a); Figure 3b reports the common linear range, 2×10^{-6}−7×10^{-4} mol/L, for Spd and Spm. These linear ranges were used for the analysis of amines in real samples.

For the DAO biosensor the sensitivity to the amines tested remained unchanged for almost 10 days of continuous analysis, whereas for the PAO probe the lifetime was ∼45 days.

Sample Analysis. DAO and PAO amine biosensors were applied for the determination of amines in sweet cherries and apricots with the purpose to study the differences of amine content in relation to storage conditions. Recovery studies were performed by adding to the samples equimolar mixtures of Put, Spm, and Spd and Spm and Spd for the analysis with DAO and PAO biosensors, respectively. With both analytical systems, we obtained recoveries between 96 and 98%, which allowed us to carry out further analysis in real samples.

Amine contents of samples at harvest stored for 20 days at 0 ± 1 °C (controls in air) and stored at 0 °C ± 1 in MAP with different polymeric films have been compared. Apricots were stored using Super L and LDPE, whereas sweet cherries were stored using Super L and MR polymeric films. Tables 2 and 3 report the amine content measured using the DAO-based biosensor

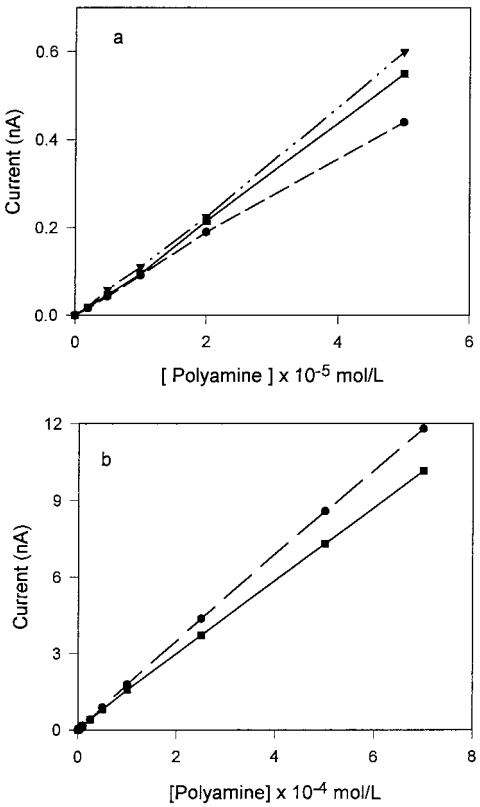

Figure 3. (a) Calibration curves in the linear range common to Put (▲), Spm (■), and Spd (●), using DAO biosensor. (b) Calibration curves in the linear range common to Spd (●) and Spm (■), using PAO biosensor.

Table 2. Total Amines (Milligrams per Liter), Expressed as Equivalents of Put, Measured in Apricot Samples Using DAO Biosensor[a]

		storage time 20 days, temp 0 ± 1 °C		
variety	at harvest	LDPE	Super L	air
Pellecchiella				
ripening time I	5.6 ± 0.5	3.0 ± 0.2	2.9 ± 0.6	3.4 ± 0.6
ripening time II	5.5 ± 0.2	3.1 ± 0.4	3.0 ± 0.7	3.6 ± 0.2
Boccuccia				
ripening time I	5.2 ± 0.2	2.2 ± 0.1	3.7 ± 0.7	4.9 ± 0.2
ripening time II	5.1 ± 0.2	4.6 ± 0.8	4.6 ± 0.4	5.1 ± 0.1

[a] Values are the means ± standard deviation of triplicate samples of fruits.

Table 3. Total Amines (Milligrams per Liter), Expressed as Equivalents of Put, Measured in Sweet Cherry Samples Using DAO Biosensor[a]

at harvest	5.4 ± 0.4
20 days at 0 ± 1 °C with MR film	3.7 ± 0.3
20 days at 0 ± 1 °C with MR film + 1 day at 20 °C in air	4.2 ± 0.2
20 days at 0 ± 1 °C with Super L film	3.1 ± 0.3
20 days at 0 ± 1 °C with Super L film + 1 day at 20 °C in air	3.9 ± 0.2
20 days at 0 ± 1 °C in air	4.5 ± 0.1
20 days at 0 ± 1 °C in air + 1 day at 20 °C in air	4.5 ± 0.3

[a] Values are the means ± standard deviation of triplicate samples of fruits.

for apricots and sweet cherries, respectively. All samples were also analyzed using the PAO biosensor. No current signal was recorded for each of the samples tested, indicating that the two polyamines content was <2.8 mg/L. Thus, the amine level measured with the DAO electrode in apricots and sweet cherries can be reasonably attributed mainly to Put. The amine content at harvest was 5−6 mg/L (measured in fruit juice) for the two varieties of apricots (Boccuccia and Pellecchiella) and for the sweet cherries (variety Ferrovia) with no significant differences between the varieties and the ripening times. After 20 days of storage in MAP, amine content decreased by ∼20% of the initial value. From the data exhibited, it can be noted that all of the polymeric films used work similarly in this respect. Both in apricots and in sweet cherries, the control samples (20 days at 0 ± 1 °C in air) exhibited an amine content slightly higher than that in MAP.

Moreover, an increase in amine content was observed in sweet cherries stored for 20 days in MAP and exposed for 1 day in air (Table 3).

Conclusions. Innovative analytical systems, based on fast and selective devices that require very little or no sample pretreatment, have been optimized and applied for the determination of amine variations in apricots and sweet cherries stored in different conditions.

The analysis carried out with the two systems in fruits stored at 0 ± 1 °C in MAP for 20 days, to preserve commercial quality and sensory attributes, showed that only the DAO biosensor measured small differences of amine content.

Finally, considering that the use of polyamines to extend the shelf life of orchard products is at present studied, we think that the biosensor technology represents an ideal analytical system for monitoring the content of amine in fruits.

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Received for review February 16, 1998. Revised manuscript received June 25, 1998. Accepted July 1, 1998.

JF980159J