A Comparison of the Efficiency of the Likens and Nickerson Extractor for Aqueous, Lipid/Aqueous, and Lipid Samples

Chi Y. Au-Yeung and Alexander J. MacLeod*

By use of a modified Likens and Nickerson apparatus, recoveries of four compounds were determined from aqueous, lipid/aqueous, and lipid media by using three different extracting solvents. Dichloromethane, with recoveries usually in excess of 80%, was the best general extractant, although efficiency did vary between solvents depending upon the solute. Similarly, solutes were recovered to different extents from different media, all other factors being constant. Recoveries generaly increased with the time of extraction but were constant over a small concentration range. A further modification to the apparatus is described. When it was adapted for steam distillation of lipid samples, much improved recoveries were obtained, which were similar to those from aqueous samples.

One of the most widely used routine methods of extracting volatile aroma components from foods and other systems for analysis is the simultaneous steam distillation and solvent extraction apparatus devised by Likens and Nickerson (1964). However, in nearly all cases the original apparatus has been modified by other workers to suit specific requirements [e.g., Maarse and Kepner (1970), MacLeod and Cave (1975), Groenen et al. (1976), Buttery et al. (1976), and Schultz et al. (1977)], but the principle remains the same. A wide range of aroma volatiles has been studied by using this technique, including those of vegetables, fruits, herbs, meat, eggs, and beer. The apparatus has many advantages including its relative simplicity and ease of operation as well as its supposed high efficiency. However, surprisingly very few basic studies have been carried out to substantiate this latter assumption, although Likens and Nickerson (1964) themselves reported recoveries between 72 and 97% for a number of unspecified hop oil constituents. Efficiency is best assessed by determining recoveries of known components from well-defined model systems so that any qualitative as well as quantitative effects can be studied. Farmer et al. (1973) conducted one such survey and determined recoveries from aqueous solutions of homologous series of alkanes, alkan-1-ols, alkanals, and alkan-2-ones. Results ranged from trace recovery to quantitative, although most components were retrieved in greater than 70% yield. Recoveries were poor for the short chain, more volatile compounds but improved with increasing chain length within a series. More recently Schultz et al. (1977) carried out a much more comprehensive survey to assess the efficiency of their modified Likens and Nickerson apparatus. They determined the recoveries of 12 compounds (esters, alcohols, and terpenes) from aqueous solution at various concentrations and at a range of different pH levels from 3.4 to 7.8. They also used the apparatus under reduced pressure and compared three extracting solvents (hexane, pentane, and ether), although the majority of the work was done with hexane. Overall the recoveries obtained were similar, or slightly better, compared with those reported by Farmer et al. (1973).

All such previous model system studies have been performed on aqueous systems, but most foods are not purely aqueous media, and it seems very probable that the nature of the food matrix would affect recovery of aroma volatiles by the Likens and Nickerson procedure. The main objective of this project was thus to compare recoveries of components from model systems in aqueous, lipid/aqueous, and lipid media. Furthermore, in previous studies only limited attention has been devoted to the nature of the extracting solvent and the length of time of the extraction process. Therefore, both of these variables were also assessed in this study.

The Likens and Nickerson apparatus used in this work was a modified version which has been described in detail before (MacLeod and Cave, 1975). The modifications were partly designed to improve efficiency, and the apparatus has been successfully employed in the past to obtain extracts of aroma volatiles of a number of plants and foods [e.g., MacLeod and Cave (1975), MacLeod and Islam (1975, 1976a,b), MacLeod and Coppock (1977), and Gil and MacLeod (1980a,b)].

EXPERIMENTAL SECTION

Apparatus. The modified Likens and Nickerson apparatus has been described in detail in a previous publication together with a drawing (MacLeod and Cave, 1975).

Model Systems and Solvents. The components of the model systems were ethyl acetate, ethyl alcohol, methylpyrazine, and allyl isothiocyanate. Purest possible samples were purchased and were purified further if necessary by distillation. Exactly 10^{-2} M amounts of each compound were mixed in the appropriate test medium. Distilled water provided the aqueous medium, milk the homogeneous lipid/aqueous medium, and pure corn oil the lipid medium. These test mixtures were also quantitatively diluted 100-fold to provide the 10^{-4} M model systems.

The extracting solvents were isopentane (2-methylbutane), dichloromethane, and trichlorofluoromethane. All were triply distilled.

Reference standards for each of the four solutes in each of the three solvents were accurately prepared at the 10^{-2} and 10^{-4} M levels.

Conventional Use of the Likens and Nickerson Apparatus. In its original form (MacLeod and Cave, 1975) the apparatus was used broadly as follows. The separation area of the extractor was first primed with appropriate amounts of medium and extracting solvent. However, in the case of the lipid medium, this was miscible with the solvents used, and therefore in order to ensure that the separator functioned properly, the extractor was primed with water instead of lipid, so that two layers were maintained. The amount of water used was not sufficient for any to be returned to the sample flask during use of the extractor. An accurate aliquot of the model system (100 mL) was placed in the sample flask and heated to boiling (or 100 or 150 °C in the case of the lipid medium). The extracting solvent (30 mL) was also heated to boiling in the other flask, and the period of extraction was taken

Department of Chemistry, Queen Elizabeth College, University of London, Campden Hill Road, London W8 7AH, England.

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Table I. Percentage Recoveries of Components from an Aqueous Medium (at a Concentration of 10^{-2} M for Each Compound) by Using a Modified Likens and Nickerson Apparatus (MacLeod and Cave, 1975)

time, h	% recoveries											
	ethyl acetate			ethanol			methylpyrazine			allyl isothiocyanate		
	isop.[c]	CH_2Cl_2	$CFCl_3$	isop.	CH_2Cl_2	$CFCl_3$	isop.	CH_2Cl_2	$CFCl_3$	isop.	CH_2Cl_2	$CFCl_3$
0.5	85	75	96	0	a	0	26	70	62	60	82	81
1.0	65	86 (78)[b]	84	0	a	0	52	94 (90)[b]	79	57	80 (81)[b]	74
3.0	74	82	91	0	a	0	54	101	78	79	52	79

[a] Ethanol unresolved from solvent on GC. [b] Figures in parentheses are recoveries at 10^{-4} M concentrations. [c] isop. = isopentane.

from the time solvent vapor and sample vapor first mixed in the condensation area of the apparatus. For optimum efficiency it is essential that the contents of both flasks boil relatively vigorously and that the CO_2/acetone trap beyond the extractor is kept well cooled. It is also usually important to ensure adequate stirring of the contents of the sample flask. Using a heavier than water extracting solvent such as dichloromethane or trichlorofluoromethane, it is merely necessary to reverse the extractor, although some workers have devised special modifications for this purpose (Groenen et al., 1976).

At the completion of extraction the contents of the solvent flask were allowed to cool and mixed with the solvent from the separator. The combined extract was then made up to an accurate volume (100 mL) and analyzed by gas chromatography.

Use of the Likens and Nickerson Apparatus with Applied Steam. The apparatus and method were exactly as described above except that the sample flask was equipped for the introduction of steam. A T-piece and clamps were inserted between the flask and the steam generator so that steam could either be passed to the apparatus or vented. The lipid test medium (100 mL) was heated to 150 °C and the extracting solvent (30 mL) heated to boiling. The system was allowed to stabilize for ~10 min and steam was then gently admitted . Flow was maintained at such a rate that steam circulated smoothly through the extractor and that excess water did not condense in the sample flask. It was essential that the contents of this flask were stirred vigorously throughout the period of extraction, which was timed from the first introduction of steam.

Analysis by Gas Chromatography. Samples were analyzed quantitatively by conventional gas chromatography using a Pye-Unicam 204 equipped with heated FID. A 1.5-m 10% PEG 20M column was used and peak areas were measured manually against an internal standard. Injection of standard solutions of the four solutes in the three solvents showed that the detector exhibited linear response in all instances over the range at which measurements were taken.

RESULTS AND DISCUSSION

All experiments and analyses were carried out in triplicate and results agreed to within ±5%. Figures quoted are the average of the three determinations.

Table I gives recoveries of four components from an aqueous medium by using the modified Likens and Nickerson apparatus (MacLeod and Cave, 1975) and three different extracting solvents. The four compounds were selected to cover a range of volatilities, polarities, and chemical class. In addition, they are all common aroma constituents of foods and are therefore representative of the range and types of components which should be efficiently extracted by using the apparatus. Of the extracting solvents, isopentane (2-methylbutane) is often used, and some time ago we found that it provided superior results to pentane, diethyl ether, and benzene (Cave, 1972). Dichloromethane is rarely used, presumably because the Likens and Nickerson apparatus is designed particularly for solvents of lesser density than water. However, in this project it was found that this modified apparatus (MacLeod and Cave, 1975) functioned equally satisfactorily with heavier than water solvents. It was necessary merely to reverse the extractor (i.e., interchange sample and solvent flasks), although others have modified the design of the apparatus specifically to cope with such solvents (Groenen et al., 1976). Trichlorofluoromethane, which also has a density greater than water, has not been employed before with the Likens and Nickerson apparatus, but it has been claimed that it is a particularly effective solvent for aroma components, and as a result it has been the subject of a number of patents [e.g., Scheide and Bauer (1967) and Stanley et al. (1963)].

From Table I it can be seen that ethanol was not extracted at all, although conclusions cannot reasonably be drawn when using dichloromethane as the solvent. This result is not unexpected with an aqueous medium, and previous workers have also reported zero recovery (Schultz et al., 1977). In food flavor analysis this seeming deficiency of the system can be considered an advantage, since ethanol is frequently a major, yet irrelevant, constituent of aroma volatiles, and it often masks more important components on subsequent gas chromatography of extracts. Of the three solvents, isopentane generally gave the poorest results, but either of the other two would be a good choice as extractant, and recoveries were usually well in excess of 70%. Their efficiency varied depending on the solute, with trichlorofluoromethane being somewhat better for the ester and dichloromethane for methylpyrazine. Dichloromethane appears to be a good general-purpose extractant. From the one experiment performed, recoveries were about the same at lower concentrations of solutes.

Increasing the period of extraction did not greatly improve efficiency, except to some extent for isopentane, and there was little to be gained by extending the time from 1 to 3 h. Excess time could result in thermal degradations or interactions with consequent artifact formation and should therefore be avoided if possible. There is some evidence that this might have been occurring with allyl isothiocyanate, for which the concentration in dichloromethane decreased appreciably between 1 and 3 h. Similar behavior was observed in experiments using other sample media, and allyl isothiocyanate is known to undergo dimerizations and other reactions in aqueous solution [e.g., Kawakishi and Namiki (1969)]. From the data in Table I, 1 h would seem to be a reasonable optimum time for extraction.

It is difficult to compare these results with those of previous surveys due to the number of variables involved. However, for the one common compound studied (apart from ethanol), Schultz et al. (1977) reported a recovery of

Table II. Percentage Recoveries of Components from a Lipid/Aqueous Medium (Milk, at a Concentration of 10^{-2} M for Each Compound) by Using a Modified Likens and Nickerson Apparatus (MacLeod and Cave, 1975)

time, h	% recoveries							
	ethyl acetate		ethanol		methylpyrazine		allyl isothiocyanate	
	isop.[b]	CH_2Cl_2	isop.	CH_2Cl_2	isop.	CH_2Cl_2	isop.	CH_2Cl_2
0.5	91	65	0	a	28	52	66	96
1.0	80	80	0	a	31	73	60	90
3.0	79	82	0	a	67	95	73	75

[a] Ethanol unresolved from solvent on GC. [b] isop. = isopentane.

Table III. Percentage Recoveries of Components from a Lipid Medium (at a Concentration of 10^{-2} M for Each Compound) by Using a Modified Likens and Nickerson Apparatus (MacLeod and Cave, 1975) Equipped for Steam Distillation

time, h	% recoveries							
	ethyl acetate		ethanol		methylpyrazine		allyl isothiocyanate	
	isop.[b]	CH_2Cl_2	isop.	CH_2Cl_2	isop.	CH_2Cl_2	isop.	CH_2Cl_2
0.5	69	85	0	a	27	69	46	77
	(6)[c]	(17)	(tr)[d]		(tr)	(6)	(tr)	(tr)
	[7]	[27]	[tr]		[tr]	[7]	[tr]	[tr]
1.0	73	81	0	a	29	63	64	81
	(10)	(18)	(7)		(tr)	(9)	(tr)	(tr)
	[38]	[56]	[11]		[11]	[22]	[8]	[17]
3.0	83	87	0	a	44	74	72	79
	(45)	(80)	(8)		(14)	(12)	(10)	(16)
	[45]	[83]	[13]		[17]	[24]	[13]	[18]

[a] Ethanol unresolved from solvent on GC. [b] isop. = isopentane. [c] Recoveries using the apparatus without applied steam at 100 °C are in parentheses and those at 150 °C are in brackets. [d] tr = trace.

59% for ethyl acetate extracted into pentane during 1 h. This agrees well with our result (Table I), so there is some evidence that in this respect the two modified Likens and Nickerson extractors have similar efficiency.

Table II gives results for the recovery of the same four components from an homogeneous lipid/aqueous medium (fresh milk). Again, ethanol was not retrieved. In agreement with results using the purely aqueous medium (Table I), dichloromethane was consistently a better solvent than isopentane, except on a short-term basis for ethyl acetate. Generally, recoveries increased with the time of extraction, although the concentration of allyl isothiocyanate in dichloromethane again decreased between 1 and 3 h, presumably due to thermally induced reactions. Overall the recoveries obtained from a lipid/aqueous medium agreed very well indeed with those from a purely aqueous medium (compare Tables I and II), and clearly the relatively small percentages of lipid, and other material, in milk does not significantly affect the efficiency of the Likens and Nickerson procedure.

Table III gives results for the recovery of the four compounds from a lipid medium (corn oil), and the figures in parentheses relate to the normal procedure and apparatus. These recoveries are much poorer than those obtained from aqueous systems and would be unacceptable for practical analyses. However, in this one instance, in the complete absence of water, it was possible to extract ethanol from the medium, although the amounts were small. In agreement with the data obtained for aqueous and lipid/aqueous media, dichloromethane was the better solvent. Thus, overall it is the nature of the solute rather than the medium which mainly determines relative efficiencies of different extracting solvents. Therefore, choice of solvent in Likens and Nickerson extractions can be important and should, if possible, take into account the types of solute to be extracted.

In contrast to the results for aqueous systems, ethyl acetate was extracted from corn oil much more efficiently than methylpyrazine or allyl isothiocyanate, but recoveries for the latter two compounds were very low. Although as usual all recoveries increased with time, these two components were retrieved in only ~15% yield even after 3 h of extraction. Therefore, the extent of recovery of a particular solute depends partly on the nature of the medium, all other factors (e.g., extracting solvent) being constant. Apart from the wider significance, these findings mean that, if the efficiency of such an extraction apparatus is to be determined with any degree of accuracy, it is essential that it be done for each compound in question and either by using a genuine model system or by the method of standard addition. Since such a procedure would be an extremely arduous task for most food flavor analyses, "quantitative" data obtained by Likens and Nickerson (and similar) extractions should in most cases be treated with reservation, although, clearly, reasonable approximations are possible.

During the above experiments the lipid medium was held at 100 °C to be comparable with boiling aqueous media. In addition, a series of experiments was carried out where the temperature was raised to 150 °C, and it can be seen from Table III (figures in brackets) that improved recoveries were obtained, as would be expected, but they were still relatively poor.

The main problem in using the Likens and Nickerson apparatus in this manner for lipid samples is that the system lacks the increased efficiency provided by steam distillation which occurs naturally with aqueous samples. To regain this inherent advantage, the modified apparatus (MacLeod and Cave, 1975) was further slightly modified, but simply to allow the introduction of steam into the sample flask from an external source. The apparatus and its use are described under Experimental Section and have been the subject of a preliminary communication (Au-Yeung and MacLeod, 1980). Data obtained by using this system are also given in Table III, and it can be seen that recoveries were very much improved and in all cases were

superior to those obtained when using the apparatus without external steam. This is excepting ethanol for which the introduction of water into the system effected zero recovery again. As with recoveries from aqueous media, dichloromethane was the better solvent and 1 h was optimum for the extraction period, although as usual recoveries did generally increase with time. Results obtained by using this system were of the same order as those obtained from aqueous media by using the original apparatus (MacLeod and Cave, 1975) (see Table I).

This further minor refinement to the modified Likens and Nickerson apparatus (MacLeod and Cave, 1975) thus provides a relatively simple system with adequate efficiency for most purposes for the ready recovery of components from a purely lipid medium. Its efficiency would seem to be comparable with more sophisticated and complex equipment specifically designed for lipid samples, although identical recovery data with regard to the latter are not available. However, the Likens and Nickerson apparatus and procedures described here possess the additional advantage of versatility, and as such they are suitable for the analysis of the volatile components of a very wide range of foods and similar samples.

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Characterization of Astaxanthin Pigments from Heat-Processed Crawfish Waste

Samuel P. Meyers* and David Bligh

Carotenoid pigments have been isolated and identified from the heat-processed exoskeleton of crawfish, *Procambarus clarkii*. Pigment concentrations as great as 153 μg/g are noted, consisting of 49.4% astaxanthin ester, 40.3% astaxanthin, and 10.3% astacene. When measured as a function of time and antioxidant treatment, using BHA and Santoquin, pigment concentrations showed a maximal decrease, i.e., 45% over 3 weeks, in untreated samples. Levels of Santoquin at 0.5% were effective in minimizing pigment loss with storage. Application of the high pigment quality meal in diets for aquaculture-raised fish is discussed.

Increasing interest is being shown in astaxanthin and astaxanthin-containing raw materials for use in dietary formulations where integument and flesh coloration is of economic importance. Attention has been directed toward pen-reared salmonid fishes with emphasis on use of a variety of crustacean meals in feed formulas (Steel, 1971; Kuo et al., 1976; Meyers, 1977; Bligh, 1978; Spinelli and Mahnken, 1978). In addition, a variety of carotenoid and carotenoid-containing materials, i.e., β-carotene, canthaxanthin, paprika, and β-apo-8′-carotenal, have been tested as flesh pigmenters in the feeds of fish such as trout, salmon, and tropical fish and in poultry (Bauernfeind, 1976; Bunnell and Bauernfeind, 1962; Fey and Meyers, 1980). Recently a number of investigators (Ellis, 1979; Simpson and Kamata, 1979) have stressed the importance of carotenoid-rich ingredients as well as extracted pigment in aquatic diets for enhancement of coloration, especially among salmonids.

The multi-million-dollar Louisiana crawfish (*Procambarus clarkii*) industry characteristically produces a significant amount of potentially usable proteinaceous waste during the harvest periods December to June, with the dominant catch occurring in the spring months of April to June. Landings in 1980 of over 25 million pounds of animals (85% waste) is indicative of the magnitude of the potentially usable byproduct generated. Disposal of this material is an increasing economic problem, adding to the processor's operating expense. Previous works from our laboratories have focused on nutritional and other properties of the meal, along with economic data on the composite crawfish processing industry. The research reported here concerns astaxanthin levels present in the dried meal and its preservation under storage conditions, along with

Department of Food Science, Louisiana Agricultural Experiment Station, Louisiana State University, Baton Rouge, Louisiana 70803.

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