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Automated sample preparation for cholesterol determination in foods

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Abstract

An automated sample preparation system has been developed for the determination of cholesterol in a wide range of matrices. Isolation of cholesterol is performed with a robotic arm coupled with a series of modular stations. Samples are introduced into the system which adds the appropriate reagents, carries out the saponification, pH adjustment, solid-phase extraction and drying steps. This system was evaluated using 15 different food matrices. The average recovery for NIST standards exceeded 97%. A solution of *n*-hexane–2-propanol was substituted for the traditional methanol–chloroform extraction. Manual pH adjustment was replaced with a buffer. Manual and automated methods were compared and no difference was observed at the 95% confidence level.

1. Introduction

Public interest in dietary cholesterol has increased due in large part to the relationship of plasma cholesterol levels with the risk of developing coronary heart disease. This emerging public consensus that limiting dietary cholesterol contributes to good health has resulted in a series of government guidelines for food labeling including for the first time specific requirements for cholesterol [1–4]. In order to ensure compliance with these guidelines the company's entire product line needed to be reexamined.

Determination of cholesterol has been the objective of numerous methods over the last three decades and has been the subject of a series of comprehensive reviews [5–7]. The most widely used method includes isolation from the sample matrix via extraction, The isolated components could then be analyzed by enzymatic determination [8,9], spectrophotometry [10,11], liquid chromatography [12,13] and gas chromatography [14,15] for free cholesterol and cholesterol esters. An alternative approach particularly for food analysis includes a saponification step before extraction [6,16]. The esters are hydrolyzed to form the free cholesterol alcohol then isolated by extraction with an organic solvent.

Evaluation of products for cholesterol content during development, building a data base for new products and monitoring marketed products resulted in an average of 3000 requests for cholesterol analysis for each of the past four years. The method used in this laboratory for the determination of cholesterol includes saponification and extraction followed by gas chromatographic analysis [17]. An experienced analyst can

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prepare and analyze 80 samples a week. Meeting this demand for increased analytical services was made more difficult because this increased demand had to be met without an increase in staff. It became clear that automation of all or part of the sample preparation would free analysts for problem solving and investigative work rather than devoting a majority of their time to preparing samples for analysis. Other benefits of automation are more consistent results, cost reduction through economies of scale and tap a potential 24-h/day capacity.

The system described below takes a sample through the entire cleanup process to include the tasks of pipetting, addition of powders and liquids, solid-phase extraction (SPE) and tube capping. The solid-phase extraction step was reexamined to explore alternative solvents to replace chloroform which is not only costly to procure but recovery and disposal costs are becoming increasingly expensive. This method was validated and can be applied to a wide variety of food products. All results reported here are expressed as mg/100 g rather than mg/serving because the serving size varies between food products. Data are presented for recoveries, repeatability and analysis of control samples.

2. Experimental

2.1. Apparatus

A five-axis CRS Plus robotic arm (CRS, Toronto, Canada) was used for this application. Robotic and automation programming language (RAPL) was used to control the robotic arm and input/output boards for control of the SPE unit and other modules. It was equipped with a dual hand having fingers and a pneumatic pipet holder. The robot was mounted on a 3-m linear track (Bohdan Automation, Mundelein, IL, USA). A configuration diagram is shown in Fig. 1. A Millilab 2A Workstation (Waters Associates, Milford, MA, USA) with an on-line personal computer was used for the SPE portion of the procedure. The two pumps on the fluidics module were equipped with a 0.5- and 1-ml syringe. The sample rack was divided into two sections: one for the incoming saponified samples (16×100 mm open tubes) and the second to receive the SPE eluate (16×125 mm capped tubes) for further processing. Solvent reservoirs of distilled water and methanol were attached to a 0.5-ml syringe for activating the solid-phase extraction cartridges and sample handling. A solution of *n*-hexane–2-propanol (85:15, v/v) or chloroform–methanol (95:5, v/v) was attached to the 1-ml syringe to elute cholesterol from the cartridges.

A series of modules to include a capper/decapper, cap dispenser, vortex-mixer, powder dispenser, tumble-mixer, chilled rack, and pipette tip actuator (Bohdan Automation) were built to KGF specifications. Reacti-Therm III heating/stirring blocks (Pierce Chemical Corp., Rockford, IL, USA), 3-position Satellite magnetic stirrer (Fisher Corp., Pittsburgh, PA, USA), 50-position Turbovap Model ZW700 (Zymark Corp, Hopkington, MA, USA) and 16-mm test tube racks (Zymark) were used without modification. Two Microlab 900 Dispensers (Hamilton Corp., Reno, NV, USA) were used to dispense potassium hydroxide, internal standard, hydrochloric acid and phthalate buffer. A third Microlab 900 was used in connection with the pneumatic pipette holder. All modules and peripherals were controlled by input/output devices on the CRS system.

2.2. Reagents

HPLC grade water, methanol, *n*-hexane, 2-propanol, potassium hydroxide, hydrochloric acid, potassium hydrogen phthalate, chloroform, sodium sulfate, culture tubes and pipet tips were purchased from Fisher Scientific (Springfield, NJ, USA). Ethanol (95%) was purchased from AAPR Alcohol and Chemical (Shelbyville, KY, USA). Cholestane was purchased from Aldrich (Milwaukee, WI, USA). BSTFA containing 10% TMS was from Regis Chemical Co. (Morton Grove, IL, USA). Cholesterol standard, coconut oil Standard Reference Material (SRM) containing 64.2 ± 0.4 mg/100 g and powdered egg

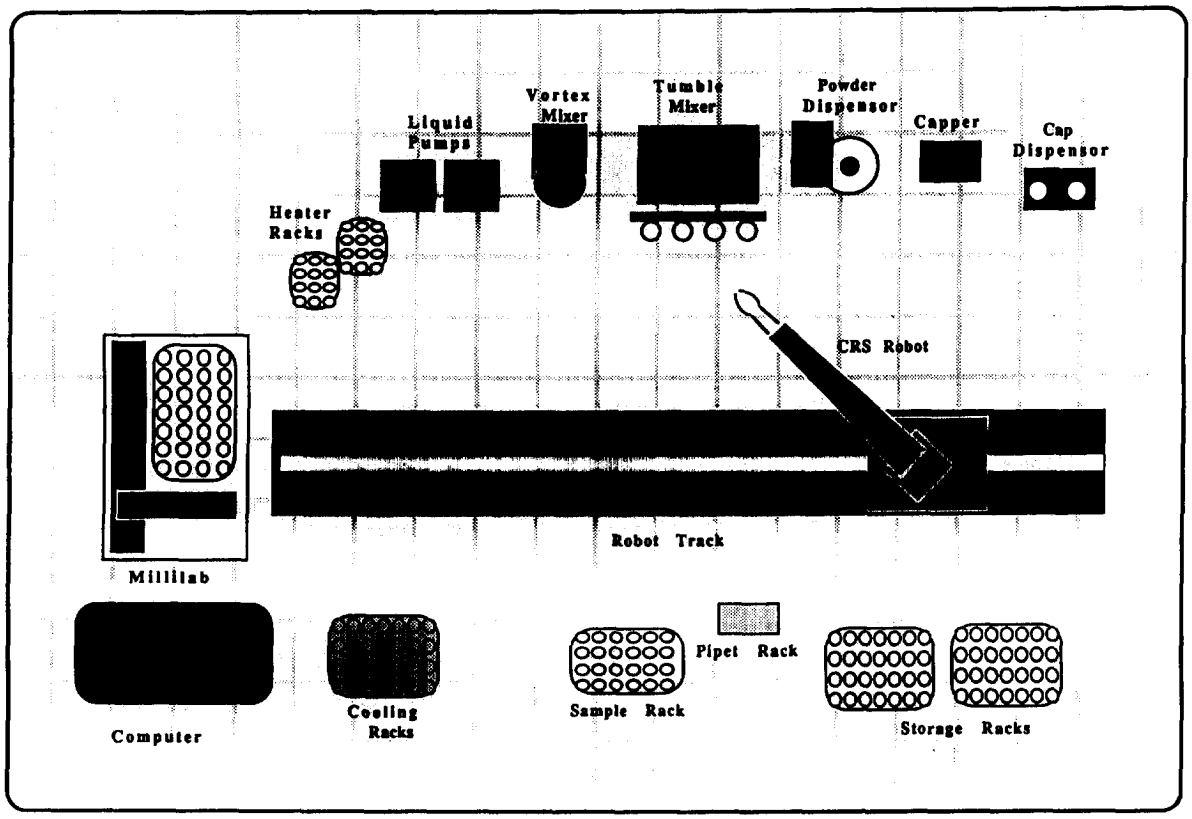

Fig. 1. Cholesterol robot diagram.

yolk SRM containing 1900 ± 20 mg/100 g were purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). All reagents were used as received. Food samples were experimental Kraft General Foods formulations or commercial products purchased from retail stores.

Solid-phase extraction cartridges containing one gram of C_{18} adsorbent in a 6 ml-barrel were purchased form Varian (Harbor City, CA, USA) and Baker Chemical Co. (Phillipsburg, PA, USA).

2.3. Sample preparation

Manual method

Samples were prepared and analyzed as previously described [17].

Automated method

Using Table 1 as a guide, an appropriate amount of sample was weighed into individual 16 × 100 mm culture tubes. A 12 × 2 mm stirring bar (Fisher Scientific) was added to each tube. Tubes were then capped and placed in the sample rack. The same number of extraction cartridges and 16 × 125 mm tubes were loaded into the Millilab Workstation. The 0.5-ml syringe of the workstation was primed with water and methanol. The second syringe was primed with chloroform–methanol (95:5, v/v) or *n*-hexane–2-propanol (85:15, v/v). The powder dispenser was charged with sodium sulfate. The Microlab dispenser reservoirs were filled with an 50 ± 5% aqueous sodium hydroxide, ethanolic solution of 0.5 mg/ml of cholestane internal standard in ethanol, 6.4 ± 0.2 *M* hydrochloric acid and 6 ± 1% potassium hydrogen phthalate solutions,

Table 1
Examples of optimum sample size as a function of matrix

Sample type	Recommended sample weight (mg)
Egg yolk	100–150
Animal oils	100–200
Vegetable oil	200–400
Butter fat	250–300
Soybean oil	400–500
Viscous dressings	300–400
Natural cheese	300–500
Process cheese	300–400
Pourable dressing	400–500
Sour cream	400–600
Ice cream	400–600
Ice milk	500–600
Milk	700–900
Cream cheese	300–400
Pasta	400–500
Frozen dinner	400–500
Luncheon meats	300–400
Breakfast sausage	200–300
Seafood	200–400

respectively. The CRS robot was initialized and the prompts for the number of samples to be processed and the saponification time set. The workstation was similarly initialized and programmed with the number of samples to be processed. A flow chart of the various steps is shown in Fig. 2.

2.4. Gas chromatographic analysis

Cholesterol analysis was accomplished using a capillary gas chromatographic system. A Hewlett-Packard Model 5890A was used with a split ratio set at 25:1 and a flame ionization detector. The column was a 30 m × 0.25 mm I.D. fused-silica capillary column, DB1 (J&W Scientific, Folsom, CA, USA) with 0.25-μm film thickness. The column temperature was programmed from 245°C at 5°C/min to 285°C and held at that temperature for 18 min. The hydrogen carrier gas was set to 1 ml/min measured at 160°C; detector gasses, hydrogen at 50 ml/min, air at 300 ml/min, injection port temperature 280°C; and detector temperature at 300°C.

2.5. Statistical analysis

SAS version 6.04 (Statistical Institute, Cary, NC, USA) was used for statistical analysis. For each comparison of methods/procedures studied, a series of different samples were taken and analyzed by both the new (automated) and established (manual) methods. The relative error was calculated for each sample as a basis for comparison between the two methods. The difference between the automated and manual sample values was divided between by the value from the manual method.

A positive relative error indicated that the automated afforded a higher result while a negative relative error indicated the manual result was higher.

A null hypothesis that the mean relative error included zero was tested by computing two-sided 95% confidence intervals for each group. If zero was included in the interval, then there was no difference between the methods. Conversely, if zero was not included in the interval, then a difference existed between the two methods and they did not yield equivalent results. The null hypothesis was also tested at the 0.05 alpha level of significance by computing the p-value for the mean relative error. A p-value above 0.05 indicated no difference between the methods while a value below 0.05, indicated a significant difference.

3. Results and discussion

In order to enhance the chances of success, the approach taken was that of parallel development. Three separate objectives were defined. The first was to determine if an off-the-shelf instrument was viable for automation of the solid-phase extraction (SPE) portion of the method. The second objective was to find an alternate way to replace the manual neutralization and pH adjustment of the samples after saponification prior to solid-phase extraction. Only after accomplishing the first two objectives a program could be designed and a system be developed of linked modules and the robot be

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CHARGE RESERVOIRS
      Millilab
      Saponification/neutralization dispensers
      sodium disulfate dispenser

FILL RACKS WITH CONSUMABLES
      Pipet tips
      16x125 mm tubes in Millilab
      SPE cartridges in Millilab

INITIALIZE SYSTEM
      Set sample number and saponification time on CRS
      Set sample number on Millilab
      Add samples
      Activate CRS and Millilab systems

SAPONIFICATION STEP
      Remove cap from sample tube
      Add 1 ml ethanolic KOH/internal standard solution
      Return cap to sample tube and place in heating  block
      Perform other pick-and-place operations while waiting for specified
      saponification time

NEUTRALIZATION STEP
      Remove tube from heating block
      Wait 30 s
      Remove cap from tube
      Remove 1-ml aliquot from tube
      Place 1-ml aliquot in 16x100 mm open tube
      Add 0.3 ml 6.4 M HCl followed by 4.0 ml phthalate solution
      Vortex for 10 s

SOLID-PHASE EXTRACTION STEP
      Place 16x100 mm open tube in Millilab rac
      Activate Millilab system
      Condition cartridge with 3 ml methanol and 5 ml water
      Add sample to cartridge, dry for 13 min
      Elute cartridge with 15 ml isopropanol-hexane into 16x125 mm tube

DRYING STEP
      Add 1 g Na2SO4 to 16x125 mm tube
      Cap tube
      Rotate tube to mix Na2SO4

SAMPLE STORAGE
      Place dried sample in cold rack

ANALYSIS
      Evaporate/derivatize with BSTFA/TMS for GC analysis
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Fig. 2. Robotic sample preparation sequence.

placed for total automation. A third objective, replacing the chloroform as the SPE eluent was not deemed a prerequisite for automation but would be useful in the manual mode because it would result in less exposure for the analysts and would also lower the overall cost of analysis.

Criteria for success included adherence to the current in-house quality assurance (QA) program. A QA sample from a lot of viscous salad dressing with an established cholesterol content of 40 ± 2 mg/100 g was analyzed with every ten samples. In addition, one of these ten samples was analyzed in duplicate. If the result for the viscous QA sample was outside the 38–42 mg/100 g limit or the results of the duplicate sample varied by more that 10%, all results from that batch were rejected. During development, each new method/procedure was also explored using coconut oil and egg yolk standard reference materials.

The automated solid-phase extraction objective included exploring the use of a Millilab system. This unit was chosen because it was compatible with the sample size, solution volumes and solid-phase extraction cartridges used in the existing method. The only difference from the manual method was that the Millilab unit pushed reagents through the cartridges rather than drawing them with house vacuum. Hardware limitations dictated a minimum flow of 2 ml/min through the cartridge rather than 1 ml/min as called for in the original method. The flow for the chloroform–methanol also had to be doubled. This increase in flow-rates did prove to be not significant. Experiments with coconut oil and egg yolk standard reference materials yielded recoveries of 97.2% and 97.9%, respectively. Experiments comparing the two procedures were then expanded to more samples and matrices.

Manual method values were compared with those obtained from the Millilab SPE unit for a set of seventy-three samples. Each sample was weighed, saponified, acidified to pH 3 and then split. One aliquot was processed using the SPE unit and the second manually. The results are shown in Table 2. For each group, with the exception of butterfat, the corresponding 95% confidence interval included zero indicating that there is no difference between the two methods. Conversely the 95% confidence level for butterfat did not include zero indicating a significant difference between the two methods. Although the differences for butterfat were relatively high, the absolute differences were small as the cholesterol content in these samples ranged between 4 and 11 mg/100 g. Hence a difference of 1 mg/100 g resulted in a 10% relative error. The same conclusions can be drawn from the p-values listed in Table 2. For each matrix except butterfat, the p-values exceed 0.05, indicating no difference. However a p-value of 0.08 and a mean relative error of −0.03447 for the butterfat, again implied that the manual method gave higher results.

The second objective to be met before the method could be automated, was to find an alternative way to bring the pH of the saponified sample within the 3–5 range without the need for measurement with pH paper as with the manual method. Initial experiments with electrodes resulted in unstable readings because the samples contained 60% ethanol which dried the electrode resulting in erratic readings. It was then decided to explore a direct means of acidifying the saponified samples.

Buffers are also a means to bring solutions to a desired pH. Potassium hydrogen phthalate, potassium tartrate and acetic acid were investigated as each has buffering capacity in the desired range. The pH of viscous QA samples after saponification and addition of buffer exhibited pH values of 4.5, 3.5 and 4.3, respectively. Further experiments with these buffers showed that 3 ml of 3 M buffer was necessary to give the desired pH range and compensate for the variations when using a Mohr pipet to draw a

Table 2
Comparison of solid-phase extraction methods: Millilab vs. manual

Group	n	Mean relative error	95% Confidence interval	p-Value
Egg yolk	16	0.00039	(−0.0271, 0.0279)	0.98
Butterfat	18	−0.03447	(−0.0648, −0.0041)	0.028
Dairy cream	9	0.00020	(−0.1071, 0.1067)	0.997
Cream cheese	15	0.03852	(−0.0298, 0.1068)	0.26
Cheese products	6	0.02904	(−0.0606, 0.1187)	0.42
Viscous dressings	9	0.00227	(−0.0085, 0.0140)	0.70
All	73	0.00221	(−0.0183, 0.0227)	0.83

1-ml aliquot of the saponified sample or an Eppendorf pipet to add 0.39 ml of 7.5 *M* hydrochloric acid.

The accuracy of the procedure with these buffers was initially demonstrated using egg yolk powder and coconut oil reference materials. The results are shown in Table 3. Both the manual results and those with phthalate and tartrate buffers exhibited comparable recoveries. Recoveries were consistently lower with acetic acid, most likely due to the formation of cholesterol acetate. Both tartrate and phthalate buffers generated a precipitate when added to saponified samples. Moreover, samples with tartrate would occasionally clog the Millilab probe. Hence phthalate buffer was chosen as it afforded an acceptable pH and was compatible with the Millilab SPE unit. The phthalate buffer procedure was then compared with the manual method using a set of eighty-five samples representing fifteen matrices. The results are shown in Table 4. In every case the 95% confidence interval includes zero and the *p*-value was greater than 0.05 indicating no difference between the buffer and manual pH adjustment techniques.

In order to meet the third objective, a study was undertaken to find a substitute for the methanol–chloroform mixture used to elute cholesterol from the SPE cartridges. Most extraction techniques described in the literature use chloroform–methanol, first described almost four decades ago [18]. Recently the cost of chloroform disposal has risen to where it approaches the cost of procurement. Another reason to pursue a new solvent mixture, was to find one which is less toxic to both the analyst and the environment. High-performance chromatographic methods for cholesterol analysis have been described using *n*-hexane–2-propanol (99:1, v/v) as a mobile phase with an octadecyl (C_{18}) column [19–21]. Since the SPE columns are also C_{18}, solutions of 2-propanol in *n*-hexane in varying proportions were investigated to see if they could be employed with the SPE step. Initial experiments with 0.1–5% of 2-propanol in *n*-hexane did not elute cholesterol from the SPE columns. Only with a solution of *n*-hexane–2-propanol (90:10, v/v) did the cholesterol recoveries become reproducible. The experiments used to determine the optimum eluting solvent mixture are summarized in Table 5. Further examination showed that the 2-propanol content could be varied between 10–25% without sacrificing recovery. Having had success with samples of known composition, a solvent system of *n*-hexane–2-propanol (85:15, v/v) was chosen. This eluting mixture and the phthalate buffer described earlier were then incorporated into the automated method for subsequent validation experiments.

An off-line approach to robot design was taken where the robot performed pick and place operations while individual work stations executed individual steps in the saponification and SPE procedure. This afforded an opportunity to prepare samples in a parallel mode. Using a four

Table 3
Comparison of buffers for acidification technique

Sample	Theory (mg/100 g)	Recovery (%)			
		Manual	Acetate	Phthalate	Tartrate
Egg powder[a]	1900	98.9	77.2	99.0	98.8
%R.S.D.[b]	±20	1.27	4.30	1.18	1.20
Coconut oil[a]	62.4	97.8	85.4	98.7	99.6
%R.S.D.[b]	±0.4	0.94	1.5	0.75	0.60
QA sample	42.0	43.4	38.5	43.3	43.2
%R.S.D.[b]	±2	0.94	1.5	0.98	0.96

[a]National Institute of Standards and Technology Standard Reference Materials.

Table 4
Comparison of phthalate buffer with manual method for sample acidification

Matrix	*n*	Mean relative error	95% Confidence interval	*p*-Value
Semi solid dressing	6	−0.01044	(−0.0779, 0.057)	0.963
Neufchatel cheese	3	0.04798	(−0.1239, 0.2198)	0.353
Processed egg yolk	16	0.02237	(−0.0227, 0.675)	0.307
Low-fat cheese	2	0.4528	(−1.0603, 1.965)	0.164
Margarine	7	0.06576	(−0.0295, 0.161)	0.142
QA sample	9	0.0045	(−0.0212, 0.0221)	0.963
Cheese curd	2	0.2000	(−2.3412, 2.7412)	0.500
Natural cheese	7	0.0611	(−0.144, 0.1762)	0.814
Cream cheese	8	0.04979	(−0.123, 0.0234)	0.152
Cheese powder	2	0.01510	(−0.453, 0.4832)	0.752
Process cheese	7	−0.0394	(−0.094, 0.0151)	0.127
Cheese sauce	3	0.03715	(−0.0431, 0.1174)	0.185
Raw pasta	5	0.01121	(−0.0462, 0.0687)	0.617
All	79	0.02338	(−0.0153, 0.0621)	0.233

position heating block, samples were rotated in and out at 15-min intervals. Thus a sample was ready for subsequent steps every 15 min, thereby reducing bottlenecks in the overall sample preparation scheme.

SPE became the rate limiting step in the automated method because the Millilab is designed to process one sample at a time. In order to achieve the desired throughput, the CRS was programmed to check the status of the SPE step before performing each subroutine to determine if a sample had completed the SPE step and thus was ready for further processing. The automated system processed 200 samples per week. The operator weighed and prepared reagents for two hours a day per week. Working full time, an analyst can manually process 80 samples per week. Because the Millilab unit is controlled by a PC, it is possible to add a second unit to the system working in tandem to cut the rate limiting

Table 5
Comparison of hexane–2-propanol mixtures with chloroform–methanol for elution of cholesterol from solid-phase extraction cartridges

Sample matrix	Recovery (%)			
	5% Methanol in chloroform	10% Propanol in hexane	15% Propanol in hexane	20% Propanol in hexane
QA sample	40.0	40.0	40.0	39.8
%R.S.D.	1.09	0.78	0.95	1.08
Egg yolk powder	1903	1899	1884	1890
%R.S.D.	0.82	.072	0.38	0.98
Coconut oil	63.9	64.7	64.6	62.8
%R.S.D.	0.94	1.08	1.13	1.21

step in half whereby the weekly output would be increased to about 350 samples per week. Several vendors have introduced SPE units since this work was undertaken which have eliminated the slow dispensing problem associated with the unit described here with a commensurate increase in efficiency. When the fully automated method was used with coconut oil reference material, recoveries ranged from 98 to 102%. The relative standard deviation for 20 determinations was 0.97%. In a similar experiment using egg yolk powder reference material, recoveries ranged from 97 to 101%. The relative standard deviation for 20 determinations was 0.81%.

The fully automated and manual procedures were compared with 103 samples in twenty matrices (Table 6). In each case, the 95% confidence interval included zero. Because the calculated p-value was greater than 0.05 for each matrix and for all 103 samples taken as a whole, there was no evidence to reject the null hypothesis that the methods gave equivalent results. In all matrices except luncheon meat, cheese sauce and cream cheese, the p-value was greater that 0.05, hence both methods were considered to be equivalent. Fig. 3. shows a scatter plot illustrating the equivalence of the two methods. The slope is 0.994 and the intercept is 0.98.

When meat, cheese sauce and cream cheese samples were examined after the saponification step, it was noted that in some cases the matrix had not dissolved after one hour. This is overcome with the manual method by stirring samples such as these in the ethanolic potassium hydroxide solution overnight or until the matrix was dissolved. Determining a saponification time at room temperature based on observation is not feasible with an automated system. Knowing this to be a potential problem, these meat cheese and cream cheese samples were placed in the rack so they would be processed last. The system was programmed to add the enthanolic potassium hydroxide to all samples before the first sample was subjected to heating at 100°C. Using this strategy, the first sample was stirred at room temperature for two hours and the last for 15 h

Table 6
Comparison of solid-phase extraction methods: Millilab vs. manual

Matrix	n	Mean relative error	95% Confidence interval	p-Value
Natural cheese	12	−0.04606	(−0.0987, 0.0066)	0.078
Processed cheese	10	0.01027	(−0.0241, 0.0447)	0.517
Low fat cheese	5	−0.01150	(−0.1345, 0.1115)	0.808
Cheese sauce	9	0.00815	(−0.0053, 0.0216)	0.200
Cream cheese	10	−0.00870	(−0.0569, 0.0394)	0.692
Mayonnaise	3	−0.00300	(−0.0613, 0.0555)	0.847
Viscous dressing	4	0.00015	(−0.0439, 0.0442)	0.992
Sour cream	2	0.30000	(−2.2412, 2.8412)	0.374
Ice cream	8	0.01256	(−0.0059, 0.031)	0.151
Pasta	6	−0.00060	(−0.0474, 0.0462)	0.975
Natural egg yolk	4	0.00103	(−0.0112, 0.0132)	0.806
Processed egg yolk	10	−0.01663	(−0.0455, 0.0123)	0.226
Cooked beef	3	0.06293	(−0.0410, 0.169)	0.121
Breakfast sausage	3	0.22990	(−0.7578, 1.2178)	0.422
Liver sausage	3	0.03717	(−0.0204, 0.0947)	0.109
Balogna	2	0.00000	(−3099, 0.3100)	1.000
Deviled ham	2	0.08177	(−0.3616, 0.5255)	0.257
Sandwich loaf	2	−0.00526	(−0.3396, 0.3291)	0.874
Chili	3	0.03064	(−0.0470, 0.1083)	0.232
Shellfish	2	0.00000	(−0.2782, 0.2782)	1.000
All	103	0.01088	(−0.0066, 0.0284)	0.220

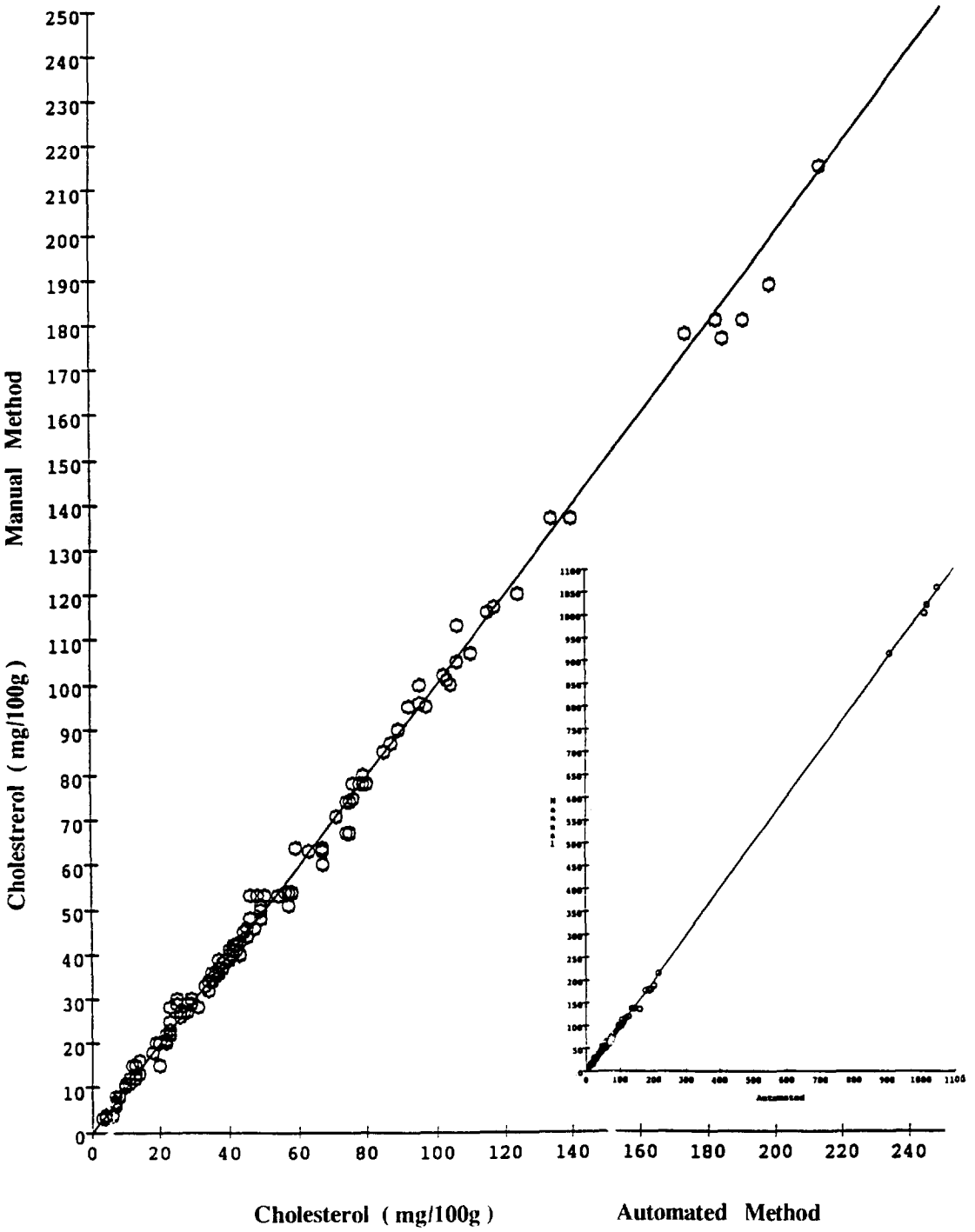

Fig. 3. Scatter plot: comparison of manual and automated cholesterol results for 103 samples spanning 20 matrices.

before heat was applied. The fortieth sample was stirred for approximately 10 h at room temperature before being heated at 100°C. When this placement of samples was incorporated into the scheme with cream cheese, meat and cheese sauce as the last samples, more consistent cholesterol values were observed for these matrices.

4. Conclusion

The system described here for automated sample preparation has reduced the analyst hours required for sample preparation by 80%. This has allowed analysts to become problem solvers rather than sample preparers for automated chromatographs. In addition to increasing productivity, this automated system provided the added benefit of isolating reagents and solvents from the analyst. Substitution of *n*-hexane–2-propanol for methanol–chloroform in the solid-phase extraction step significantly reduced solvent procurement and disposal costs by 30%. Laboratories employing the manual method [17] can also employ the phthalate buffer for acidification of the saponified samples and substitute *n*-hexane–2-propanol to reduce costs and analyst exposure as well.

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