13. KJELDGAARD, N. O. AND C. G. KURLAND. 1963. The distribution of soluble and ribosomal RNA as a function of growth rate. J. Mol. Biol. 6: 341–48.
14. MATSUSHITA, S. 1958. Studies on the nucleic acids in plants. I. Nucleic acid contents of cereal and pulse seeds and their nucleic acid containing fractions. Kyoto University Res. Inst. Food Sci. Mem. 14: 14–23.
15. MICHELSON, A. M. 1963. The Chemistry of Nucleosides and Nucleotides. First Edition. Academic Press, New York. p 343–51, 380–81, 530–34, and 562–67.
16. OFENGAND, E. J., M. DIECKMANN, AND P. BERG. 1961. The enzymic synthesis of amino acyl derivatives of ribonucleic acid. J. Biol. Chem. 236: 1741–47.
17. PABST LABORATORIES. 1956. Ultraviolet absorption spectra of 5'-ribonucleotides. Circ. OR-10. Division of Pabst Brewing Company, Milwaukee, Wisconsin. 21 pp.
18. SANTER, M., D. C. TELLER, AND W. ANDREWS. 1960. Environment-induced changes in the base ratios of ribonucleic acid of *Escherichia coli*. J. Mol. Biol. 2: 273–75.
19. SANTER, M., D. C. TELLER, AND L. SKILNA. 1961. Variation in base composition of ribonucleic acid in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 47: 1384–92.
20. SMILLIE, R. M. AND G. KROTKOV. 1960. The estimation of nucleic acids in some algae and higher plants. Can. J. Botany 38: 31–49.

Isolation, Yield, and Fatty Acid Composition of Intracellular Particles from Ripening Fruits [1, 2]

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Introduction

Our interest in quantitative isolations of intracellular particles results from earlier experiments (13) in which the capacity of avocado mitochondria to metabolize α-keto acids was enhanced by the addition of a denatured supernatant fraction. The response of the mitochondria to supernatant fluid increased with ripening of the tissues from which they were extracted. This fact coupled with an apparent decrease in mitochondrial yield from riper fruit led to the suggestion by one of us (12) that the senescense of fruit may be accompanied by a partial dissolution of the particulates with the resultant loss of specific metabolic capacities. Added support for this observation was gained from preliminary investigations of the quantitative intracellular changes accompanying radiation stress in which a decreasing yield of particulate material was noted with ripening of the unirradiated fruit (14).

As discussed by Hanson (7), attempts at quantitative isolations of intracellular particles are beset with serious operational limitations. With fruit tissues the problem is further aggravated by the textural changes that accompany ripening. As progressively less firm fruit is macerated, the viscosity of the homogenate varies in ways which may affect both shearing forces and subsequent sedimentation characteristics. This paper describes, in part, our attempt to ameliorate these difficulties by lyophilizing the homogenate and reducing it to powder form prior to its reconstitution and isolation of particulates. The technique was then applied to a study of mitochondrial yields from ripening fruit.

Additional experiments have been based on the premise that changes in mitochondrial numbers, forms, or functions would involve changes in membrane composition. Supporting evidence is presented for the existence of subcellular membrane changes in ripening fruit based on fatty acid composition of mitochondrial lipids.

Materials and Methods

Homogenization and Lyophilization. Bartlett pears, New Yorker and Bing cherries, and Yellow Newtown apples were obtained from local orchards and stored temporarily at 0°. In the course of the experiment fruits were kept at 20° (cherries at 15°) for several days extending through the period of ripening and senescence. At the indicated intervals representative samples of fruit were removed to a

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cold room. All subsequent isolation procedures were carried out at 0° to 2° with prechilled fruit and solutions.

For the lyophilization experiments pears were first peeled, grated, and combined with 1:3 to 1:5 (v/w) of 0.25 M sucrose plus sufficient 2 M dibasic phosphate to neutralize the vacuolar acids as predetermined by titration of a boiled and homogenized pear sample. Cherries were pitted and combined with adequate 2 M phosphate to again neutralize the acids. To prevent excessive browning 1×10^{-3} M cysteine was also incorporated in the cherry homogenate as suggested by Catlin (2). A partial homogenization was obtained by blending for 30 seconds at speeds sufficiently slow to prevent the formation of a suction cone. Manual control was achieved with a voltage regulator. Pear homogenates were immediately distributed in appropriate flasks, shell-frozen, and lyophilized. Cherry homogenates were passed through 1 layer of cheesecloth to remove a major part of the skins prior to freezing and lyophilization. After lyophilization the dry homogenates were finely ground in a mortar and the resultant powders stored at −20° in tightly sealed jars.

When the homogenates were prepared for the immediate extraction of particulates rather than lyophilization the initial maceration was more thorough with the addition of 2 parts (v/w) of sucrose-buffer solution and a longer (1 min) controlled blending period.

Isolation of Particulate Matter. Lyophilized homogenates were reconstituted with 10 parts (v/w) of 0.25 M sucrose, containing 0.01 M potassium phosphate buffer, pH 7.4, and ground for 1 minute in a mortar. The amounts of buffered sucrose solution used either for the immediate extraction of fresh homogenates or for reconstitution of lyophilized preparations resulted in approximately similar ratios of tissue to suspending medium with each method. Thereafter, standard differential centrifugation techniques were used for separation of the intracellular particles. A low-speed centrifugation (10 min at $1000 \times g$) to remove cellular debris was followed by a high-speed centrifugation (15 min at $18{,}000 \times g$) to collect the particulates. The pellet was resuspended in 0.25 M sucrose containing 0.01 M potassium phosphate buffer, pH 7.4, and centrifuged again at low speed to remove additional small amounts of debris. This was followed by the second high-speed centrifugation. The pellets were then resuspended in prescribed amounts (3–6 ml) of the buffered 0.25 M sucrose solution.

Larger volumes of the supernatant fraction from the slow speed centrifugation of apple or pear homogenates were processed through a Szent-Gyorgyi continuous-flow apparatus to collect sufficient amounts of particulates for subsequent fatty acid analyses. A centrifugal force of $18{,}000 \times g$ and a flow rate of 20 ml per minute were used. The pellet surfaces were first rinsed with distilled water, suspended in a small volume of water, and then lyophilized.

Electron Microscopy. Mitochondria sedimented from the washing suspension were fixed with equal parts of a 2% solution of osmic acid and .05 M Tris (pH 7.4) in 0.25 M sucrose. Following dehydration in a graded series of ethyl alcohol the specimen were embedded in epoxy resin and post-stained with saturated uranyl acetate. Sections were observed with an RCA-EM 43 electron microscope.

Analyses. Acid digestion and the Nessler colorimetric assay were used for the determination of nitrogen. In some of the later experiments a modified Lowry technique (9) was used for protein assay.

Lipids were extracted from lyophilized particulate preparations by the method of Bligh and Dyer (1). The procedure was appropriately scaled down and further modified by withdrawing the nonpolar phase with a pipette after centrifugation at approximately $1500 \times g$ thus omitting the filtration step.

Samples of the liquid extract were esterified for gas chromatography by trans-esterification in anhydrous HCl-methanol for 12 hours at 50°. The reagents and subsequent purification steps were as described by Stoffel et al. (15) except for omission of the sublimation step.

Gas chromatographic analyses of the fatty acid methyl esters were performed under the following conditions: column, 2 m stainless steel, 6.4 mm OD; substrate, 25% DEGS (Wilkens Instrument Company, Walnut Creek, California) on 60-80-mesh firebrick; temperature, 210 to 215°; carrier gas, helium at 100 ml/min; detector, thermal-conductivity cell calibrated with a fatty acid methyl-ester mixture of known composition.

The fatty acid methyl esters in the samples were identified by cochromatography under the above conditions as well as on another substrate, 25% Lac-2-R 446 (Wilkens Instrument Company, Walnut Creek, California). Identifications were further verified by plotting retention time versus chain length or degree of unsaturation (10).

Absolute measures of extracted lipids were not obtained other than in initial experiments when each of two 300-g samples of fresh pears yielded approximately 200 mg of lyophilized particulates which in turn rendered 15.2 and 16.9 mg of lipid. These amounts were found quite adequate for chromatographic analysis and similar or larger sized samples were used in all subsequent experiments.

Results

Use of Lyophilized Homogenates. Since fresh deciduous fruit are available for only a few weeks during the year there is an obvious advantage in preparing stable, lyophilized homogenates for subsequent extraction. A second advantage sought in these experiments was the fact that dry, powdered homogenates of similar physical characteristics could be obtained from fruit which differed in texture due

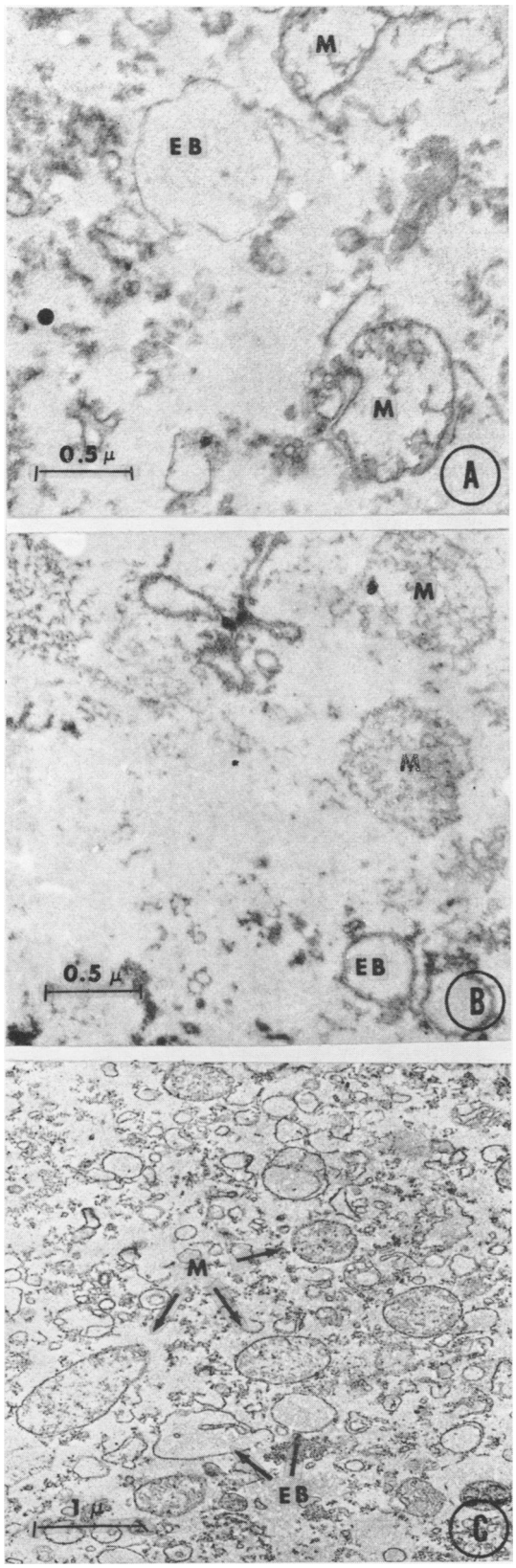

Table I. *Effect of Lyophilization on the Yield of Particulate Material from Pear Homogenates*

Treatment of homogenate	Yield*
Centrifuged immediately	0.044
Lyophilized	0.085

* Particulate N per gram fresh tissue.

to the ripening process. Table I illustrates the particulate yields obtained from fresh and lyophilized homogenates prepared from similar fruit. The additional maceration achieved both in preparation of the dry powder and in its reconstitution, resulted in a considerably higher particulate yield per given amount of original fruit tissue.

Electron micrographs of final pellets are shown in figure 1, A, B, and C. A and B are sections from fresh and lyophilized portions of the same pear homogenate. In so far as could be discerned similar structures are obtained from both fresh and lyophilized homogenates. Part C is a pellet cross-section with a much higher density of mitochondria illustrating one of the better yields from a fresh pear homogenate. In addition to mitochondria, parts of endoplasmic reticulum, and other unidentified inclusions, all preparations contained bodies surrounded by a distinctly stained membrane but having no internal structure. It is not known if these "empty bodies" were artifacts produced during the isolation.

Particulate Yield with Increasing Fruit Ripeness. Experiments with New Yorker cherries completed in 1961 are summarized in table II. The particulate yield values for the progressively riper fruit are the averaged results of 5 replicate isolations from each of the lyophilized homogenates. The experiments were repeated in 1962 with Bing cherries (table III) with 4 replicate isolations from homogenates prepared at each ripening date. In both years, particulate yields declined with advancing senescence of the tissue. Definitive data on the stage of ripeness could not be derived from the respiration rates. However, in both years the indicated number of days at 15° span the sequence of physiological changes from firm, harvest mature to soft, overripe cherries.

Particulate yields are given as percent of the combined soluble and particulate nitrogen present in the supernatant after the first low speed centrifu-

FIG. 1. Electron micrographs of cross-sections of once washed mitochondrial pellets isolated from pears. A and B are isolations from a fresh and a lyophilized portion respectively of the same pear homogenate. C is a more inclusive view of a pellet from fresh homogenate. M, mitochondria; EB, empty bodies. Magnification: A and B, 25,000 X; C, 15,000 X.

Table II. *Yield of Particulate Material from Ripening New Yorker Cherries*

	Days at 15°		
	0 and 1	5	7
Yield* (avg of 5 isolations)	2.28	1.62	1.56
Yield as % of 0 and 1 day	100	76	70
Range		(32–110)	(51–81)
Student's t value**		2.0	10.0
		(Not sig.)	(Sig.–0.01)

* Yield = particulate N as percent of total N remaining in the supernatant fraction after the first low-speed centrifugation.

** Student's t distribution test was applied after the yields in each extraction were expressed as percent of the 0 and 1 day period to allow for differences among the separate series of extractions performed by different individuals ($t_{0.05} = 2.36$, $t_{0.01} = 3.355$, 8 df).

Table III. *Yield of Particulate Material from Ripening Bing Cherries*

	Days at 15°			
	0 and 1	3	7	9
Yield* (avg of 4 isolations)	6.34	5.75	5.10	4.6
Yield of % of 0 and 1 day	100	87.5	79.5	70.5
Range		(64–105)	(65–95)	(57–88)
Student's t value**		1.56	2.93	4.91
		(Not sig.)	(Sig. 0.05)	(Sig. 0.01)

* Yield as described for table II.

** Student's t test applied as described for table II ($t_{0.05} = 2.447$, $t_{0.01} = 3.707$, 6 df).

Table IV. *Yield of Particulate Material from Ripening Bartlett Pears*

	Stage of the climacteric			
	Min	On rise	Peak	Post
Avg yield*	7.2	7.5	6.4	4.9
No. of isolations	16	16	17	7
Range of values	5.8–8.4 %	5.4–9.2 %	4.9–7.7 %	3.4–5.9 %
Student's t value**		0.88	2.39	5.52
		(Not sig.)	(Sig. 0.05)	(Sig. 0.01)

* Yield as described for table II.

** Student's t test was run following an analysis of varience. The values given are for the levels of significance between yield at the climacteric minimum and those obtained at the more advanced stages of ripeness.

Table V. *Fatty Acid Composition of Particulates Isolated from Ripening Pears and Apples*

Days at 20°	Approx stage of ripeness	Fatty acid methyl ester (% of total)				
		Palmitic	Stearic	Oleic	Linoleic	Linolenic
Pears						
0	On rise	22 ± 1	2.2 ± 0	6 ± 1	62.3 ± 0.4	7.5 ± 0
2	Near peak	26 ± 2	2.2 ± 0	7 ± 1.8	59 ± 1.7	5.4 ± 0.9
7	Post-	25 ± 1	3 ± 1	20 ± 0	49 ± 0.3	3 ± 1
Apples*						
0		23 ± 1	5.2 ± 2.7	4 ± 2	48 ± 5	19 ± 1
2		24 ± 1	3.2 ± 0.5	1.8 ± 0.4	51 ± 2	21 ± 2
7		23 ± 2	2 ± 0	7.6 ± 0.5	56 ± 2	11 ± 1

* Respiratory activity of apples was not diagnostic of physiological state. Progressive ripeness was only that which can be assumed to have taken place during the given interval of time at 20°.

gation. This mode of expression obviates anomalies that would arise from occlusion or initial denaturation of protein, from a variable moisture content of the lyophilized powders, the occasional discarding of improperly dried portions of the lyophilizate, and other discrepancies that could result in attempts to relate yields to original fresh weight.

Changes in the course of senescence are shown by expressing the particulate yields from the ripening fruit as a percent of the yields obtained on the first and second days of the experiment. This is done to facilitate the statistical evaluation of a series of extractions performed by several different workers where the absolute yields may have differed, though similar effects of senescence pertained.

The yields of particulate material from ripening pears are given in table IV. In these experiments the physiological age of the fruit was accurately assessed by measurements of the respiratory rates and isolations were made from fruits which were lyophilized at the indicated stage of the climacteric. The experimental results of numerous particulate isolations performed by 3 separate individuals were combined and analyzed statistically.

A significant decrease in particulate yield was apparent with fruit at the climacteric peak followed by a more pronounced decrease in yield from the post-climacteric pears. A slight though not statistically verifiable increase in mitochondrial yield during the climacteric rise is perhaps related to a phase of protein synthesis as discussed by Hulme (8).

Changes in the Fatty Acid Composition of Particulates. Table V lists the principal fatty acids in the lipids extracted from the fruit particulates. The values, given as percent of the total fatty acids extracted, are the averages of 2 separate experiments with apples and 1 with pears. Two or more chromatographic analyses were run on each sample of extracted fatty acids. An increase in oleic acid with a concomitant, but not equivalent reduction in linolenic acid, was noted as mitochondria were isolated from increasingly more senescent pears. This trend was present but less well defined in the mitochondria from apples. Minor changes in the other major fatty acids were not considered significant under the experimental conditions. Several other fatty acids, including 2 tentatively identified as lauric and myristic, were present in trace amounts in both the pear and apple particulates.

Discussion

Observations with the electron microscope suggest that the particulate matter isolated from either lyophilized or fresh homogenates of cherries and pears consists principally of broken segments of mitochondria, whole mitochondria (with cristae apparent), "empty bodies" and endoplasmic reticulum. Of the particulate preparations discussed in this paper only those from fresh pear homogenates demonstrated Kreb's cycle activity and limited phosphorylative capacity. In accord with Hanson (7), a more thorough biochemical and morphological characterization would be required to affirm the cytological derivation of the particulates. However, for the comparative aspects of these present experiments it is sufficient that both fresh and lyophilized homogenates yield particulate fractions composed of similar structural entities.

Transitions in mitochondrial structures are known to occur in detached leaves (16), and during the aging of other tissues (3, 5, 6). Striking evidence for the presence of cytoplasmic reorganization in the placental cells of ripening tomato has also recently been presented by Cocking and Gregory (4). The presence of cytoplasmic transitions during the senescence of fruit tissues is indirectly supported by the particulate yields and changes fatty acid composition presented in this paper.

Statistically significant decreases in particulate yields with senescence of the tissues have been obtained in several experiments comprising over 100 separate isolations performed by 6 different individuals. Further substantiation of these data may be derived from density gradient experiments as indicated in a preliminary study (11), from shifts in metabolic capacities of the particulates, and from a more extensive use of electron microscopy. At present, extrapolation to cytoplasmic events must remain speculative.

Summary

A decrease in intracellular particulate matter was shown to accompany the senescence of cherry and pear fruit. The estimation of particulate yields was facilitated by lyophilization of homogenates to preserve the material for subsequent and multiple extractions and to mitigate some of the physical changes accompanying ripening. As indicated by electron micrographs, comparable particulate material could be isolated from fresh and lyophilized homogenates.

A minor shift to the more saturated fatty acids was noted in the lipid fraction of mitochondria from ripening pears and apples.

Note. Delineation of the structures and introduction of the term "empty bodies" in our corrected manuscript was influenced largely by the description of somewhat similar entities, termed "empty zones," in muscle cells (Shy et al. Science 145:493–96, July, 1964). Evidence that these structures were not anomalous products of our isolation procedures has recently been offered by Dr. Virginia Sjaholm of our department (unpublished data) who has noted similar bodies in the cells of peach callus.

Acknowledgment

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Literature Cited

1. BLIGH, E. G. AND W. J. DYER. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911–17.
2. CATLIN, P. B. 1963. Active cytoplasmic particulates from developing apricot fruits. Proc. Am. Soc. Hort. Sci. 83: 217–25.
3. CHERRY, J. H. 1963. Nucleic acid, mitochondria, and enzyme changes in cotyledons of peanut seeds during germination. Plant Physiol. 38: 440–46.
4. COCKING, E. C. AND D. W. GREGORY. 1963. Organized protoplasmic units of the plant cell. I. Their occurrence, origin, and structure. J. Exptl. Botany 14: 504–11.
5. DEMPSEY, E. W. 1956. Mitochondrial changes in different physiological states. Ciba Found. Colloq. Aging, Vol. 2, Aging in Transient Tissues. J. A. Churchill Ltd., London.
6. ELLIOT, A. M. AND I. J. BAK. 1964. The fate of mitochondria during aging in *Tetrahymena pyriformis*. J. Cell Biol. 20: 113–29.
7. HANSON, J. B. 1963. The yield of mitochondrial material from maize roots. Physiol. Plantarum 16: 814–21.
8. HULME, A. C. 1963. The respiration climacteric in apple fruits. Proc. Royal Soc. (London) B. 158: 514–35.
9. MILLER, G. L. 1959. Protein determinations for large numbers of samples. Anal. Chem: 31: 964.
10. PECSOK, R. L. (ed.) 1959. Principles and Practice of Gas Chromatography. Chapman Hall Ltd., London. p 135–50.
11. PRASAD, R. AND R. J. ROMANI. 1963. Separation of intracellular components of fruits by density gradient centrifugation. A paper presented at the Western Section, AAAS, Stanford, California.
12. ROMANI, R. J. 1955. Intracellular metabolism of the avocado fruit. Ph. D. thesis. University of California, Los Angeles, California.
13. ROMANI, R. J. AND J. B. BIALE. 1957. Metabolic processes in cytoplasmic particles of the avocado fruit. IV. Ripening and the supernatant fraction. Plant Physiol. 32: 662–68.
14. ROMANI, R. J. AND J. G. VAN KOOY. 1962. Effect of large doses of gamma radiation on the intracellular particles of fruit. Abstr. Second Intern. Cong. Rad. Res., Harrogate, England p 138.
15. STOFFEL, W., F. CHU, AND E. H. AHRENS. 1959. Analysis of long-chain fatty acids by gas-liquid chromatopraphy. Micromethod for preparation of methyl esters. Anal. Chem. 31: 307–08.
16. WEBSTER, G. C. 1959. Nitrogen Metabolism in Plants. Chapter 5. Row, Peterson and Company, Evanston, Illinois.

Activity of Mitochondrial Preparations Obtained from Faris Sweet Lemon Fruit [1]

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Active mitochondrial preparations have been obtained from a wide variety of plants (1) but apparently have not been reported for citrus[2]. Some of the difficulties which may have interfered with citrus fruit preparations are believed to be the highly acid cell sap, the low concentration of proteins, and the high concentration of pectins.

By modifying the usual methods of obtaining active mitochondrial preparations (1, 5), it was possible to obtain preparations of citrus fruit which oxidized citric acid cycle intermediates, esterified P_i, and formed and accumulated some organic acids.

[1] Revised manuscript received November 16, 1964.

[2] After submission of this paper, an abstract on mitochondrial preparations from citrus appeared (7).

Materials and Methods

Young Faris sweet lemons (*Citrus limon* Linn. Burmann), about 4 cm in diameter, were picked and cooled to 1° for 2 to 3 hours before extraction of the mitochondria. About 200 g of cold, peeled fruit were grated in 300 ml of cold 0.6 M sucrose solution containing 0.25 M Tris buffer adjusted to pH 7.8 with KOH. It was necessary to grate the fruit directly in the buffer to neutralize immediately the acids in the cell sap. During the grating procedure, the pH of the buffer was determined with pH indicator paper occasionally, and was adjusted with 5% KOH in 0.6 M sucrose solution.

Although this was the preferred method, it was also possible to infiltrate cold buffer into the chopped fruit at a reduced pressure (10–15 cm Hg) in an