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Quantitative Evaluation of Lachrymatory Factor in Onion by Thin-Layer Chromatography

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A thin-layer chromatographic technique and an incubation method have been developed for quantitative evaluation of lachrymatory factor in onion. Both these methods were essentially based on color forming reaction of lachrymators with glycine–formaldehyde reagent followed by measurement of the color at 520 mμ. A linear relationship between the amounts of lachrymator and corresponding optical densities has been ascertained by both these methods. Total lachrymator content of white and red globe as well as Madras varieties of onion was determined by the incubation method, while the relative abundance of individual lachrymators within the varieties was quantitized by thin-layer chromatography.

Within the last few years, especially since the recent advances in chromatographic techniques, increasing attention has been given to an objective method of evaluating the pungent and lachrymatory components of onion. Both these factors arise as a result of the interaction of S-substituted L-cysteine sulfoxide derivatives and enzyme of the alliinase type when the integrity of the onion tissue is destroyed by comminution (Schwimmer et al., 1960; Virtanen and Matikkala, 1959). Pungency of onion is attributed mainly to various disulfides (Bernhard, 1968), while the lachrymatory factor (LF) is a thiopropanal *S*-oxide (Brodnitz and Pascale, 1971).

In recent years several approaches have been made to assess the volatile flavor compounds of onion and onion products by gas–liquid chromatography (Boelens et al., 1971; Saghir et al., 1964) as well as thin-layer chromatography (Bandyopadhyay et al., 1970; Lukes, 1971). However, there have been no objective methods for quantitative evaluation of LF in onion. Thin-layer chromatographic separation of LF in onion in the form of a cysteine derivative on a cellulose plate (Lukes, 1971) and the gas–liquid chromatographic method of isolation of thiopropanal *S*-oxide (Brodnitz and Pascale, 1971) from onion extract have been reported. Recently, Bandyopadhyay and Tewari (1973) have investigated the color-developer (Lukes, 1959; Shannon et al., 1967) involved in pinking of onion purees by thin-layer chromatography, where they have shown for the first time that LF of onion is in fact the color-developer compound(s) consisting of at least three components, all of which are indeed related to the tear-causing factor of onion. Although Shannon et al. (1967) have demonstrated that the color-developer compound reacts with glycine–formaldehyde reagent resulting in a pink color having an absorption maximum at 520 mμ, quantitative data regarding this aspect are not available.

In the present investigation the color-forming reaction of lachrymators with glycine–formaldehyde reagent has been utilized to develop primarily a thin-layer chromatographic (TLC) technique and also an incubation method for quantitative evaluation of LF in onion.

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EXPERIMENTAL SECTION

White and red globe (Nasik, Maharashtra) as well as small Madras variety onions believed to have been stored at ambient temperature (30–34°) for about 4 months after harvest were purchased from a local market. All solvents and reagents were analytical grade. The solvents were redistilled before use.

Glycine–Formaldehyde Reagent. Glycine (0.11 *M*) and formaldehyde (3×10^{-3} *M*) solutions were prepared in distilled water. Freshly prepared solutions were used.

Onion Extract. Onion extract containing the flavor compounds as well as lachrymatory agents was obtained by extracting the respective variety of onions with cold peroxide-free diethyl ether at 0°. Prior to extraction the onions were kept at 0° overnight. Skin- and disk-free onions (200 g) were cut into pieces and macerated with 200 ml of distilled water in a Waring Blendor and the juice was collected by filtering through four layers of mull cloth. The juice was then extracted repeatedly with cold ether (3 × 200 ml) in a separating funnel. The residual pulp was macerated twice with 100 ml of ether each time. Ether layers from both pulp and juice were pooled together after centrifugation, dried over anhydrous sodium sulfate, and filtered. The bulk of the ether was removed in a rotary evaporator at room temperature and the crude extract was then transferred quantitatively into a small glass stoppered container, from which the solvent was finally removed by blowing a stream of nitrogen at room temperature. A 2% chloroform solution of each extract was prepared and kept at −20° under nitrogen until further use.

Reference Lachrymator. A lachrymatory compound identified as thiopropanal *S*-oxide was isolated from the ether extract of white onion purees by high vacuum distillation at 40° and purified as a single component by preparative silica gel TLC according to the method described elsewhere (Bandyopadhyay and Tewari, 1973). This served as a reference compound since a synthetic compound of

Table I. Optical Density Values Measured by Both Incubation and TLC Method from a Solution Containing a 1:1 Proportion by Weight of Reference Lachrymator and Garlic Extract[a]

Sample, μg	OD values obtained by	
	Incubation	TLC
100	0.05 ± 0.003	0.05 ± 0.003
150	0.075 ± 0.003	0.08 ± 0.002
200	0.098 ± 0.002	0.096 ± 0.003

[a] Mean values ± standard error of the mean in five independent determinations.

Table II. R_f Value and Composition of Major Lachrymators in White, Red, and Madras Varieties of Onions Stored for about Four Months at Ambient Temperature (30–34°)

R_f	Amount, mg/100 g of wet onion[a]		
	White	Red	Madras
0.11	1.9 ± 0.20	2.0 ± 0.35	6.9 ± 0.79
0.33	2.2 ± 0.30	2.2 ± 0.10	10.6 ± 1.29
0.57	1.8 ± 0.35	1.9 ± 0.35	9.8 ± 0.71

[a] Mean value ± standard error of the mean in five independent determinations.

similar nature was not available during the course of the experiments. A 0.5% solution of this reference compound in chloroform was prepared and kept under nitrogen at −20° before use.

Estimation of Lachrymator by Incubation. The experimental procedure employed here for quantitative estimation of lachrymators was essentially based on the observations reported by Shannon et al. (1967). Definite volumes in microliters of reference lachrymator solution were taken in test tubes (16 × 1.5 cm) and to each tube 4.5 ml of glycine solution was added. The tubes were then incubated for 15 min in a boiling water bath. After the tubes had cooled to room temperature 0.5 ml of formaldehyde solution was added in each tube. They were then incubated again for 30 min at 50 ± 1° in a water bath. After cooling the tubes to room temperature the optical densities (OD) of the pink colored solution were measured at 520 mμ against a reagent blank in a Bosch and Lomb spectronic 20 spectrophotometer. A reference curve was obtained by plotting increasing amounts of reference lachrymator against optical density. Aliquots of onion extracts were subjected individually to similar treatment and optical densities of the respective samples were measured.

Estimation of Lachrymator by TLC. Silica gel G plates of 400-μ layer thickness were prepared from a slurry of silica gel G (E. Merck) and distilled water (1:2, w/v) drawn on glass plates (10 × 20 cm) with the aid of an adjustable applicator. The plates were activated at 110° for 1.5 hr before use.

Aliquots of different concentrations of reference lachrymator and extracts of white, red, and Madras variety onion were spotted on a TLC plate. The plate was developed at room temperature in a chromatographic tank containing petroleum ether (40–60°)–diethyl ether–acetic acid (60:40:1, v/v) solvent system. Within a few minutes after development and subsequent removal of solvent at room temperature, the plate was uniformly sprayed with glycine–formaldehyde reagent prepared by mixing 9 ml and 1 ml of glycine and formaldehyde solution, respectively, until the plate became just translucent. The whole plate was then carefully covered with a cleaned glass plate and heated in an oven at 98 ± 2° for 30 min in order to develop the pink colored spots. The plate was then cooled to room temperature. Each pink spot from respective samples was carefully scraped off by means of a sharp-edged stainless steel spatula and transferred into a small column (10 × 0.8 cm) fitted with a grease-free stopcock having a filter bed (1–1.5 cm) of Celite (80–120 mesh) over a cotton plug. The column had been previously flushed with 80% methanol and the filter bed was always kept under a few milliliters of the same solvent. Each pink colored compound was eluted separately in 5-ml fractions using 80% methanol as the solvent. The optical density of each fraction was measured at 520 mμ as described above against an experimental control which was a similarly treated blank spot on the chromatogram.

Estimation of Fortified Reference Lachrymator in Garlic Extract Medium by Incubation and TLC. Garlic extract was prepared similarly to onion extracts according to the procedure described above. Equal amounts of each 0.5% chloroform solution of garlic extract and reference lachrymator were mixed together and from this fortified solution the lachrymatory content was quantitatively estimated by incubation and the TLC method reported above.

RESULTS AND DISCUSSION

The reference curve is linear within a concentration range up to 150 μg of reference lachrymator and the optimum range of its determination was found to be within 50 to 100 μg. The lachrymator content of white, red, and Madras variety onions, determined by the incubation study, has been derived from the reference curve by comparing the corresponding optical density values measured in each case from a known concentration (750 μg) of the respective samples, and is found to be 8.8, 9.0, and 36.4 mg/100 g of wet onions, respectively. In this case, reproducible results were obtained when the sample concentrations were chosen within 500 to 1000 μg.

Occasionally, during incubation of onion samples at higher concentrations (1000 μg) of the sample, the solution was observed to be somewhat turbid. Thus, turbidity could be eliminated without affecting optical density values by filtering the solutions through a Whatman No. 1 filter paper previously moistened with glycine–formaldehyde reagent.

TLC estimation of reference lachrymator gave identical values to those obtained by the incubation method. The efficacy of estimating the reference lachrymator in fortified garlic extract by both these methods has been compared. The results of such a fortification are represented in Table I, where both the methods appear to be equally sensitive. Moreover, Table I reveals that there is practically no effect of other ether extractable components of garlic on the color forming reaction of lachrymator with glycine–formaldehyde reagent. A similar observation, though qualitative, in garlic and onion extract was reported earlier by Shannon et al. (1967). A proportional increase in optical density with increasing amounts of reference lachrymator in garlic extract further shows that silica gel is unaffected by spray reagent and thus prewashing of the plate with any organic solvent is not advocated.

Table II summarizes the major lachrymator composition of white, red, and Madras variety onion by TLC. The components having R_f values of 0.11, 0.33, and 0.57, respectively, were estimated from the reference curve by comparing optical density values of the corresponding spots being separated on a TLC plate from a known concentration of the respective extracts. Among these the compound having an R_f value of 0.33 was thiopropanal *S*-oxide as reported earlier (Bandyopadhyay and Tewari, 1973). For quantitation of individual lachrymators present in all the samples,

amounts of 2000 μg of each sample of white and red variety and 750 μg in the case of Madras variety have been found to be suitable for spotting on the TLC plate. With these concentrations all the extracts give rise to identical chromatograms consisting of several pink colored spots of varying intensities detected by glycine–formaldehyde reagent. Among these the major three components of each sample having the same R_f values with respect to each other were estimated. From Table II it appears that the amount of individual lachrymators in both white and red varieties of onion is nearly the same, whereas in the Madras variety these individual lachrymators are present in higher amounts. The small Madras onion is believed to be a "strong" variety in terms of pungency and lachrymatory factor. The present data show that the total lachrymator content in this variety is considerably higher than the other two varieties studied.

The results of the incubation study described previously could give an overall estimation of total lachrymators in onion. This method is, however, not suitable when quantitative information on the relative abundance of individual lachrymator components of onion is required.

The present TLC method of separation of lachrymators followed by estimation of the individual component gives a better understanding of various onion lachrymators, which in turn reflects the quality of onions in respect to their flavor strength.

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Effect of Free and Bound Gossypol on the Absorption of L-[^{14}C]Lysine, L-[^{14}C]Methionine, and L-[^{14}C]Valine from the Rat Small Intestine

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Studies were conducted to determine the relationship of any alteration in amino acid transport and an observed decrease in nitrogen uptake from the gut due to gossypol. The amino acids used were ^{14}C-labeled L-lysine, L-methionine, and L-valine. The effects of free and bound gossypol on the in vivo removal of these amino acids from surgically produced sacs were studied. In vitro determinations of the effect of two forms of gossypol on the kinetic values K_t and V_{max} for these amino acids using everted intestinal sacs were used to evaluate transport. Transport of gossypol alone was studied. In vivo removal of lysine was not significantly altered; methionine was reduced by free gossypol and showed an increase in the sac wall; bound gossypol increased the removal of valine, lessened the amount in the sac wall, and increased deposition in the liver. In the in vitro studies, K_t and V_{max} were increased above the control for lysine-bound gossypol and with free gossypol were increased over the controls for methionine. K_t was unchanged from the control for valine-bound gossypol, while the V_{max} was increased. Gossypol alone was not actively transported.

The nutritive quality of cottonseed meal is affected by a toxic polyphenol, gossypol (Withers and Carruth, 1915), which is largely detoxified during processing by its combination with the free amino groups of the protein with a concomitant reduction in nutritive quality (Smith et al., 1958). Research on cottonseed meal as a nutrient has been directed toward determining the level of the residual free gossypol that may be safely fed to production animals (Heywang and Bird, 1955; Hollon et al., 1958; Sharma et al., 1966), and on the mode of gossypol toxicity in the animal body (Smith, 1957; Albrecht et al., 1968; Skutches et al., 1973).

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Little attention has been given to the interaction of gossypol with the absorptive surface of the gut which is the true interface of nutrients with the body. Fecal nitrogen from rats fed diets with and without bound gossypol was higher for the former as were the individual amino acids in the small intestinal chyme of rats an hour after the diet was consumed (unpublished data). An increased rate of passage of digesta, inhibition of proteolytic enzymes (Lyman et al., 1959; Wong et al., 1972; Finlay et al., 1973; Tanksley et al., 1970), and the action of gossypol on amino acid absorption are all possible factors involved in such observations. Possibly, gossypol in cottonseed protein could affect all amino acid transport systems to effectively reduce the protein level fed, or it could affect one system to effect an amino acid imbalance (Delhumeau et al., 1962).

To determine if gossypol alters the intestinal absorption of amino acids, two experiments were conducted, one in vivo and the other in vitro, using, in both experiments, lys-