COMBINED EXTRACTION AND PAPER CHROMATOGRAPHY OF FOOD PRESERVATIVES

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If a foodstuff is applied direct on paper for the chromatographic separation of food preservatives, food components interfere with the migration, and by masking the spots on the chromatogram, make this rather difficult to interpret. For this reason, a preliminary extraction step usually precedes paper chromatography of ether-soluble preservatives. A large number of extraction funnels and other items of glassware are needed for the extraction, if several analyses are to be made simultaneously. Further trouble is occasioned by emulsions; these are formed easily and can make the funnel extraction of fruit juices and jellies a rather difficult matter.

The purpose of this study is to find a remedy for these difficulties. Use is made of the well-known effect—normally detrimental in chromatographic separation—of compounds migrating with the solvent front if the solvent is too powerful. This phenomenon can be regarded as a kind of extraction on paper, as it permits the direct

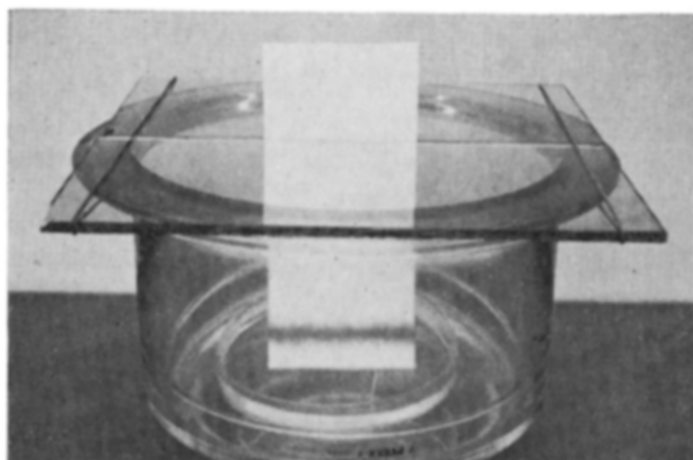

Fig. 1. Extraction vessel.

separation of ether-soluble compounds from interfering substances on chromatographic paper. The procedure is simple and rapid and does not call for any complicated apparatus. The extracting solvent concentrates the preservatives in a narrow line, well suited as a starting line for chromatography on the same paper. Moreover, some preservatives can be separated quickly and efficiently during the course of the extraction, with the help of arresting reagents. A modification of the method has been developed for fat-containing foods.

EXPERIMENTAL

Apparatus and materials

Extraction vessel (see Fig. 1). The lower part of a desiccator is used for this purpose. The arrangement allows easy regulation and change of the extraction distance. The rim of the extraction vessel should be smooth so that a saturated atmosphere is maintained within the vessel, otherwise ether evaporates from the paper before it reaches the slit between the glass plates.

Bromination chamber (see Fig. 2). This is an arrangement which resembles the extraction vessel, but a shallow dish (height about 4 cm) replaces the desiccator. Only the lowest part of the paper (starting line) comes inside the chamber through a slit between the glass plates.

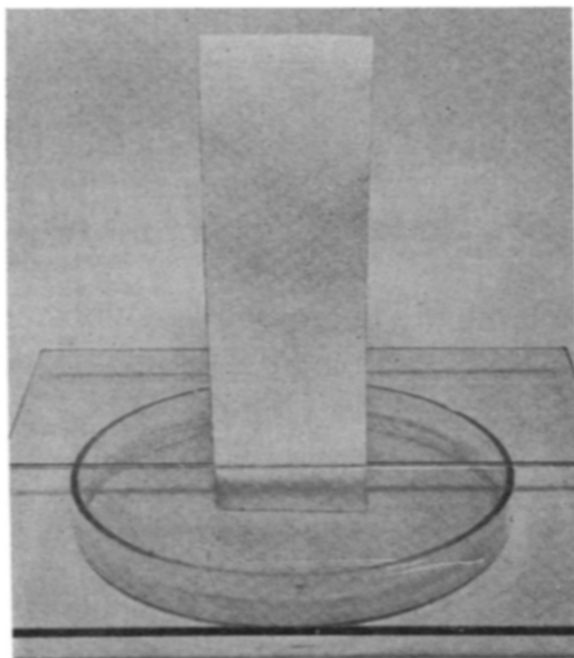

Fig. 2. Bromination chamber.

Chlorination chamber. A 2-l decanter glass used for this purpose is covered with a saran-membrane attached by means of a rubber band. Chlorine is led from a chlorine generation bottle into the chamber through a plastic tube.

U.V.-lamp. The maximum wavelength was 254 nm.

Chromatographic paper. Whatman 3 MM, or a corresponding thick paper, is cut into strips 5 cm in width; the length is varied from 15 to 30 cm according to the needs of the analysis.

Reagents

Extraction. Ethyl ether.

Impregnating solution: 1 part of 0.5 *N* sodium hydroxide, aqueous solution, and 2 parts of acetone. The acetone is added immediately before use.

Alkaline "arresting reagent": a saturated sodium bicarbonate solution.

Chromatography. Solvent system: isoamyl alcohol, ethanol and (1/10 diluted) ammonium hydroxide (6:3:2).

Identification reagents. Potassium permanganate, 0.05 % solution, 1 N with respect to sulphuric acid.

Manganese dioxide, for the development of chlorine gas.

Hydrochloric acid, concentrated and 1 N.

Sodium hypochlorite: an 11 % commercial solution, diluted to 1/15 with water.

Extraction procedure

Low fat foods. A juice of low sugar concentration (10 to 20 %) can be applied as such on to the paper by pipetting 50 to 100 μl across a strip of chromatographic paper as a broad band at a distance of about 1 cm from the end of the paper. The material does not need concentration in a narrow area as in ordinary chromatographic separation; it is spread across the paper quickly, without any intermittent drying. Juices with a higher sugar content, as well as jellies and jams, should be diluted with the same or double the amount of water.

In other cases, a sample of homogenized and diluted material, corresponding to 50 to 100 mg of the original product, is transferred to the paper as described above. Application has also been effected by weighing about 50 to 100 mg of the food in a small decanter glass, diluting with double the amount of water, and transferring the total weighed and diluted material to the paper with the aid of a glass capillary.

After the sample has been applied to the paper, it is left to equilibrate in the air for a short period (3 to 7 min), so that the moisture becomes equalized at the starting line; however, it must not be allowed to dry. The presence of moisture is essential if the extraction is to succeed. With new material for analysis, some experimentation may be needed to determine the correct equilibration time. The extraction is also dependent on a certain degree of acidity, but most food materials are sufficiently acidic to make the addition of acid seldom necessary.

The strip is now pressed between the edges of two glass plates so that the lower end of the paper (about 5 to 8 cm) reaches through the slit between the plates into a dish, filled with ethyl ether, in the extraction vessel (Fig. 1). The ether is adsorbed through the paper, and evaporates on the slit, concentrating the ether-soluble material as a narrow line in the solvent front (Fig. 3). The extraction is complete within 15 min, and the paper is removed from the extraction vessel.

The evaporation line may serve as a starting line for the chromatographic separation. The paper strip is cut off one to two centimetres below the evaporation line, and the paper is transferred into a chromatographic solvent. *p*-Hydroxybenzoic acid, and its esters, are separated from sorbic and benzoic acid within an hour; 30 min may be adequate if the sample is small enough. The solvent system was described by JOUX[1].

Fat-containing foods. About 50 mg of the homogenized food is weighed into a small decanter glass. A material with a medium fat content, such as fish, is mixed with double the amount of water; a fatty substance, such as margarine, is mixed with double the quantity of butanol. If the acidity of the material is insufficient, acidifi-

cation with hydrochloric acid may be necessary. The weighed and mixed material is transferred to the paper strip as described above.

The paper is placed into the extraction vessel as above, but instead of the preservatives being extracted to the solvent evaporation front, they are now arrested by alkaline arresting reagents and thus separated from the fat, which is extracted by the ether over the alkaline area up to the evaporation front. Two types of arresting reagents are used: sodium bicarbonate for free acids, and sodium hydroxide for esters.

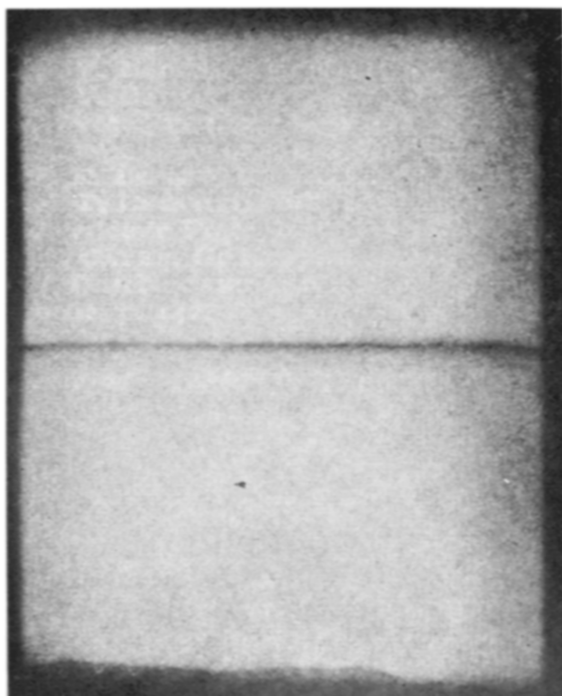

Fig. 3. The line of sorbic and benzoic acids photographed in U.V. light on the ether evaporation front.

Sorbic, benzoic and *p*-hydroxybenzoic acids are arrested in a line of saturated sodium bicarbonate which had previously been streaked across the paper with a glass capillary at a distance of 2 to 3 cm from the start line where the food material is applied. Fat, and such neutral substances as the esters of *p*-hydroxybenzoic acid, are extracted through the bicarbonate line to the solvent front. The distance between the evaporation line and the starting line should be at least 10 cm, to leave space for paper chromatography. The long migration distance, and the need to extract an appreciably large amount of fat to the evaporation line, make a longer extraction time necessary —at least half an hour, or even more. When the extraction is completed, the paper is cut off between the foodstuff and the bicarbonate lines, and transferred to the chromatographic solvent, which directly dissolves out benzoic and sorbic acids from the bicarbonate line; *p*-hydroxybenzoic acid is retained by the bicarbonate, however.

Chromatography of PHB esters. The efficient separation of the *p*-hydroxybenzoates from each other and from free acids is possible by a single direct ether extraction. After the food has been applied to the paper as described above, the strip is dipped into a freshly prepared mixture of aqueous sodium hydroxide and acetone,

which has been added to ensure uniform impregnation. The lower border of the alkaline region is one to two cm from the foodstuff line, so that ether may collect the preservatives into a narrow line before the alkaline region where the separation take place, is reached. With foods of moderate fat content, the paper is transferred into the ether extraction vessel 5 to 7 minutes after dipping into the impregnation solution; with fats, the equilibrating time is even shorter (1 to 3 min). The migration of the esters is slower on wet paper; this facilitates the separation from the fats. An acidic milieu on the extraction line is required also in this case.

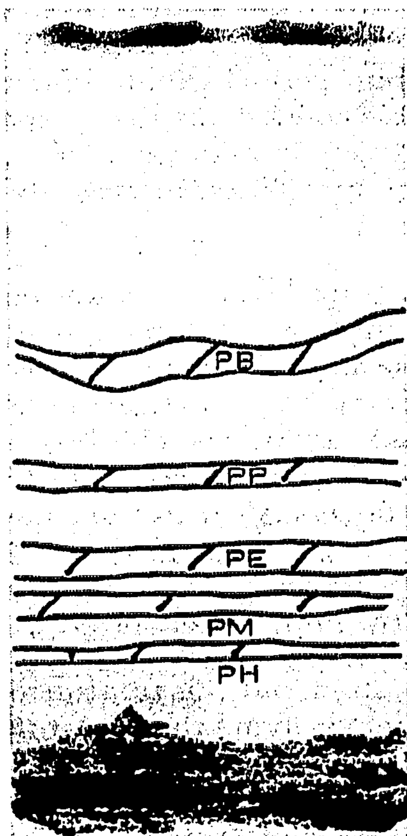

Fig. 4. 50 mg of fish homogenate (the broad dark line at the start) have been extracted with ether for 30 min on NaOH impregnated paper. Concentrations of 12 % NaCl and 12 % sugar, pH 6.2. To the material has been added *p*-hydroxybenzoic acid (PH) and methyl, ethyl, propyl and butyl *p*-hydroxybenzoates, PM, PE, PP and PB respectively, 0.02 % of each compound. The extracted fat shows as a dark line on the upper part of the chromatogram marking the evaporation front. The preservatives have been outlined with pencil in U.V. light.

While the PHB-esters are separated from each other as distinct lines in the alkaline region, the fats are extracted to the evaporation line; acid substances, such as benzoic, sorbic and *p*-hydroxybenzoic acid, are arrested at the lower border of the alkaline region (see Fig. 4).

Detection and identification

All the acids and esters mentioned above are detectable as dark blue bands in short wave, ultra-violet light. This is the simplest method of detection, and makes quantitative determination possible after elution.

Most of these compounds are distinguishable by differences in the migration rate, either in the chromatographic solvent, or simply after the ether extraction. However, two pairs of compounds could not be resolved: the butyl and benzyl *p*-hydroxybenzoates migrated at the same speed in the extraction–separation procedure for esters; and benzoic and sorbic acids had R_F values which were too close, so that separation within a short period of time was impracticable in the chromatography of these acids. However, these acids can be distinguished by their differences in reactivity with the visualization reagents.

Permanganate reaction. Sorbic acid is detectable as a white band against a pink background after the paper has been sprayed with 0.05 % potassium permanganate in 1 N sulphuric acid. Benzoic acid does not decolorize potassium permanganate under the same conditions. PHB and PHB esters are also discernible as white bands on the chromatogram, although they are distinguished by their different migration rates. The pink colour of the background disappears within a few minutes, but if desired, the spraying may be repeated a number of times.

Chlorination. For detection of the possible presence of benzoic acid in addition to sorbic acid, the chromatogram is cut lengthwise into two parts, and one half is put into a chamber containing chlorine gas for five minutes. Sorbic acid is no longer visible in U.V. light after the chlorination. Benzoic acid does not undergo any change, and is also visible after the chlorine treatment as a blue band in U.V. light. The chlorine is generated in a reaction bottle by the addition of concentrated hydrochloric acid to manganese dioxide, and the application of gentle warmth. The gas is led to the chlorination chamber through a plastic tube.

The chlorine treatment should be carried out while the paper is still wet after chromatography. If the paper has dried, it should be wetted by a fine water spray.

Bromination. Bromination has the same effect as chlorination, but this treatment must be effected before the chromatography. Immediately after applying the foodstuff to the paper, it is pressed between the edges of the two glass plates, so that the lowest part of the paper strip, with the still moist line of food, reaches into a shallow glass dish filled with bromine vapour—generated by a small drop of bromine at the bottom of the dish (Fig. 2). Five minutes of this bromine treatment is adequate to ensure disappearance of the U.V. absorption of sorbic acid.

Hypochlorite treatment. Disappearance of the dark blue band of sorbic acid in U.V. light is instantly achieved by spraying with 0.1 N sodium hypochlorite solution, to which has been added immediately before spraying two drops of concentrated hydrochloric acid per 10 ml. A similar effect is obtained by spraying first with 0.1 N sodium hypochlorite solution, and then with 1 N hydrochloric acid. Benzoic acid remains stable during this treatment.

DISCUSSION

Experiments have been made with fruit and berry material, such as juices, jams and jellies, and with foods of medium fat content, such as herring preserves. Margarine was studied as an example of fat.

To date, the preservatives studied have been: benzoic and sorbic acids, *p*-hydroxybenzoic acid and methyl, ethyl, butyl, propyl and benzyl *p*-hydroxybenzoates. After 15 min extraction on paper a chromatographic run lasting half an hour was capable of separating these compounds into three groups: (I) *p*-hydroxybenzoic acid (PH); (II) benzoic and sorbic acids (BH + SH); and (III) *p*-hydroxybenzoates (PM).

This short running time is feasible by virtue of the favourable form of the new starting line caused by evaporation of the ether front, and the absence of interfering agents.

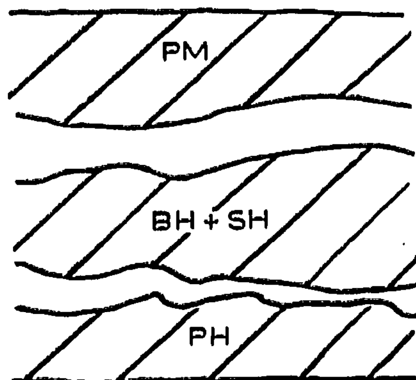

Fig. 5. Separation of extracted preservatives after a ½ h chromatographic run. The compounds have been outlined with pencil in U.V. light.

Half an hour was sufficient for the simultaneous extraction and chromatographic separation of *p*-hydroxybenzoates from non-fatty foods and foods of medium fat content on sodium hydroxide impregnated paper. One hour, or occassionally $1^1/_2$ h, was required for separation from fats, such as margarine.

Stress is laid upon the critical nature of the degree of moisture of the starting line and the impregnated paper—if the paper is dried too extensively after impregnation, the esters migrate too fast, and the lines become so diffuse that the substances are not detectable, or may even be pushed into the evaporation line. Conversely, if the paper is too wet, the separation of the esters from each other may not be achieved within a reasonable length of time. An increasing amount of fat calls for less drying time, and consequently a longer extraction time.

In Fig. 6, the PHB esters were isolated from margarine, and separated by three successive 30 min runs with ether. Drying time in air after impregnation was 1 minute.

The chromatography of acid substances, and separation of PHB esters, has been effected on a single paper as follows: food material was applied to a strip of paper 25 cm in length as described above. A line of bicarbonate was drawn across the paper, 2 cm from the starting line. Fats and *p*-hydroxybenzoates were extracted by ether over a distance of 14 cm to the evaporation front. The paper was cut 2 cm below the evaporation front. The lower part of the strip was transferred to the isoamyl alcohol solvent mixture for chromatography of the acids, and the upper part was impregnated with sodium hydroxide solution and subjected to ether extraction for separation of the PHB esters.

Observations on the identification treatments

The method of generating bromine vapour is very simple, but the treatment is practicable only when conducted before the chromatographic stage; the background of the paper is dark coloured, due to adsorbed bromine, in U.V. light, and some hours are required before bromine has totally evaporated. In the arrangement employed here, the effect is limited to the starting line. This treatment is well suited to the quantitative determination of benzoic acid in the presence of sorbic acid. The examination of U.V. spectra after chromatography and elution has shown that under the conditions stated sorbic acid undergoes complete reaction in 5 min, whereas benzoic acid is practically unaffected[2].

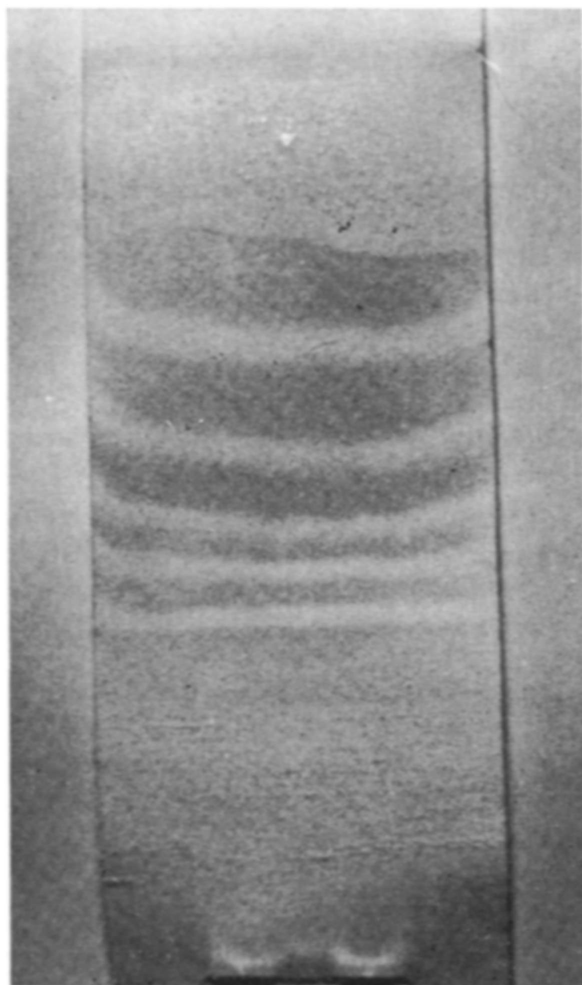

Fig. 6. The separated compounds are in a similar sequence to those in Fig. 4. The extracted fat shows as a broad light band in the upper part of the chromatogram. The compounds have been made visible by permanganate spraying.

Chlorine generation is slightly more complicated than that of bromine, although the advantage here is that chlorination can be effected after chromatography. Chlorine does not impart a colour to the paper such as would hamper U.V. observations. Chlorination may be introduced before or after chromatography—and the same thing applies to hypochlorite treatment.

Treatment with hypochlorite has the advantage that ordinary spraying techniques can be applied. The spraying must not be too generous, as benzoic acid is easily eluted from the chromatogram, and may thus become undetectable. Pre-chromatographic treatment of sorbic acid has been effected not only by spraying, but also by streaking hypochlorite solution on both sides of the line containing sorbic acid, so that the reactant is brought to the action site by adsorption. The reaction has also been effected by directly mixing hypochlorite solution with the food to be analysed. The small amount of hydrochloric acid added to hypochlorite is insufficient to make the solution acidic, but activates the reagent so that it is capable of reacting with sorbic acid; in fact, if too much acid is added, the hypochlorite loses effectiveness in a very short time.

Some foodstuffs may yield interfering reaction products with the sorbic acid decomposition reagents, and thus it might be advisable to carry out the pre-chromatographic treatments not on the extraction line, but on the ether evaporation front, or on the alkaline arresting line.

CONCLUSIONS

It seems to be a reasonable assumption that modifications of this technique of extraction combined with chromatography on paper should also be suitable for other analytical applications, in cases where preliminary extraction before chromatography is necessary.

ACKNOWLEDGEMENT

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SUMMARY

Food preservatives—sorbic, benzoic, *p*-hydroxybenzoic acid and *p*-hydroxybenzoates—have been extracted from foods directly on to chromatographic paper with ethyl ether. Paper chromatographic separation of the extracted compounds was effected on the same paper.

Methyl, ethyl and propyl *p*-hydroxybenzoates have been separated from each other during ether extraction and from the critical pair, the butyl and benzyl *p*-hydroxybenzoates, on a paper impregnated with sodium hydroxide. An examination was made of the reactions which distinguish sorbic and benzoic acids when both are present.

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