Histochemistry of fleshy fruits polyphenols, cryostabilization and GMA embedding. Histotopochemical preservation and detection of polyphenols in fruits

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Abstract

In the process of wax embedding the pulp of fleshy fruits is subsequently subjected to the action of the fixatives, the dehydrating agents (ethanol, acetone), the clearing agents (xilene) and finally to hot melted wax.
Polar resin embedding is now proposed to preserve enzyme activity in semithin sections for high resolution optical microscopy.
In our experience the polar resin embedding (Glycol methacrylate, Kulzer 7100) process can be modified to avoid the action of chemical fixatives, dehydrating agents and apolar clearing solvents. In the process of "cryosubstitution" tissue water is gradually substituted, working at -20°C, by solutions of Ethylene Glycol; then the tissue is infiltrated by the hydrophilic monomer, always working at subzero temperatures, the polimerization is obtained at about 10°C.
The procedure has been tested on several fleshy fruits and at present we are working to confirm the preservation of lipids and waxy components, but also some water soluble components, notably polyphenolic substances, such as tannins. In fact lipids are easily extracted by apolar clearing solvents, but the dehydrating solutions (ethanol or acetone) may extract more polar components, or displace them from their original location.

Introduction

Epidemiologic studies support the view that diets rich in fruits and vegetables are associated with a reduced incidence of chronic and degenerative pathologies such as cancer, diabetes, obesity and cardiovascular diseases. Benefits of these diets must be imputed to the synergistic effect of a complex mixture of various phytochemicals. In fact are present several thousands of these compounds, differing in molecular size, polarity and solubility; but now phenol compounds (polyphenols) are proposed as the active phytochemicals of fruits.
The evaluation of phytochemicals dietary intake still lacks precision as most of the data on their content in food originate from scattered and non standardized sources. Moreover their bioavailability is affected by the distribution in different subcellular organules, cells, tissues and organs. Significant differences in phytochemicals among fruit species, *cultivars* of the same fruit, region and orchard productions but also during storage and food processing are reported. Thus the chemical, qualitative and quantitative information on polyphenols has to be supported by the precise localization in the fruit tissues, and also in their cellular and extracellular compartments. Until now the application of histochemical reactions to fruit tissues appears seldom applied to study components distribution in watery fruit flesh. In fact cellular organization is based on the interactions between water, ions and organic molecules; thus the high water content in cells and tissues represents a relevant problem during the processing of fleshy fruit samples for light microscopy.
In this paper we confirm the "cryostabilization" as a new easy and performing technique for the histochemical study of tannin like substances (condensed polyphenols) in flesh of fruits during ripening and post-harvest. To reveal the precise localization of polyphenols our cryostabilization method – based on Ethylene Glycole (at –20°C) and Glycol Methacrylate embedding, at low temperatures (6-10 °C) - has been applied to some fruits.

Materials and methods

This study was operated on different fruits: apple (*Malus domestica* Bork., *cv* Braeburn) unripe and ripe, grape (*Vitis vinifera* L., *cv* Chardonnay), sweet cherry (*Prunus avium* L.), kiwi (*Actinidia chinensis*) and tomato (*Lycopersicon esculentum*).

Small samples (few mm^3) of this fruits were cut and placed in water /Ethylene Glycol (EG) solution of increasing concentration (50%, 75%, 90%, 95%), each step 24 hs, at -20°C. In this process -called by us "cryosubstitution"- water tissue is gradually substituted by EG. Then the samples were placed in a 1:1 EG and polar resin Glycol Methacrylate (GMA Technovit 7100-Kulzer) monomer solution, during 24 hours at + 4°C. After this steps the samples were embedded in polar resin GMA.
Sections (2.5 µm) were obtained on glass "Ralph" knives, by a rotary microtome, stretched on water and then examined after some staining procedures: Toluidine Blue O (Debeaujon *et al*, 2000; O'Brien *et al*, 1964; Clark, 1981) and Fe^{++} reaction. Moreover, Fast Blue B reacts specifically with polyphenolic compounds (O'Brien and McCully, 1981), resulting in the reddish-brown reaction production (Bilkova *et al*, 1999).
The content of polyphenols was observed under light microscope, and assessed by visual estimation from independent observers.

Results and discussion

Sample preparation is a critical step in analysis, especially in biological samples. The substitution of cellular water with any other solvent unavoidably causes some alterations, thus the modification, or the loss, of chemical components or biological properties (such as antigen and enzyme activities), are frequently observed. Besides, chemical fixatives (metal ions, acids, alcohols) acts by the alteration of water/molecules interactions and by harsh proteins denaturation. Not coagulative fixatives, such as cross-linking aldehydes, likewise interact strongly with the most reactive groups of the tissue native components (Pearse, 1978).
In our experience the use of Ethylene Glycole, an antifreeze and cryoprotectant agent, if associated with polar resin embedding, preserve diffusible and labile components (such as starch, glycogen, lipids and possibly also metal ions) and meanwhile a good morphology. Because of the use of polar substituents, at low temperatures, the method preserves enzyme activities and lipid and wax components (Ponso *et al*, 2003). In facts, lipids as many other components are easily extracted or displaced by apolar dehydrating and clearing solvents and enzyme activities severely inhibited (Dore, 1992).
The process proposed is easy to perform and does not require specialized instruments. The polar resin, harder than paraffin, allows thinner sections, so a higher optical microscopy resolution may be reached. Notably, the possibility of substituting chemical fixatives, toxic and carcinogenic, with the lower toxicity of Ethylene Glycol is interesting: it brings lower risks for the worker and the environment.
In traditional embedding processes, tannins-like substances are easily solubilized in water/ethanol and water/acetone solution, so they can be easily displaced from the original localization (predominantly the vacuole), and then react to cell such as cellulosic walls structures. Moreover, the action of chemical fixatives on the vacuolar fluid soluble proteins may form aggregates of insoluble protein-tannin complexes (Vallania *et al*, 2004).
Here we report the preliminary results of a test over some fleshy fruits, in which the histotopochemical presence and distribution of tannin like substances (polyphenols) is compared with the analytical bibliographic data.
Phenolic compounds are not uniformly distribuited within the tissue of fruits. At the subcellular level, phenolics are deposited in the cell wall and stored in vacuoles, whereas, at the tissue level, phenolics are concentrated in the epidermal and subepidermal layers of fruit (Asami *et al*, 2003), as reported in the table (Tab. 1).

	References	EG
Apple	total polyphenols: 66,2 mg/100g and 211,9 mg/100g depending on variety (Podsedek *et al*., 2000). Condensed tannins are 71-90% (Vrhosek *et al*., 2004; Lotito *et al*., 2004) and they are localized in flesh and peel of apple (Wolfe *et al*., 2003).	The reaction of Fast Blue B in unripe apples is strong and diffused in vacuoles of the cells of six-seven layers of pericarp (Photo 1 a). In ripe apples the reaction is limitated only in the vacuoles of cells that constitute the first and the second layer of pericarp.
Kiwi	total content of phenols: 274,4 mg/100g (Imeh *et al*., 2002).	The reaction is stronger in cell saps and in cell walls, the reaction is present also in deeper layers than the other fruits examined (Photo 1 b).
Grape	content phenolic acids, flavonoids, anthocyanins, proanthocyanins (Yusuf and Toledo, 2004).	reaction of Fast Blue B is visible, as soluble compounds, in vacuoles of cell of the first and the second layers of pericarp (Photo 1 c, d).
Sweet Cherry	contained: phenolic acids, flavonoids, anthocyanins (Gonçalves *et al*, 2004).	condensed polyphenols are located in cells of first layers of pericarp, inside the superficial cell were visible anthocyanins whit a stronger reaction.
Tomato	total contents: 13,15 mg/100g and 1,3 mmol/g (Minoggio *et al*, 2003), is rich in phenolic acids and flavonoids but condensed tannins are absent.	In this fruits we can not reveal the reaction. This result point out that in tomatoes are accumulated soluble phenolic substances of low molecular-weight.

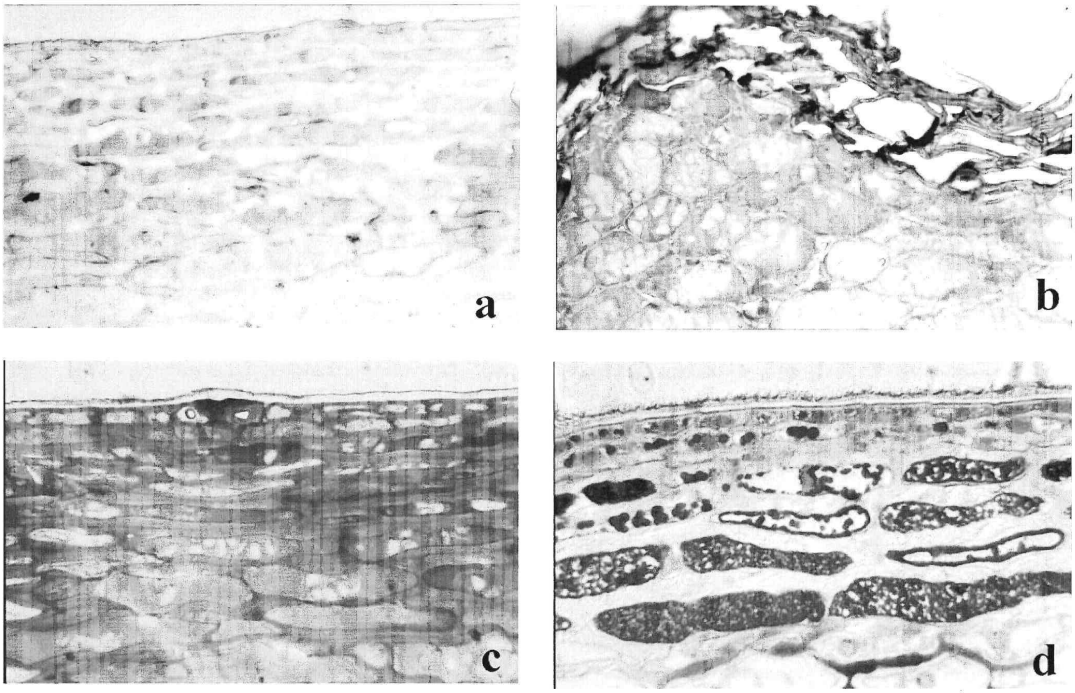

Fig. 1 - a) Apple, cv Breaburn, unripe. EG cryostabilization, Fast Blue B. Reaction in the vacuoles of superficial layers.
b) Kiwi, unripe. EG cryostabilization, Fast Blue B. Reaction in cell sap and walls. Starch granules appear as unstained.
c) Grape, cv Chardonnay, ripe. EG cryostabilizazion. Toluidine Blue. Diffuse reaction in cell vacuoles.
d) Grape, cv Chardonnay, ripe. Formaldehyde fixation. Toluidine Blue. Condensed heavily reactive granules in the vacuoles.

Obviously more work is due to asses and confirm the possibility to observe and discriminate between the "tannin-like substances" here evidenced and other polyphenols molecules reported in the fruits.

References

Asami D K, Hong Y J, Barrett D M, Mitchell A E. 2003. Processing induced changes in total phenolics and procyanidins in clinstone peaches. J Sci Food Agric. 83: 56-63.

Bilkova J, Albrechtova J, Opatrna J. 1999. Histochemical detection and image analysis of non specific esterase activity and the amount of polyphenols during annual bud development in Norway Spruce. J Exp. Bot. 35: 1129-1138.

Clark G. 1981. Staining procedures. William and Wilkins. Baltimore, London. Pp 35.

Debeaujon I, Leon-Kloosterziel KM, Koornneef M. 2000. Influence of the testa on seed dormancy germination and longevity in *Arabidopsis*. Plant Physiol. 122: 403-414.

Dore B. 1992. Appunti di tecniche istochimiche per il laboratorio di tecniche microscopiche istologiche ed istochimiche. CLU, Torino. Pp 216.

Gonçalves B, Landbo AK, Let M, Silva AP, Rosa E, Meyer AS. 2004. Storage affects the phenolic profiles and antioxidant activities of cherries (*Prunus avium* L.) on human low-density lipoprotein. J Sci Food Agric. 84: 1013-1020.

Imeh U. and Khokhar S. 2002. Distribution of conjugated and free phenols in fruits: antioxidant activity and cultivar variation. J Agric Food Chem. 50: 6301-6306.

Lotito SB. and Frei B. 2004. Relevance of apple polyphenols as antioxidants in human plasma: contrasting in vitro and in vivo effects. Free Radic Biol Med. 36: 201-211.

Minoggio M, Bramati L, Simonetti P, Bardana C, Iemoli L, Santangelo E, Mauri PL, Spigno P, Soressi GP, Pietta PG. 2003. Polyphenol pattern and antioxidant activity of different tomato lines and cultivars. Ann Nutr Metab. 47: 64-69.

O'Brien TP, Feder N, McCully ME. 1964. Polychromatic staining of plant cell walls by Toluidine blue. Protoplasma. 59: 368-373.

O' Brien TP and McCully ME. 1981. The study of plant structure principles and selected methods. Termarcarphi, Melbourne, Australia.

Pearse AGE. 1978. Trattato di istochimica. Piccin Ed, Padova.

Podsedek A, Wilska-Jeszka J, Anders B, Markowski J. 2000. Eur Food Res Technol. 210: 268-272.

Ponso A, Ruffa E, Dore B, Me G. 2003. The ripening process studied using histochemistry on cryostabilized apple tissues. Eur J Histochem. 30th national congress of the Italian society of histochemistry. Rapallo.

Vallania R, Ruffa E, Miaja ML, Me C, Caramiello R, Dore B. 2004. Molecular and histochemical analysis of *Vitis vinifera* L. cultivars. Acta Horticulturae 663: 651-655, ISHS.

Vrhosek U, Rigo A, Tonon D, Mattivi F. 2004. Quantitation of polyphenols in different apple varieties. J Agric Food Chem. 52: 6532-6538.

Wolfe K, Wu X, Liu RH. 2003. Antioxidant activity of apple peels. J Agric Food Chem. 51: 609-614.

Yusuf Y, Toledo RT. 2004. Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin and gallic acid. J Agric Food Chem. 52: 255-260.