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INTERNATIONAL COLLABORATIVE STUDY CONCERNING THE IMPROVED GAS CHROMATOGRAPHIC DETERMINATION OF ADIPATE IN STARCH

Abstract

A draft protocol in ISO format for the improved gas chromatographic method for the determination of the total and the free adipate content in acetylated adipyl cross-linked starches is evaluated by an international collaborative study. The improvements in the method provide an analytical protocol in which the amount of organic solvent needed for each determination was reduced tenfold and the daily capacity of the analyses was increased threefold.

This international collaborative study led to the following results: (1) for the total adipate determination the repeatability (r) and reproducibility (R) were respectively 50 and 90 ppm adipic acid and (2) for the free adipate determination the repeatability (r) and the reproducibility (R) were respectively 12.6 and 27.2 ppm adipic acid.

Introduction

Acetylated adipyl cross-linked starch is a modified starch used in food applications. The adipyl content in these cross-linked starches can be determined by gas chromatography as described by Mitchell et al. [1] in 1982. According to this method the sample is saponified with alkali in the presence of an internal standard glutaric acid (pentanedioic acid). During the saponification the adipyl group is hydrolyzed from the starch and forms free adipate. After acidifying the hydrolysate, the resulting adipic acid (hexanedioic acid) and the internal standard, glutaric acid, are extracted with ethyl acetate. After removal of the ethyl acetate, the organic acids are silylated to their corresponding trimethyl silylesters. These are quantified by gas chromatography using a packed column with silicone oil as the active phase.

This method is laborious and uses large quantities of ethyl acetate (300 ml per determination).

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For environmental and economic reasons, and for improved efficiency, we have miniaturized the analytical method, especially with respect to the amount of organic solvent needed for each determination [2]. Provided that the samples of cross-linked starch to be analyzed are homogeneous, the sample weight can be decreased considerably. Consequently, the amount of organic solvent can be decreased, resulting in a considerable reduction of time needed for the evaporation to dryness of the ethyl acetate extracts.

Moreover, we have used pimelic acid (heptanedioic acid) as an internal standard instead of glutaric acid. The solubility of pimelic acid in water, and its extraction behaviour, is more similar to adipic acid than glutaric acid.

This improved methodology results in a reduction of about 90 % in the use of ethyl acetate needed for each determination, and in an increase of about 200 % in the daily capacity of analysis.

This improved method has been discussed in ISO/TC93 WG3 "Starch (including derivatives and by products) – Chemical functions". A draft protocol in ISO format of this method was prepared and an international collaborative test study has been started to evaluate this method for the determination of adipic acid content of acetylated distarch adipates.

In this paper the results of this international collaborative study are presented and discussed.

Material and methods

Chemicals

The following chemicals were used:

- concentrated hydrochloric acid (Merck, Darmstadt)
- sodium hydroxide (Merck, Darmstadt)
- ethyl acetate (Merck, Darmstadt)
- adipic acid (hexanedioic acid) (Merck, Darmstadt)
- pimelic acid (heptanedioic acid) (Merck, Darmstadt)
- acetonitrile (Lab-Scan)
- bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) which includes 1 % trimethylchlorosilane (TMCS) (Pierce)
- nitrogen gas (Hoek Loos, Schiedam)

Apparatus

- glass reaction tubes (100 x 16 mm) with screw cap fitted with PTFE covered rubber seals were used for the saponification, the extraction, the evaporation, and the silylation of the sample, respectively, the analyte.
- rotary shaker.
- adjustable Finn pipettes 0.1 – 1.0 ml
- waterbath adjusted to 30 °C
- evaporation device, based on solvent removal with a stream of nitrogen (e.g. Pierce Reacti-Vap III)
- ultrasonic bath
- gas chromatograph, accomodating capillary columns, fitted with a flame ionisation detector, on-column injector, and a (computer) integration system. Typical chromatographic conditions are as follows:
  Carlo Erba Vega gas chromatograph equipped with a cold on-column injection system, and a flame ionisation detector (temperature 300°C, hydrogen pressure 0.5 bar, air pressure 1.0 bar). The separation was performed on a WCOT fused silica CP-sil 5CB capillary column (length 50 m, internal diameter 0.32 mm, film thickness 0.12 mm) with helium as the carrier gas (pressure 0.7 bar). During the separation the temperature of the column oven was programmed as follows: after injection the temperature was kept constant at 130°C for 1 minute, then the temperature was raised at 5°C/min. up to 190°C, immediately followed by a fast temperature rise of 25°C/min. to 290°C. The temperature was kept at 290°C for 5 minutes, and then the oven was cooled down to 130°C in order to get the instrument ready for the next injection. The retention times of adipic and pimelic acid derivatives are 10.3 min. and 12.2 min. respectively.

Analytical Methods

Total adipate

Sample preparation

50 mg of the acetylated adipic cross-linked starch sample is weighed accurately in a glass reaction tube, and 1.5 ml distilled water, and 1.0 ml aqueous solution containing 0.05 mg pimelic acid/ml are added. The reaction tube is shaken to disperse the sample and 2.5 ml of 4 M sodium hydroxide solution are added. Agitation of the reaction tube is continued in order to dissolve the starch sample. The reaction tube is closed and the adipyl-starch ester bond is saponified by continuous rotating the tube with the rotary shaker during at least 5 minutes. Then 1.0 ml of concentrated hydrochloric acid is added and the mixture is homogenized. 5 ml of ethyl acetate are added, the tube is closed, and shaken vigorously for at least 1 minute to extract the adipic and

pimelic acid into the ethyl acetate. After phase separation the upper, ethyl acetate layer is transferred with a glass Pasteur pipette into a clean glass reaction tube. The ethyl acetate extraction of the aqueous solution is repeated three times and the ethyl acetate fractions are collected. These collected fractions are evaporated to dryness with a nitrogen stream in a Pierce Reaction-Vap Evaporator at a temperature of 30°C in a water bath. Then 0.3 ml of acetonitrile is added to the dry residue, and the reaction tube is placed in an ultrasonic bath for several minutes to dissolve the residue. 0.3 ml of BSTFA/1% TMCS solution is added, and the mixture is homogenized again in the ultrasonic bath for several minutes. After a reaction time of at least 30 minutes in a water bath at a temperature of 30°C, 0.3 ml of the reaction mixture is injected in the capillary gas chromatograph.

Calibration

Four 50 mg samples of waxy corn starch are weighed into four glass reaction tubes. 1.0 ml aqueous pimelic acid solution containing 0.05 mg pimelic acid/ml is added into each tube followed by the addition of 0.25, 0.50, 0.75, and 1.00 ml aqueous adipic acid solution, containing 0.05 mg adipic acid/ml, into the respective tubes. The volume is adjusted to 2.50 ml with distilled water and the procedure as described in the sample preparation section, beginning with "The reaction tube is shaken to disperse the sample..", is carried out.

Free adipate

Sample preparation

100 mg of the acetylated adipic cross-linked starch sample is weighed accurately in a glass reaction tube, and 4.0 ml distilled water, and 1.0 ml aqueous solution containing 0.05 mg pimelic acid/ml are added. The tube is closed and the free adipate is extracted by agitating the closed reaction tube for 16 hours using a rotary shaker. Then the tubes are centrifugated for 5 minutes at 1100 g in a laboratory centrifuge. The clear supernatant liquid is transferred into a clean glass reaction tube and 50 ml 12 M aqueous solution hydrochloric acid and 5 ml ethyl acetate are added. The tube is closed and shaken thoroughly for 1 minute. After phase separation the upper, ethyl acetate layer is transferred with a glass Pasteur pipette into a clean glass reaction tube. The ethyl acetate is evaporated completely under a steam of nitrogen. Then the silylation and gas chromatographic determination are conducted as described for the total adipate beginning with "Then 0.3 ml of acetonitrile is added to the dry residue..".

Calibration

Four 500 mg samples of waxy corn starch are weighed into four glass reaction tubes. 1.0 ml aqueous pimelic acid solution containing 0.05 mg pimelic acid/ml is added into each tube followed by the addition of 0.25, 0.50, 0.75, and 1.00 ml aqueous adipic acid solution, containing 0.05 mg adipic acid/ml, into the respective tubes. The volume is adjusted to 2.50 ml with distilled water and the procedure as described in

the sample preparation for the determination of free adipic acid content section, beginning with "The tube is closed and the free adipate is extracted by agitating the closed reaction tube for 16 hours..", is carried out.

Expression of results

The peak areas for the pimelic acid and the adipic acid in the prepared calibrant solutions are determined. A graph with the different amounts of adipic acid (mg) added to the waxy maize starch on the x-axis and the corresponding ratios of the area of the adipic acid peak to the pimelic acid peak on the y-axis is plotted. The best fitting curve is derived by using linear regression analysis.

For each sample analyzed, the ratio of the area of the adipic acid peak to the pimelic acid peak is calculated and the corresponding amount of adipic acid is derived from the graph.

The adipic acid content in the samples is expressed in ppm (mg/kg) of adipic acid in the dry matter of the sample. The bound adipic acid content is obtained by the difference between the total adipc acid and free adipic acid content in the sample. The dry substance content in the starch samples is determined by using the oven drying method according to ISO 1666.

Experimental set-up of the collaborative study

To meet ISO requirements eleven randomly numbered samples, being five blind duplicates and a test sample of known content for practising the method, were sent to the participants of this study. For the statistical evaluation single analysis were required on each sample of the five blind duplicates. The participants were in alphabetical order: Amylum in Belgium, AVEBE in The Netherlands, Cerestar R & D in Belgium, National Starch and Chemicals in the USA, Netherlands Institute for Carbohydrate Research TNO in The Netherlands (NIKO-TNO), Roquette Frères in France, and Zolltechnische Prüfungs- und Lehranstalt in Germany. Both at Cerestar and National Starch a double set of samples have been analyzed by different persons at different days.

In accordance with resolution 35 of the 8th meeting of ISO TC93 WG3 on May 1993, the samples were analyzed according to the draft protocol entitled "Determination of adipic acid content of acetylated di-starch adipates", based on a proposal of NIKO-TNO [2]. The results were reported on the form provided together with the method.

Results and discussion

Determination of the total and free adipate content

As described before [2] the most laborious and time consuming steps in the analytical procedure are the extractions with ethyl acetate followed by the complete evaporation of the organic phase.

By decreasing the sample weight from the original 1 gram to just 50 mg for the determination of the total adipate content, the saponification and the extractions can be carried out in small volumes of a few ml in screw-cap glass reaction tubes.

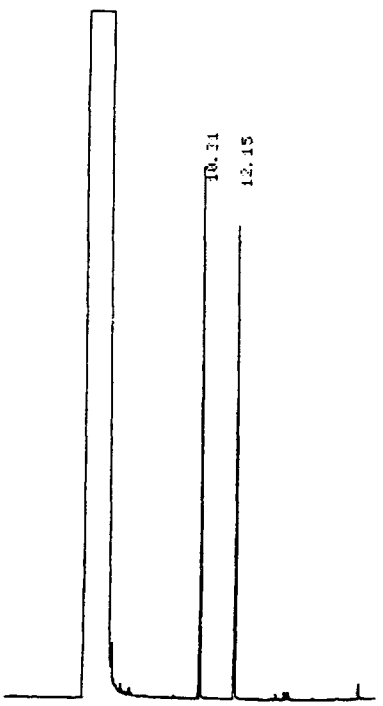

Fig. 1. Chromatogram of a calibration standard to which 1.00 ml adipic acid and 1.00 ml pimelic acid solution was added, measured with the typical chromatographic conditions as given in the protocol. Retention time of the adipic acid derivative is 10.3 min. and of pimelic acid derivative 12.2 min.

Total extraction of the analyte and the internal standard pimelic acid into the organic phase was achieved by four successive extractions with 5 ml of ethyl acetate. Moreover by performing the extractions in closed reaction tubes placed in a rack, it is possible to do 20-30 extractions simultaneously. 27 samples can be evaporated to dryness in a nitrogen stream simultaneously by applying a Reaction-Vap Evaporator (Pierce). Then the adipic acid and the pimelic acid in the residue are dissolved in acetonitrile (instead of pyridine), derivatized with the BSTFA/TMCS reagent to form their corresponding trimethylsilyl derivatives, and then separated and quantified by capillary gas chromatography (Figure 1). A calibration graph based on standard addition of adipic acid to waxy corn starch is used for quantification.

Advantages of the improved method [2] with respect to the original method are:

1. increase in daily analysis capacity of about 8 samples to about 25 samples,
2. considerable decrease in the consumption of organic solvent per determination; instead of 300 ml, just 20

Table 1

Applied GC conditions by the various laboratories

LAB nr.	Column type, length, ID film thickness	Temp. program	Injection
1	HP 1, 12 m, 0.2 mm 0.33 µm	1 min. 100°C 25 °C/min. to 250°C 8 min. 250°C.	Gerstel CI S3, cooled injection system
3	Quadrex, 15 m, 0.25 mm 0.1 µm	not reported	injector 200°C
4	DB1, 30 m, 0.32 mm 0.25 µm	init. 100 °C 7 °C/min. to 290 °C.	injector 300 °C, split 50 ml/min.
5	not reported	not reported	not reported
6	CP-SIL 5CB, 10 m, 0.32 mm, 0.12 µm	1 min. 100 °C 25 °C/min. to 290 °C.	on-column
7	HP 1, 5 m, 0.53 mm, 2.65 µm	2 min. 60 °C 15 °C/min. to 300 °C 2 min. at 300 °C.	not reported
8	not reported	not reported	split injector splitless mode
9a	not reported	not reported	not reported
9b	not reported	not reported	not reported

ml ethyl acetate is needed per determination,

3. improved repeatability.

It should be noted that, as summarized in Table 1, most participating laboratories used somewhat different GC conditions than the typical chromatographic conditions as given in the protocol. An example of such a chromatogram is given in Figure 2.

Total adipic acid

The contents of total adipate in the five blind duplicate samples of acetylated adipyl cross-linked starches as measured by the participating laboratories are presented in Table 2. In this table the duplicate difference and the average duplicate value of the blind duplicates are given also. At the bottom of this table for each participating laboratory is calculated the value of the average of all data and the sum of the duplicate averages for that laboratory.

With the Dixon Q-test no outliers could be detected in these calculated averages of all data and sums of the duplicate averages for each laboratory, indicating that no severe systematic errors are present.

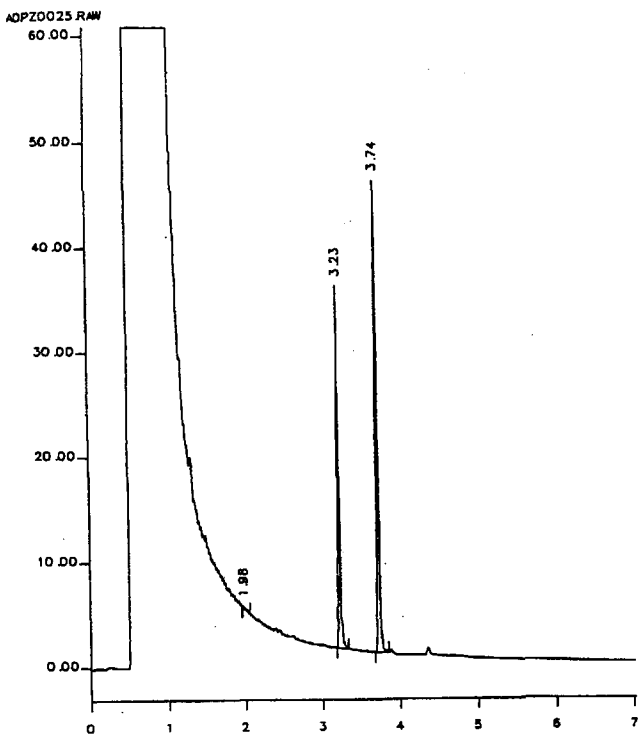

Fig. 2. Chromatogram of an acetylated adipyl cross-linked corn starch sample, measured with a modified temperature program and 10 m column (Table 1, lab nr. 6). Retention time of the adipic acid derivative is 3.2 min. and of pimelic acid derivative 3.7 min.

The Cochran maximum variance test ($p = 0.05$) and the one-tailed Dixon Q-test were used to evaluate the within sample duplicate differences and the within sample duplicate averages of the laboratories. It appeared that the duplicate difference of the samples 545/949 of laboratory 7 and 041/551 of laboratory 8 were outliers. And by one-tailed Dixon Q-test ($p = 0.05$) it was shown that the duplicate averages of samples 556/161 of laboratory 5 and of sample 041/551 of laboratory 8 were outliers also. Therefore these results have been rejected before further statistical evaluations were made.

The within laboratory standard deviation was calculated using the duplicate differences and is given also at the bottom of Table 2. With the Cochran maximum variance test ($p = 0.05$) no outliers could be detected in these within laboratory standard deviation.

The analytical results of each blind duplicate as measured by the different laboratories were statistically evaluated by one-way analysis of variances. The results of this evaluation are presented in Table 3. Of each duplicate sample (excluding the outliers) the following parameters have been calculated:

- the average measured total adipate content,
- the total standard deviation (S_{Tot}) in the average,
- the within sample standard deviation (S_r),
- the between sample standard deviation (S_R).

Table 2

Measured content of total adipate (in ppm adipic acid) in the five blind duplicate samples of acetylated adipyl cross-linked starches

sample		Laboratory number								
		1	3	4	5	6	7	8	9a	9b
557		333	414	400	529	430	394	421	454	412
664		404	433	390	539	418	403	467	471	428
	difference	71	19	10	10	12	9	46	17	16
	average	368	423	395	534	424	399	444	463	420
455		313	377	400	435	399	370	326	422	386
006		381	373	400	410	399	358	369	421	404
	difference	68	4	0	25	0	12	43	1	18
	average	347	375	400	423	399	364	348	422	395
556		631	633	620	849	666	676	607	692	653
161		572	656	625	731	642	615	635	705	645
	difference	59	23	5	118	24	61	28	13	8
	average	602	645	623	790	654	647	621	699	649
041		86	74	100	102	79	87	124	105	91
551		84	69	85	108	85	89	185	103	98
	difference	2	5	15	6	6	2	61	2	7
	average	85	72	93	105	82	88	155	104	95
545		369	384	390	482	380	354	396	434	392
949		359	374	400	499	390	434	394	436	398
	difference	10	10	10	17	10	80	2	2	6
	average	364	379	395	490	395	394	395	435	395
average all data		353	379	381	468	389	378	392	424	391
sum average data		1766	1894	1905	2342	1944	1890	1962	2122	1954
within lab standard deviation		36	10	8	12[a)]	9	22[b)]	24[c)]	7	9

[a)] excluding outlying data laboratory 5,
[b)] excluding outlying data laboratory 7,
[c)] excluding outlying data laboratory 8.

The overall total, within laboratory, and between laboratory standard deviations have been calculated by pooling the respective standard deviations of the duplicates. The results are given at the bottom of Table 3.

According to ISO 5725 the repeatability (r) and the reproducibility (R) can be calculated by multiplying both the corresponding pooled within laboratory standard deviation and the pooled between laboratory standard deviation by a factor 2.8. Thus this collaborative study results for the total adipate determination in a repeatability r = 50 ppm and the reproducibility R = 90 ppm adipic acid.

Table 3

Statistical evaluation of the data of Table 1

Sample duplicate	outlying laboratory	average	S_{Tot}	S_r	S_R
557/664	-	430	50	22	45
455/006	-	386	32	21	25
556/161	5	642	34	24	23
041/551	8	90	11	5	12
549/949	7	405	40	7	40
Pooled Standard Deviation			36	18	32

The free adipic acid content

The contents of the free adipic acid in the five blind duplicate samples of acetylated adipyl cross-linked starches as measured by the participating laboratories are presented in Table 4.

Just as in Table 2, also the duplicate differences and the average duplicate values of each blind duplicate for all the laboratories are given. The average of all data and the sum of the duplicate averages for the individual laboratories are given at the bottom of Table 4. The Cochran maximum variance test and the one-tailed Dixon Q-test were used to evaluate the within sample duplicate differences and the within sample duplicate averages of the laboratories. The duplicate difference of sample 545/949 of laboratory 8 appeared to be an outlier ($p = 0.05$) and the duplicate average values of the samples 557/664, 556/161, and 545/949 of laboratory 5 are outliers. Although the within laboratory standard deviation of laboratory 5 is very good, the duplicate averages are systematically much too high. This is clearly demonstrated by the values of the average of all data and the sum of the duplicate averages as listed at the bottom of Table 4. Looking at the analytical data of laboratory 7, it has to be concluded that these data are systematically much too low. Possibly a dilution error of a factor 2 has been made.

The within laboratory standard deviation was calculated by using the duplicate differences and is given at the bottom of Table 4 also. With Cochran maximum variance test ($p = 0.05$) no outliers in these within laboratory standard deviation could be detected.

For the above mentioned reasons all the analytical results of laboratory 5 and 7 were rejected just as duplicate 545/949 of laboratory 8. Also the analytical results of

Table 4

Measured content of free adipic acid in the five blind duplicate samples of acetylated adipyl cross linked starches

sample		Laboratory number								
		1	3	4	5	6	7	8	9a	9b
557		26	43	35	56	32	17	29	21	37
664		35	32	35	56	33	17	27	21	37
	difference	9	11	0	0	1	0	2	0	0
	average	31	38	35	56	33	17	28	21	37
455		110	120	110	164	104	60	120	80	126
006		110	102	100	169	110	58	113	91	125
	difference	0	18	10	5	6	2	7	11	1
	average	110	111	105	167	107	59	117	86	126
556		10	18	20	31	19	10	15	14	17
161		14	19	20	29	19	10	20	13	22
	difference	4	1	0	2	0	0	5	1	5
	average	12	19	20	30	19	10	18	14	19
041		10	17	15	26	16	8	9	10	17
551		14	10	20	28	17	8	11	10	17
	difference	4	7	5	2	1	0	2	0	0
	average	12	14	18	27	17	8	10	10	17
545		98	110	105	154	103	53	101	66	121
949		93	104	100	153	102	54	83	63	120
	difference	5	6	5	1	1	1	18	3	1
	average	96	107	103	154	103	54	92	65	121
average all data		52	58	56	87	56	30	53	39	64
sum average data		260	288	280	433	278	148	264	195	319
within lab standard deviation		3.7	7.3	3.9	1.8	2.0	0.7	3.2[a)]	3.6	1.6

[a)] excluding outlying data laboratory 8

laboratory 9a seem systematically too low. Statistically it is on the edge of significance. Therefore these data are given the benifit of the doubt.

The analytical results of each blind duplicate as measured by the different laboratories were statistically evaluated by one-way analysis of variances. The results of this evaluation are presented in Table 5.

Of each duplicate sample (excluding the outliers) the following parameters have been calculated:

- the average measured total adipate content,
- the total standard deviation (S_{Tot}) in the average,
- the within sample standard deviation (S_r),
- the between sample standard deviation (S_R).

Table 5

Statistical evaluation of the data of Table 3

Sample duplicate	outlying laboratory	average	S_{Tot}	S_r	S_R
557/664	5, 7	31.6	6.4	3.8	5.1
455/006	5, 7	108.6	13.2	6.7	11.3
556/161	5, 7	17.1	3.5	2.2	2.7
041/551	5, 7	13.8	3.7	2.6	2.7
549/949	5, 7, 8	98.8	18.8	5.5	18.6
Pooled Standard Deviation			10.5	4.5	18.6

The overall total, within laboratory, and between laboratory standard deviations are calculated by pooling the respective standard deviations of the duplicates. The results are given at the bottom of Table 5.

According to ISO 5725 the repeatability (r) and the reproducibility (R) can be calculated by multiplying the corresponding pooled within laboratory standard deviation and the pooled between laboratory standard deviation both by a factor 2.8.

Thus, this collaborative study results for the free adipic acid determination in a repeatability r = 12.6 ppm and the reproducibility R = 27.2 ppm adipic acid.

Considering the fact that most participating laboratories used somewhat different GC conditions than the typical chromatographic conditions as given in Table 1 and that they had no experience with the applied method, the results are very promising.

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MIĘDZYNARODOWA WSPÓŁPRACA PRZY ULEPSZONYM OZNACZANIU ADYPINIANU W SKROBI METODĄ CHROMATOGRAFII GAZOWEJ

Streszczenie

Międzynarodowa współpraca pozwala ocenić wartość roboczego protokołu w formacie ISO ulepszonego oznaczania metodą chromatografii gazowej całkowitego i wolnego adypinianu w acetylowanych skrobiach sieciowanych łańcuchem adipylowym. Ulepszenia metody pozwalają dziesięciokrotnie zmniejszyć ilość organicznego rozpuszczalnika stosowanego w oznaczeniach i trzykrotnie zwiększyć dzienną ilość oznaczeń.

Międzynarodowa współpraca dała następujące wyniki: (1) w oznaczaniu całkowitego adypinianu osiągnięto powtarzalność (r) na poziomie 50 ppm kwasu adypinowego i odtwarzalność (R) na poziomie 90 ppm tego kwasu; (2) w oznaczeniach wolnego kwasu adypinowego osiągnięto powtarzalność (r) na poziomie 12.6 ppm i odtwarzalność (R) na poziomie 27.2 ppm.