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Note

Differences in retention of protein amino acids by C_{18} Sep-Pak cartridges

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Manufacturers of amino acid analysers usually recommend that only "clean" samples are injected on to the column. The resins used in ion-exchange instruments are particularly expensive, but peptides, intact protein, lipids, etc., are likely, in time, to cause a deterioration in performance of both ion-exchange and reversed-phase columns. After some years' experience with ion-exchange/ninhydrin systems for amino acid analysis, we have recently changed to the Waters Pico-Tag system, which uses reversed-phase separation of phenylthiocarbamyl amino acids obtained by derivatisation with phenylisothiocyanate (Edman's reagent).

The Waters Pico-Tag system was recommended for amino acid analysis of pure proteins; as we were intending to use it for food analysis, some clean-up of hydrolysates seemed desirable and the manufacturers recommend the use of Sep-Pak C_{18} cartridges to remove lipids and high-molecular-weight proteins from hydrolysates.

One of our first tasks, after commissioning the new instrument, was the analysis of whey protein isolate. This paper describes the effect of Sep-Pak clean-up on the analyses and an experiment designed to explain the observations.

EXPERIMENTAL

Whey protein isolate was obtained from Bio-Isolates (Swansea, U.K.). Its protein content (Nx 6.38) was 93.8% (mean of triplicate determinations by automated Kjeldahl analysis).

Hydrolysis of whey protein isolate was performed by reflux in 6 *M* hydrochloric acid after the addition of norleucine as internal standard.

Amino acid analysis was performed using a Waters Pico-Tag system, according to the manufacturer's instructions. A standard mixture of amino acids (+ norleucine) at 2 μmol/ml was used for calibration. This was obtained by dilution of four parts of a proprietary calibration solution containing most amino acids at 2.5 μmol/ml (LKB) with one part of a 10 μmol/ml solution of norleucine in 0.1 *M* hydrochloric acid. Cystine was present at 1.25 μmol/ml, so its concentration became 1 μmol/ml after dilution. Sep-Pak clean-up was performed according to Waters' instructions. The following solutions were used: solution 1: 0.1% trifluoroacetic acid (TFA) in

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distilled water; solution 2: 0.1% TFA in water–methanol (80:20); solution 3: 0.1% TFA in water–methanol (70:30). For each sample, a new Sep-Pak C_{18} cartridge was activated with two 10-ml volumes of methanol, washed with two 10-ml volumes of solution 1 and then with 10 ml of solution 2. A 1-ml sample was then mixed with 2 ml of solution 3 and passed through the activated cartridge. The first 1 ml of eluent was discarded and the next 2 ml were collected. This fraction contained the amino acids, ready for derivatisation.

To wash off retained amino acids, the Sep-Pak cartridge was subsequently washed with 2 ml of a solution of 0.1% TFA in water–methanol (50:50).

RESULTS AND DISCUSSION

The results of analysis of whey protein isolate both with and without Sep-Pak clean-up prior to derivatisation are shown in Table I. Whey protein isolate is approximately a 3:1 mixture of β-lactoglobulin and α-lactalbumin and the calculated values[1] for such a mixture are also shown. Three points can be made about the values obtained after Sep-Pak treatment: (1) With few exceptions they are higher than the calculated values. (2) They are higher than the values obtained without Sep-Pak treatment; the latter are generally close to the calculated values, with the exception of cystine. Performic acid oxidation was not used in this study. (3) The total amino acid content obtained after Sep-Pak treatment is higher than is theoretically possible for a protein.

TABLE I

AMINO ACID COMPOSITION OF WHEY PROTEIN ISOLATE

Results are expressed as milligrams per gram of protein.

Amino acid	*Composition from Pico-Tag without sample clean-up**	*Approximate value from ref. 1*	*Apparent composition from Pico-Tag after sample clean-up**
Asp	124	132	167
Glu	196	177	258
Ser	43	42	69
Gly	19	19	28
His	19	19	24
Arg	34	24	49
Thr	60	51	87
Ala	64	58	91
Pro	52	42	69
Tyr	44	41	49
Val	59	57	79
Met	22	26	30
Cys	18	41	27
Ile	59	69	65
Leu	151	145	160
Phe	40	37	35
Lys	112	117	152
Total	1116	1097	1439

* Mean of four determinations.

TABLE II

EFFECT OF SEP-PAK CLEAN-UP ON A STANDARD MIXTURE OF AMINO ACIDS

Results are given as percentage composition in molar terms.

Amino acid	*Initial mixture*	*1st Eluate*	*2nd Eluate*
Asp	5.7	6.0	3.8
Glu	5.7	5.6	3.8
Ser	5.7	7.4	3.8
Gly	5.7	7.9	3.8
His	5.7	6.0	3.8
Arg	5.7	6.0	3.8
Thr	5.7	6.5	3.8
Ala	5.7	6.5	3.8
Pro	5.7	6.5	3.8
Tyr	5.7	4.6	7.7
Val	5.7	6.0	5.8
Met	5.7	5.6	5.8
Cys	2.9	2.8	3.8
Ile	5.7	5.1	7.7
Leu	5.7	4.6	9.6
Nor	5.7	4.6	9.6
Phe	5.7	2.8	11.5
Lys	5.7	5.6	3.8

A possible explanation for these results is that the elevated results for most amino acids were associated with the use of norleucine as internal standard. If norleucine, with its non-polar side chain, were retained in the C_{18} phase of the cartridge, then the non-retained amino acids would appear to be present at higher concentrations. If this were the case, other amino acids with similar non-polar side chains might also be expected to be retained, and therefore to give more or less correct values. Such amino acids would be leucine, isoleucine, phenylalanine and, possibly, tyrosine. Inspection of the results in Table I shows that this is, indeed, the case and, in particular, the result for phenylalanine is actually lower after Sep-Pak treatment than without. This would imply that phenylalanine is even more strongly retained than is norleucine. To investigate this possibility, a brief experiment was performed using a standard amino acid mixture. An aliquot of this was passed through a Sep-Pak cartridge as before, but the cartridge was then washed with a further 2 ml of 0.1% TFA in water–methanol (50:50) and the eluate analysed. The results of these analyses (Table II) show that the first eluate is deficient in tyrosine, isoleucine, norleucine and (particularly) phenylalanine. The second eluate is enriched in the same amino acids, indicating that they were preferentially retained on the Sep-Pak cartridge.

Some of the amino acids in the second eluate may well represent the cartridge's residual content of eluate 1 rather than retention of these amino acids in the C_{18} phase; what is important is that not all amino acids are affected in the same way. The use of an internal standard (like norleucine), which is retained by the cartridge, obviously accentuates the problem by affecting the apparent concentration of all the

other amino acids, but whatever choice of internal standard is made, the difference in behaviour between, say, phenylalanine and aspartic acid makes the procedure unsound.

CONCLUSION

From these experiments we conclude that the use of the recommended Sep-Pak C_{18} clean-up procedure for protein hydrolysates gives incorrect results. In our experience, the Pico-Tag system performs well, without the use of sample clean-up, for the analysis of food hydrolysates. In addition, the reversed-phase columns are sufficiently cheap to be regarded as consumable.

REFERENCE

1 E. O. Whittier and W. G. Loordon, in B. H. Webb and A. H. Johnson (Editors), *Fundamentals of Dairy Chemistry*, AVI Publishing Co., Westport, CT, 1965, p. 60.